

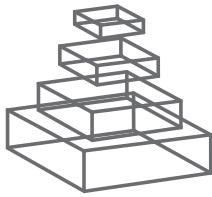
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M1/M2 MACROPHAGES: THE ARGININE FORK IN THE ROAD TO HEALTH AND DISEASE

Topic Editors

Charles Dudley Mills, Laurel L. Lenz
and Klaus Ley



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ISSN 1664-8714

ISBN 978-2-88919-499-5

DOI 10.3389/978-2-88919-499-5

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M1/M2 MACROPHAGES: THE ARGININE FORK IN THE ROAD TO HEALTH AND DISEASE

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The Fork in the Road to Health and Disease

specific killer molecule) or to Ornithine (a precursor of polyamines and collagen for repair). The dual Arginine metabolic capacity of macrophages provided a functional explanation for their ability to kill or repair. Macrophages predominantly producing NO are called M1 and those producing Ornithine are called M2. M1 and M2 – dominant responses occur in lower vertebrates, and in T cell deficient vertebrates being directly driven by Damage and Pathogen Associated Molecular Patterns (DAMP and PAMP). Thus, M1 and M2 are Innate responses that protect the host without Adaptive Immunity. In turn, M1/M2 is supplanting previous models in which T cells were necessary to “activate” or “alternatively activate” macrophages (the Th1/Th2 paradigm). M1 and M2 macrophages were named such because of the additional key findings that these macrophages stimulate Th1 and Th2 – like responses, respectively. So, in addition to their unique ability to kill or repair, macrophages also govern Adaptive Immunity. All of the foregoing would be less important if M1 or M2 – dominant responses were not observed in disease. But, they are. The best example to

Macrophages have unique and diverse functions necessary for survival. And, in humans (and other species), they are the most abundant leukocytes in tissues. The Innate functions of macrophages that are best known are their unusual ability to either “Kill” or “Repair”. Since killing is a destructive process and repair is a constructive process, it was stupefying how one cell could exhibit these 2 polar – opposite functions. However, in the late 1980’s, it was shown that macrophages have a unique ability to enzymatically metabolize Arginine to Nitric Oxide (NO, a gaseous non –

date is the predominance of M2 macrophages in human tumors where they act like wound repair macrophages and actively promote growth. More generally, humans have become M2 – dominant because sanitation, antibiotics and vaccines have lessened M1 responses. And, M2 dominance seems the cause of ever - increasing allergies in developed countries. Obesity represents a new and different circumstance. Surfeit energy (e.g., lipoproteins) causes monocytes to become M1 dominant in the vessel walls causing plaques. Because M1 or M2 dominant responses are clearly causative in many modern diseases, there is great potential in developing the means to selectively stimulate (or inhibit) either M1 or M2 responses to kill or repair, or to stimulate Th1 or Th2 responses, depending on the circumstance. The contributions here are meant to describe diseases of M1 or M2 dominance, and promising new methodologies to modulate the fungible metabolic machinery of macrophages for better health.

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Macrophages at the fork in the road to health or disease

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Edited and reviewed by:

Claudia Kemper, King's College London, UK

Keywords: macrophage, innate immunity, M1, M2, wound, cancer, infection, atherosclerosis

Macrophages are the epicenter of all immune systems (1). The first and the most abundant leukocyte observed (2), macrophage have long been relegated to the role of “servants” of T or B cells/adaptive immunity. This view is now known to be backward. Macrophages necessarily initiate and direct virtually all immune responses from simple multicellular animals to humans.

There is good news and bad news in the newly recognized importance of macrophages/innate immunity. The well-known “double-edged sword” nature of the immune system can largely be attributed to macrophages’ unique ability to make polar-opposite repair/heal (M2) or kill/inhibit (M1) type responses (3).

In health, M2-type macrophages maintain homeostasis by helping repair and replace lost or effete cells. Ever-present in tissues, macrophages are also the primary host defense against pathogens (or altered self cells) because their unique physiology allows them to rapidly switch from their M2/heal mode to an M1/inhibit mode: both powerful responses; both potentially dangerous.

In disease, over expression of M2/heal macrophages contributes to chronic infections, fibrosis, allergy, and cancer (3). Conversely, M1/inhibit-dominant activity plays a major role in atherosclerosis, autoimmunity, and other chronic inflammatory conditions.

Of fundamental importance is that both the routine M2/heal and the induced M1/inhibit macrophage functions occur in all animals whether they have T cells or not. Furthermore, M1 and M2 macrophage responses play necessary roles in causing T cells to make Th1 or Th2-type responses if pathogens or altered self are present (4). Hence, the renaming of macrophage responses M1 and M2.

This new knowledge about the central role of macrophages in immune systems brings great promise for increasing health/decreasing disease. In this regard, the ability of macrophages to exhibit the polar-opposite M2/heal and M1/kill functions result, in part, from their unique ability to metabolize one amino acid – arginine – to either growth-promoting ornithine or growth-inhibiting nitric oxide (NO) (5). Hence, the title of this Topic, “M1 and M2 Macrophages: The Arginine Fork in the Road to Health and Disease.” We hope that the articles assembled here help illuminate the basic functions of macrophages referred to as SHIP [sample, heal, inhibit, and present (antigen)]. Such knowledge is critical for developing the means to modulate the direct M2/heal or M1/inhibit responses of macrophages, or their indirect abilities to initiate and direct T and B cell responses. One can properly say macrophages are the “chicken and the egg” of immunity (1).

ORIGIN OF M1/INHIBIT AND M2/HEAL MACROPHAGES AND THE SCOPE OF THE TOPIC

As an introduction to M1 and M2 macrophages, a chronology of results (and publications) that led to their discovery is summarized below:

- Macrophages have the unique ability to produce a growth-inhibiting molecule (NO) or a growth-promoting molecule (ornithine), through the enzymatic conversion of L-arginine in different ways (6–8).
- Macrophages in normal tissues, healing wounds, or in growing tumors metabolize arginine primarily to ornithine via arginase (later to be named M2-type). Macrophages can switch to producing NO via iNOS (to be named M1-type) that is necessary to kill cancer or many pathogens. Arginine is the source of both ornithine and NO (9–12).
- Macrophages were specifically renamed M1 and M2 to highlight that innate immunity controls adaptive immunity. M1 (NO) or M2 (ornithine)-type macrophage responses are T cell independent and they stimulate Th1-type and CTL responses, or Th2-type responses, respectively (1, 3–5). Thus, M1/M2 represents a sea change in our understanding of how immune responses occur.

These studies have stimulated thousands of publications that have enhanced our knowledge of the importance of M1/inhibit and M2/heal functions, and other cytokines and factors that accompany these responses (1). Here, we have assembled papers by contributors that focus on basic aspects of macrophage biology, their roles in various diseases, and how they are regulated. Macrophages evolved long before other immune cell types and are the foundation of all animal immunity (13). Therefore, we begin with a series of “introductory” articles where readers can find basic information about macrophage biology and functions. These articles also trace the evolutionary origins of macrophages to aid in understanding their central role in immune systems. Next, articles detail the roles of macrophages in protection against (or causation of) various diseases including wounds, cancer, infections, atherosclerosis, obesity, hypertension, and other conditions. Finally, we look to the future with several articles detailing how macrophage M1/inhibit and M2/heal functions might be modulated for therapeutic benefits. We hope that the articles enhance your knowledge of this singularly multitalented and remarkable leukocyte.

INTRODUCTION TO MACROPHAGE BIOLOGY AND FUNCTIONS

To better appreciate macrophages, it is useful to know about their unique biochemistry, functions, and central role in all immune systems. Drs. Rath and Munder provide a comprehensive biochemical introduction to macrophage arginine metabolism, and how these cells can take “the fork in the road” to make either an arginine-based M1/inhibit or M2/heal response (14). Because “Nothing makes sense except in the light of evolution” (Theodosius Dobzhansky), Dr. Buchmann traces the evolutionary origins of both innate and adaptive immunity, and shows when new macrophage (and other) immune functions evolved, culminating in humans (15). Dr. Dzik importantly reveals that the macrophage M2/heal function (arginases) preceded the M1/inhibit function in animal evolution (16). Drs. Mills, Thomas, Lenz, and Munder describe the basic “SHIP” functions of macrophages [sample, heal, inhibit, and present (antigen)], and why it is important to study these functions to understand macrophage biology *in vivo* (17). In a similar vein, Drs. Italiani and Boraschi elucidate why examining macrophages by functions versus “phenotypes” can be critical for understanding how they affect health (18). Dr. Harris’ piece colorfully describes the two-edged sword nature of macrophages as “The Good the Bad and the Ugly” phases of inflammation. He also illuminates that M1/inhibit or M2/heal-type macrophage activities vary enormously in different microenvironments of lungs or other sites of inflammation (19). In turn, important local functions can be lost if one grinds up whole organs as is common. Drs. Thomas and Mattila provide an in-depth look at macrophage arginine metabolism in different vertebrate species (20). Importantly, they show that, contrary to some reports (21, 22), macrophages in mice and humans are quite similar, as one might expect from analyzing evolution (e.g., Drs. Buchmann and Dzik, mentioned earlier).

MACROPHAGE INFLUENCES IN DIFFERENT DISEASES WOUNDS AND CANCER: STARK EXAMPLES OF THE TWO-EDGED SWORD NATURE OF MACROPHAGE RESPONSES

Wound healing requires M2/heal-type responses (9). If M1/inhibit responses occur (e.g., infected wounds) wound healing is delayed until the infection is cleared (5). M2/heal-type macrophages also dominate inside tumors in experimental animals and humans (5, 11, 23). In marked contrast to their beneficial effect in wounds, M2-type macrophages actively promote tumor growth [reviewed in Ref. (3)], in part, by secreting growth factors (11, 24, 25).

Regarding the relative roles of M1 or M2-type macrophages in wounds or implanted biomaterials, Drs. Brown, Sicari, and Badylak demonstrate that there is 2–3 day dominance of M1-type macrophages (26). These data support that the first biologic priority of hosts following injuries is to prevent infections (5). However, if an injury is sterile, the priority switches to M2-type dominant macrophages that are necessary for proper healing (17). Interestingly, they note that biomaterials with larger pore sizes have less scarring/fibrosis. Thus, the physical properties of implanted materials seem important in allowing functional regeneration over typical imperfect wound healing found in adult humans (27). Drs. Beljaars, Schippers, Smit, Martinez, Helming, Poelstra, and Melgert compared M1- and M2-types of macrophages inside

chemically damaged livers in mice and cirrhotic livers in humans (28). The liver is one of the few organs able to regenerate (though not perfectly) (29). So, it was interesting that they found a predominance of M1 macrophages during resolution of damage, which contrasts with M2 macrophages that dominate in wounds elsewhere, and which results in scarring/healing, as described. Also, interesting was that the authors observed distinct M1 and M2-type macrophages in close proximity to each other with little overlap in markers. These findings do not support the hypothesis that macrophages only resemble a “color wheel” with multiple overlapping characteristics (30).

In contrast to the beneficial effects of M2-type macrophages in wounds, these same types of macrophages promote cancer growth and metastases as mentioned [reviewed in Ref. (3)]. Why is there cancer, and why does the immune system help it grow?

Species successfully evolve by acquiring traits that provide survival advantages, and losing undesirable traits. Environmental and societal influences aside, the way animals (any species) change heritable traits is through producing progeny: breeding. Cancer in humans occurs mainly after breeding age. So, there has been little evolutionary pressure (or advantage) for humans to acquire traits that prevent cancer, or that could stop it if it appears. The same lack of evolutionary pressure applies to atherosclerosis, or many other “adult” diseases that mainly occur during post-breeding (to be discussed later). Too, mate selection (important in breeding success) is mostly unaffected by knowledge of whether parents or grandparents died of cancer or other late-appearing diseases (3).

Once it appears, cancer can be eliminated. How? Modulating macrophage functions. It is now known that the majority leukocytes in tumors are macrophages: sometimes >50% of a tumor mass. However, as mentioned, these tumor-associated macrophages (TAM) are primarily M2-type and actively promote tumor growth: Cancer is often referred to as “a wound that doesn’t heal” (31). But, a wealth of recent evidence indicates that decreasing M2/heal-type and increasing M1/inhibit-type macrophages can slow or reverse tumor growth (11, 32). This is an exciting development because conventional immunologic thinking purports that tumors need to be recognized as “foreign,” like a pathogen. But, most tumors are not “foreign.” So, it falls on the shoulders of innate immunity, not adaptive immunity (T and B cells), to stop cancer. Importantly, recent antitumor effects being observed seem primarily (or solely) mediated by macrophages/innate immunity, not T cells (32). Even if a human cancer is recognized as “foreign,” it is still critical to switch M2- to M1-like macrophages. This is so because of the new knowledge, discussed earlier, that M1-type macrophages are necessary to stimulate T cells to make tumoricidal Th1-type cellular killer responses such as CTL and further amplify M1/inhibit macrophages (1, 4). In a related connection, significant prolongation of survival in human cancer has recently been observed by inhibiting immunoregulatory molecules, such as PD-1 and CTLA4 (33). The effects observed have been postulated to involve specific anti tumor T cell activity. Such effects likely depend on modulating macrophage responses. Thus, increasing our knowledge of M1 and M2 polarization in cancer, and how to modulate it, is very important.

Drs. Laoui, Van Overmeire, Baetselier, Van Ginderachter, and Raes review the evidence that M2-type macrophages predominate in most human tumors with the notable exception of colorectal cancer (34). They also describe new evidence that colony-stimulating factors are important players in determining the quantity and type of macrophages that populate tumors. MCSF is normally present in tissues and plasma, and is associated with M2-type macrophages. In contrast, GM-CSF is only present following injury or during infections, and is associated with M1-type responses. Interestingly, the authors highlight findings suggesting inhibition of MCSF by various means in humans does not only simply decrease M2-type TAM but also increases the M1/M2 ratio. Thus, altering macrophage differentiation signals can affect macrophage polarization beneficially in clinical settings. Using a different approach, Drs. Fritz, Tennis, Orlicky, Lin, Ju, Redente, Choo, Staab, Bouchard, Merrick, Malkinson, and Dwyer-Nield show that treatment of lung cancer in mice with a macrophage-depleting agent (clodronate-encapsulated liposomes) significantly decreases tumor burden (35). They also show that this treatment stimulates lung TAM that have a mixed M1- and M2-type phenotype suggesting that this depletion modality (like MCSF inhibition) may also increase the M1/M2 TAM balance. Together, these studies, like many others, are indicating that there are real and important clinical benefits from immunologically manipulating macrophage functions in cancer.

MACROPHAGES IN INFECTIONS

In the context of animal models of bacterial infection, M1-type macrophages and NO production are often, but not exclusively (36), associated with host protection (17). Conversely, M2-type macrophages are typically associated with bacterial persistence. An article by Drs. Ka, Daumas, Textoris, and Mege reviews macrophage polarization in infectious diseases (37). They discuss some difficulties encountered when trying to extend these concepts to bacterial infections in humans. In humans suffering from leprosy or Whipple's, macrophage, M2-type polarization can be readily observed. However, as mentioned earlier, analyzing whole organs can overlook microenvironmental differences in inflammation (19). Also, studies with human patients often utilize peripheral blood monocytes that lack the polarized M1- or M2-type functions associated with tissue macrophages. They also review that many pathogens, such as *Leishmania*, can survive or spread by blocking or subverting the process of macrophage development toward an M1/inhibit phenotype (27). An article from Drs. Burrack and Morrison discusses how macrophage activation and arginine metabolism by M1- or M2-type macrophages can have diverse effects on health and disease during viral infections (38). Macrophage NO production during viral infections, as in other settings, can be induced independent of lymphocytes (4). The production of NO can have direct anti-microbial effects on certain bacteria, fungi, and viruses. Hence, the M1/inhibit phenotype in these settings plays an immune protective role. However, because NO kills non-specifically, it can also have immunopathologic or immune suppressive effects during infections by influenza, herpes simplex virus-1, and cytomegalovirus. Similarly, the two-edged sword nature of M2/heal macrophage responses can cut both ways. For example, M2/heal responses

(via arginase and growth-promoting ornithine) usefully promote tissue repair in some viral infection models, and via stimulating protective antibody responses. However, M2/heal responses are also associated with viral persistence or immune pathology during many infections, such as coronavirus-induced sudden acute respiratory syndrome (SARS), hepatitis B or C viruses, Ross river virus, HIV, and influenza. These articles indicate that it is important to understand the infectious disease type, stage, and severity in order to properly modulate M1- or M2-type responses to optimally eliminate pathogens and decrease untoward pathology.

MACROPHAGE RESPONSES IN ATHEROSCLEROSIS AND OTHER NON-PATHOGEN-INDUCED INFLAMMATORY CONDITIONS

In contrast to the primarily protective role of M1/inhibit-type responses against infection agents, described above, these killer/damaging activities are often associated with bad outcomes in chronic inflammatory conditions.

Regarding atherosclerosis, Drs. Thomas and Mattila show that both M1- and M2-types of macrophages are found during foam cell formation, a hallmark of atherosclerosis (20). Drs. Hayes, Tsaousi, Gregoli, Jenkinson, Bond, Johnson, Bevan, Thomas, and Newby show that there is an altered expression of certain matrix metalloproteinases in atherosclerosis (39). Interestingly, they also show that M1 and M2 macrophage polarization in atherosclerotic ApoE null mice occurs in the absence of T- and B-lymphocytes, again highlighting the independence of innate immunity from adaptive immunity discussed earlier. Drs. Murphy, Dragoljovic, and Tall review the recent evidence that cholesterol efflux pathways regulate myelopoiesis (40). Traditionally, cholesterol efflux was considered as a safeguard against foam cell formation, but Tall's group has shown that knocking out cholesterol efflux molecules like ABCA1 and ABCG1 cause profound changes in hematopoiesis associated with more Ly6C+ inflammatory monocytes and more neutrophils. This shift could lead to altered macrophage function. Drs. Peled and Fisher review dynamic aspects of macrophage polarization during atherosclerosis progression and regression: progression is associated with macrophage M1 polarization and regression with M2 polarization (41). The article by Drs. Yang and Ming looks at the less commonly studied arginase II enzyme. They show that, unlike arginase I, that is typically inversely related to macrophage NO production, arginase II seems under different regulation. Also, Arg II expression in endothelial cells tends to uncouple eNOS (or NOS1) causing loss of vascular tone (42).

Regarding other non-pathogen associated inflammatory conditions, Drs. Vlahos and Bozinovski review the role of alveolar macrophages in chronic obstructive pulmonary disease (COPD) (43). COPD is a widespread chronic inflammatory condition with immense medical and societal impact. Interestingly, in COPD, there is an accumulation of airway macrophages that show a transcriptome skewed toward wound healing M2 markers suggesting defective resolution of inflammation (as occurs in wound healing). Drs. Kraakman, Murphy, Jandeleit-Dahm, and Kammoun review macrophage polarization in obesity and type 2 diabetes (44). M1 "pro-inflammatory" macrophages are enhanced compared with M2 "anti-inflammatory" macrophages,

leading to chronic inflammation and the propagation of metabolic dysfunctions. The brain and spinal cord are primarily populated by macrophage-like microglial cells, which are derived from yolk sac precursors under resting conditions. Drs. Cherry, Olschowka, and O'Banion show that these microglia are normally M2-polarized, although the microglia transcriptome is different from that of M2 macrophages in other organs (45). This observation fits well with an emerging concept that M1 and M2 polarization varies between organs (46). In this connection, Drs. Brown, von Chamier, Allam, and Reyes report on M1/M2 macrophage polarity in normal and complicated pregnancies and find that the balance and location of M1- and M2-type responses show significant variation (47). In general, over expression of M1-type macrophages is associated with untoward outcomes during pregnancy.

Finally, this Topic focuses on macrophage polarization. Little is currently known about how the origin of macrophages in tissues (e.g., yolk sac, Ly6C-high, Ly-6C-low monocytes) influence M1/M2 polarization. Drs. Dey, Allen, and Hankey-Giblin begin to explore how the ontogeny of monocytes and macrophage can influence M1- and M2-type responses in different tissues (48).

REGULATION OF MACROPHAGE DIFFERENTIATION AND FUNCTIONS

The importance of macrophage M1/inhibit and M2/heal imbalances in various disease or protective processes being clear, immunologists and clinicians are interested in how they might therapeutically intervene to shape macrophage differentiation or modulate various macrophage functions to restore health. Drs. Wang, Liang, and Zen provide an overview of the various molecular mechanisms known to impact macrophage M1 and M2 polarization (49). Several of these mechanisms are expanded upon in subsequent articles. Cytokines such as interferon gamma (IFN- γ) have profound impacts on development of M1 and M2 functionality in macrophages. Regulators of cytokine receptor signaling can importantly impact macrophage functions. Dr. Wilson details in her article evidence that various suppressor of cytokine signaling (SOCS) protein family members shape M1/M2 macrophage functions in several disease settings (50). Colony-stimulating factors (CSFs) and macrophage stimulating protein (MSP) also impact macrophage responsiveness to polarization. Drs. Hamilton, Zhao, Pavicic, and Datta introduce the concept that the myeloid colony-stimulating factors MCSF and GM-CSF act not only to promote the development and maintenance of various myeloid populations but can also shape the responsiveness of macrophages to stimuli that direct the M1 or M2 phenotype (51). The effects of MSP and its receptor, a tyrosine kinase known as RON, on macrophage polarization are clearly presented in an article by Dr. Chaudhuri (52). Other extrinsic factors that regulate macrophage polarization include components of the complement cascade as well as extracellular nucleotides. The contrasting effects of various complement components on M1 and M2 functions are described in an article by Drs. Bohlson O'Conner, Hulsebus, Ho, and Fraser (53). Drs. Desai and Leitinger go on to detail how purinergic receptors for extracellular ATP and other nucleotides couple with calcium signaling to modulate macrophage activities and resolution of inflammation (54). An improved understanding of how

these extrinsic factors and intrinsic signaling pathways regulate the acquisition of M1 and M2-type functions will lead to improved methods for fine-tuning of macrophage polarization to promote health.

As mentioned above, a key difference between M1 and M2 macrophages is in their processing of L-arginine. NO can not only kill susceptible microbes but also has a variety of signaling and regulatory effects on macrophages and other cell types. NO is generated from L-arginine through the activities of three NO synthase (NOS) enzymes. In contrast to canonical views, Drs. Mattila and Thomas present the perspective that "constitutive" enzyme activities (NOS1 and 3) can be induced, and "inducible" NOS2 is constitutively expressed in several tissues (55). The different functions of M1 and M2 macrophages are also associated with changes in the metabolic pathways they use to produce ATP. Drs. Galván-Peña and O'Neill discuss the differences in metabolism between M1 and M2 macrophages and how these differences impact other aspects of macrophage function (56). M1 macrophages mainly rely on glycolysis for energy, while M2 macrophages primarily use oxidative phosphorylation. Accumulation of succinate in M1 macrophages can stimulate HIF1 α to sustain production of factors such as IL-1 β and thus can impact the ability of M1 macrophages to prolong inflammation. Manipulation of NOS enzyme expression and activities as well as the products and consequences of the different metabolic processes in M1 and M2 macrophages should further our ability to shape the outcome of infections and other diseases.

SUMMARY

The "Fork in the Road" that macrophages take in making either M1/inhibit or M2/heal-type responses define "immunity" throughout the animal kingdom. In all animals, M1/inhibit-type responses are the primary host defense, and M2/heal-type responses help repair and replace lost or effete tissue to maintain host homeostasis. In humans (and other higher animals), macrophage M1/inhibit or M2/heal-type responses necessarily direct T (and B) cells/adaptive immunity to make Th1 or Th2-like responses. Thus, whether acting directly or indirectly, which "fork" macrophages take is the central controlling element that promotes health (as in pathogen control or wound repair) or impedes health (as in atherosclerosis, autoimmunity, or cancer). By illuminating the biochemical underpinnings, evolution, diseases, and regulation of macrophage functions, the papers in this Topic advance our understanding of how to modulate this most important of all leukocytes: the chicken and the egg of immunity (1).

ACKNOWLEDGMENTS

We are grateful for the many good papers and discussions that have occurred in creating this Topic and EBook.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 20 January 2015; accepted: 30 January 2015; published online: 16 February 2015.

*Citation: Mills CD, Lenz LL and Ley K (2015) Macrophages at the fork in the road to health or disease. *Front. Immunol.* 6:59. doi: 10.3389/fimmu.2015.00059*

This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology.

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Metabolism via arginase or nitric oxide synthase: two competing arginine pathways in macrophages

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Macrophages play a major role in the immune system, both as antimicrobial effector cells and as immunoregulatory cells, which induce, suppress or modulate adaptive immune responses. These key aspects of macrophage biology are fundamentally driven by the phenotype of macrophage arginine metabolism that is prevalent in an evolving or ongoing immune response. M1 macrophages express the enzyme nitric oxide synthase, which metabolizes arginine to nitric oxide (NO) and citrulline. NO can be metabolized to further downstream reactive nitrogen species, while citrulline might be reused for efficient NO synthesis via the citrulline–NO cycle. M2 macrophages are characterized by expression of the enzyme arginase, which hydrolyzes arginine to ornithine and urea. The arginase pathway limits arginine availability for NO synthesis and ornithine itself can further feed into the important downstream pathways of polyamine and proline syntheses, which are important for cellular proliferation and tissue repair. M1 versus M2 polarization leads to opposing outcomes of inflammatory reactions, but depending on the context, M1 and M2 macrophages can be both pro- and anti-inflammatory. Notably, M1/M2 macrophage polarization can be driven by microbial infection or innate danger signals without any influence of adaptive immune cells, secondarily driving the T helper (Th)1/Th2 polarization of the evolving adaptive immune response. Since both arginine metabolic pathways cross-inhibit each other on the level of the respective arginine break-down products and Th1 and Th2 lymphocytes can drive or amplify macrophage M1/M2 dichotomy via cytokine activation, this forms the basis of a self-sustaining M1/M2 polarization of the whole immune response. Understanding the arginine metabolism of M1/M2 macrophage phenotypes is therefore central to find new possibilities to manipulate immune responses in infection, autoimmune diseases, chronic inflammatory conditions, and cancer.

Keywords: macrophage, M1 and M2, arginine, arginase, nitric oxide synthase, immunoregulation, amino acid transporter

INTRODUCTION: ARGININE IN THE CENTER OF M1/M2 MACROPHAGE DICHOTOMY

Macrophages are highly versatile cells, which are (i) crucial for infection control (“kill/fight mode”) and tissue homeostasis (“default mode”, phagocytosing cellular debris) and (ii) involved in disease pathophysiology in cancer, autoimmunity, metabolic, and fibrotic disorders (1). Macrophages react to a wide variety of external stimuli and are able to produce a multitude of effector molecules for intercellular communication, microbial defense, and modulation of inflammatory reactions (1). They induce, suppress, or modulate both innate and adaptive immune responses. Considering this enormous complexity it is reasonable to deduce classification schemes to create order and sense in the experimental results of macrophage research (2, 3). Potential macrophage diversity, both in terms of activation states, surface marker expression, metabolic phenotype, and interspecies differences clearly requires rigid standards for experimental set-up and reporting (4). While undue simplification hampers the comparability between studies

(4), a reductionist approach tries to avoid getting lost in the complexities of macrophage biology and has both enormous power for the explanation of reality and can be the basis for experimentally testing of hypotheses. One should never forget that even the most sophisticated modern computers are based on the “0–1” dichotomy!

One of the most fruitful and reasonable classification of macrophages relates to their two main functions, namely, to kill/fight or to heal/fix. Within this classification view, macrophage biology is driven by two phenotypes (M1 for killing/fighting versus M2 for healing/fixing), which are also relevant in an evolving or ongoing immune response (5). M1 or M2 dominant macrophages then direct T lymphocytes to produce Th1 or Th2 responses, respectively, to further amplify M1 or M2 type responses in positive feed-back loops stabilizing the predominant immune phenotype in the respective setting of infection, tumor, or inflammation. The M1/M2 macrophage classification can be condensed into two opposing pathways for the metabolism of one

amino acid: the preference of macrophages to metabolize arginine via nitric oxide synthase (NOS) to NO and citrulline or via arginase to ornithine and urea defines them as M1 (NOS) or M2 (arginase) macrophages (5). NOS or arginase are enzymes that catalyze a “reaction,” but we will use “pathway” here to illuminate that they are part of multi-enzyme pathways producing other physiologically important products.

In this introductory review, we will describe macrophage arginine metabolism, its functional consequences and how the macrophage arginine metabolic phenotype defines the two opposing M1 and M2 types of macrophages. While various molecules and features of macrophages are reciprocally or mutually exclusively associated with the M1 versus M2 phenotype, the dichotomous regulation of arginine metabolism is at the center of the different functions that are associated with M1 and M2 macrophages.

ARGININE: ONE SMALL AMINO ACID FOR MACROPHAGE METABOLISM, A GIANT CONTROLLER FOR MAMMALIAN (PATHO-)PHYSIOLOGY

Mammalian arginine metabolism is complex both at the level of the whole organism (6, 7) and at the level of the individual cell types (8) and we would like to adopt the term “argenomics” that was suggested by Sidney M. Morris Jr (7) for the regulation of gene expression via arginine availability in an even broader sense for the whole fascinating complexity of arginine-driven cellular regulation. Before focusing in on arginine metabolism of macrophages and its determining role for the M1/M2 dichotomy, let us first have a short overview on some historical facts relating to arginine in mammalian physiology. The story started nearly 130 years ago, arginine was first isolated in 1886 and was identified as a component of animal proteins in 1895 (8). The role of arginine in metabolic physiology was first demonstrated in 1932, when Krebs and Henseleit discovered the urea cycle. In 1981, Windmueller and Spaeth reported that the small intestine is the major source of citrulline for synthesis of arginine by the kidneys, now called the intestinal–renal axis for arginine synthesis on an organismal level (9). In 1987, it was shown that arginine is the precursor for macrophage citrulline and nitrite synthesis (10) and that arginine-derived NO is the elusive endothelium-derived relaxing factor (EDRF) (11, 12). Soon afterwards, NO was categorized as physiologically active intermediate of the arginine to nitrite (+nitrate) pathway in macrophages (13, 14) and endothelial cells (15). The discovery of the fundamental role of arginine-derived NO for human cardiovascular physiology already led to the award of the Nobel Prize in 1998 to Robert F. Furchtgott, Louis J. Ignarro, and Ferid Murad. The importance of arginine has still risen since then, it is now clear that immune cell arginine metabolism is fundamentally involved in cancer, inflammation, infections, fibrotic diseases, pregnancy, and immune regulation in general (16–21). A huge responsibility for a small molecule!

MACROPHAGE ARGinine AVAILABILITY: SEVERAL ROADS LEAD TO ONE AMINO ACID

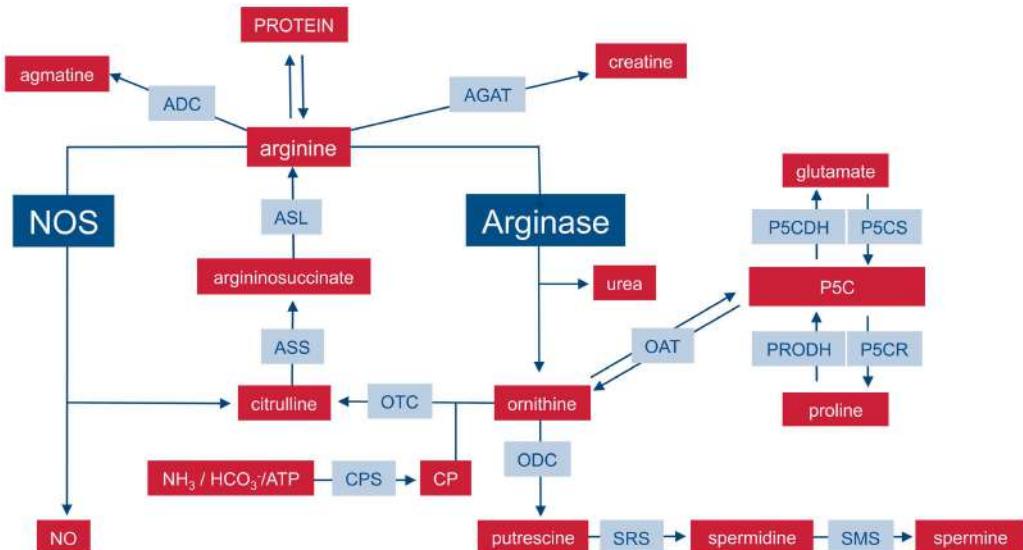
On the level of the whole organism, arginine is a non-essential amino acid for healthy adult humans, but it has to be supplemented during growth or various disease states (6, 7) and has thus been

characterized as a semi- or conditionally essential amino acid. Average arginine ingestion with a Western style diet is around 4–5 g/day and the normal plasma arginine level is 100–200 μM. Besides dietary intake, arginine is derived from cellular protein break-down or endogenous *de novo* arginine production. Mammalian arginine biosynthesis (Figure 1) involves mainly the amino acids glutamine, ornithine, and citrulline and the involved enzymatic steps are compartmentalized in different tissues and also on the subcellular level so that not all reactions can take place in each individual cell type or tissue. For a more detailed description of the chemical pathways of arginine metabolism, the reader is referred to the excellent review by Wu and Morris (8). (i) Glutamine can be converted to ornithine via glutaminase (yielding glutamate), pyrroline-5-carboxylate synthetase (P5CS), which is almost exclusively expressed in the intestinal mucosa, and ornithine aminotransferase (OAT). (ii) Ornithine transcarbamylase (OTC) and carbamoyl phosphate synthetase (CPS) are involved in the formation of citrulline from ornithine. The enzymes are restricted to the mitochondrial matrix of hepatocytes and epithelial cells of small and (to a minor extent) large intestine. This reaction is therefore a part of the hepatic urea cycle and also involved in intestinal synthesis of citrulline, which is released into the circulation. The proximal tubules of the kidneys take up most of the circulating citrulline, which is then converted within the kidney to arginine and again released into the circulation. (iii) Argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) are cytosolic enzymes responsible for the biosynthesis of arginine from citrulline (and aspartate as a co-substrate). While ASS and ASL are expressed constitutively or inducibly in many different cell types, their degree of expression, and the efficiency of their catalytic pathways vary between different tissue types. On a whole body level, the latter enzymatic steps form the basis of the so-called intestinal–renal axis with intestinal production of citrulline (see above) and renal synthesis of arginine (7).

M1 macrophages can also synthesize arginine in a cyclic fashion (Figure 2): during NO synthesis, arginine is converted to NO and citrulline via N^ω-OH-arginine (22, 23). Murine macrophages have long been known to (i) upregulate ASS and constitutively express ASL when stimulated with the NOS-inducing agents lipopolysaccharide (LPS) and IFN-γ (24) and (ii) to partially rescue NO synthesis via citrulline uptake and ASS-mediated recycling to arginine (25). This set of reactions via ASS and ASL forms the so-called citrulline–NO cycle (26). The importance of this pathway for the resynthesis of arginine to ensure sufficient substrate supply for prolonged NO synthesis under arginine limitation has been recently demonstrated *in vivo* in murine mycobacteria infection (27). Despite the upregulation of ASS1, availability of arginine remains a rate-limiting step for synthesis of NO and cellular uptake of arginine also determines the amount of NO synthesized in case of NOS and ornithine in case of arginase (28, 29).

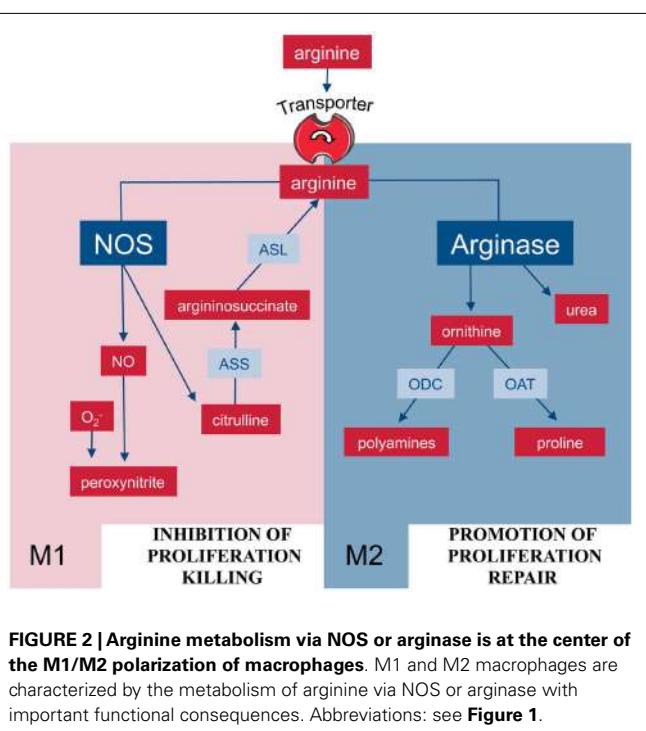
CELLULAR UPTAKE OF ARGinine

The transmembranous arginine transport is one essential component of cellular arginine metabolism and important for the cells to perform their tasks. Amino acids do not pass membranous lipid bilayers freely, but are transported via specialized proteins. These amino acid transporters show different transport

**FIGURE 1 | Important pathways of mammalian arginine metabolism.**

M1 and M2 macrophages are characterized by the metabolism of arginine via NOS or arginase with important functional consequences. This dichotomy is put into the context of other important pathways that can lead to the synthesis or degradation of arginine in mammalian cells. For sake of clarity, the diagram focuses on relevant enzymes (gray-shaded boxes), metabolites (red boxes), and the position of NOS and arginase within the network is highlighted. Various intermediate steps, by-products, or substrates are

omitted and the reader is referred to more extensive chemical reviews (see text). ADC, arginine decarboxylase; AGAT, arginine:glycine amidinotransferase; ASL, argininosuccinate lyase; ASS, argininosuccinate synthase; CP, carbamoyl phosphate; CPS, CP synthase; NOS, nitric oxide synthase; OAT, ornithine aminotransferase; ODC, ornithine decarboxylase; OTC, ornithine transcarbamylase; P5C, pyrroline-5-carboxylate; P5CDH, P5C dehydrogenase; P5CR, P5C reductase; P5CS, P5C synthase; PRODH, proline dehydrogenase; SRS, spermidine synthase; SMS, spermine synthase.

**FIGURE 2 | Arginine metabolism via NOS or arginase is at the center of the M1/M2 polarization of macrophages.** M1 and M2 macrophages are characterized by the metabolism of arginine via NOS or arginase with important functional consequences. Abbreviations: see Figure 1.

characteristics and specificities for the various amino acids, based on their physicochemical properties. Arginine, like the other cationic amino acids ornithine and lysine is preferentially taken

up via members of the solute carrier family 7 (SLC7) (30–32). The subfamily of cationic amino acid transporters (CAT1–3, i.e., SLC7A1–3) recognizes exclusively cationic amino acids, while members of the subfamily of heteromeric amino acid transporters, γ^+ LAT1 (SLC7A7) and γ^+ LAT2 (SLC7A6), and $b^{0,+}$ AT transport also neutral amino acids. CAT and γ^+ LAT proteins are widely distributed in various tissue types. CAT proteins are the main transporters for arginine uptake into cells, where the amino acid is then used for protein synthesis and all arginine-dependent metabolic pathways (7). CAT-1 is constitutively expressed in most tissues, with the exception of the liver, while CAT-2B is cytokine-inducible. CAT-2A is predominantly expressed in liver while CAT-3 is widely expressed during embryonal development but largely restricted to central nervous system and thymus in adults (30). In contrast to CAT, γ^+ LAT proteins exchange primarily extracellular neutral against intracellular cationic amino acids and are therefore responsible for arginine export rather than import (30, 32). $b^{0,+}$ AT is expressed in epithelial cells of small intestine and kidney where it is responsible for the (re)absorption of cationic amino acids and cysteine (32). ATB $^{0,+}$, a member of the SLC6 family, also transports both cationic and neutral amino acids and is expressed in the apical membrane of epithelial cells in various tissues (30).

The complex regulation of CAT expression has been studied quite extensively (30, 32, 33), whereas comparatively little is known about arginine transporter expression in most cells of the hematopoietic system in general or the immune system specifically. Induction of CAT-2 has been shown in murine macrophages upon both Th1 and Th2 cytokine stimulation (28), in murine dendritic cells (DCs) by retinoic acid (RA) (34) and in murine

microglia upon stimulation with IFN- γ +/- LPS (35). In contrast, CAT-1 is either expressed constitutively (28) or even downregulated upon activation (35). Coordinated induction of CAT-2 and arginase (partially dependent on CAT-2 expression) has been demonstrated in macrophages during murine allergic lung inflammation, forming the basis of bleomycin-induced fibrosis (36) and also in RA-activated DCs (34). On the other hand, sustained production of NO in murine macrophages is also based on CAT-2 expression and CAT-2-mediated arginine uptake (37). The induction of CAT-2 in both M1 and M2 type murine macrophages has been shown to differ quantitatively between different mouse strains with important pathophysiological consequences: a deletion in the SLC7A2 promoter of C57BL/6 mice leads to impaired CAT-2 expression, reduced arginine uptake, and decreased susceptibility to *Leishmania* infection as compared to BALB/c mice (38). In contrast to murine macrophages, arginine transport is based on system y^+L in IFN- γ -activated human primary monocytes (39) or LPS-stimulated alveolar macrophages (40), another example of interspecies differences, which are so prominent in various aspects of arginine metabolism in the immune system (17, 41, 42).

A crucial principle of immune cell signaling is the constitutive preexistence or the activation-induced assembly of multi-protein complexes that facilitate efficient transduction of stimuli. In murine myeloid cells, enhanced arginine import via CAT-2 is coupled to the induction of the arginine-catabolizing enzymes arginase I (28) and NOS (28, 34, 35, 43). It remains to be analyzed if (i) further similar higher-order structures or coordinated enzyme induction, combining arginine transporters with specific arginine-metabolizing proteins, can be found in macrophages and if (ii) differences in M1 and M2 type cells do exist. This is a valid hypothesis since amino acid transporters are not only final elements of distinct signal transduction pathways, which immune cells need for nutrient supply, but are also rather intricately involved in complex metabolic networks in which they influence further downstream signaling nodes and metabolic pathways (44). Interestingly, the ornithine-derived polyamine spermine enhances the expression of CAT-1 mRNA in human retinal pigment epithelial cells (45). This observation leads to an interesting question regarding macrophages: do M2 type macrophages increase their arginine transport capacity via endogenous arginase-mediated synthesis of polyamines, which would then further amplify arginase-based metabolism in a positive feed-back loop? An alternative scenario might result from the extracellular synthesis of ornithine after arginase has been secreted (46) or liberated unspecifically during cell death of myeloid cells (47). As ornithine is a cationic amino acid and substrate of cationic amino acid transporters, it (i) competitively inhibits arginine uptake and (ii) can be exchanged for intracellular arginine via CAT transporter proteins (31), potentially leading to a cellular depletion of arginine.

ARGININE CATABOLISM: A BIPOLAR WORLD IN MACROPHAGES

In mammalian cells, arginine can be catabolized by four classes of enzymes (Figure 1): NOS, arginase, arginine decarboxylase (ADC), and arginine:glycine amidinotransferase (AGAT) (8). Although the enzymes are of course regulated and expressed in a cell-type-specific manner, the metabolism of arginine is

potentially complex since its downstream metabolites encompass NO, urea, ornithine, citrulline, creatine, agmatine, glutamate, proline, and the family of polyamines (7, 8). In macrophages, arginine is a precursor for mainly two important metabolic pathways: it is metabolized either by inducible nitric oxide synthase (iNOS) to NO and citrulline or it is hydrolyzed by arginase to ornithine and urea (Figure 2). This fundamental dichotomy of macrophage arginine metabolism has wide ranging implications for their function as well as for the type and outcome of immune responses in which these innate immune cells are involved in. Before these consequences are discussed (see below), let us first look at the two important distinct arginine enzymatic pathways in macrophages in more detail.

NITRIC OXIDE SYNTHASE: ARGININE – NITRIC OXIDE PATHWAY

In 1980, Furchtgott and co-workers discovered (i) the necessity of an intact endothelium for relaxation of isolated blood vessels and (ii) the presence of an endothelial cell-secreted unknown soluble relaxing factor (EDRF) (48). In 1987, this factor was found to be identical with NO (11) and arginine was revealed as the precursor for NO synthesis in cardiovascular physiology (15). In parallel studies, NO_2^- and NO_3^- were measured as end products of a novel oxidation pathway expressed in macrophages upon stimulation with LPS (49) or IFN- γ (50). Arginine was recognized as the biological precursor molecule of nitrite/nitrate released from activated macrophages (10). Further studies demonstrated that NO is an intermediate of macrophage arginine oxidation to the final end products nitrite/nitrate (14) and that NO synthesis is required for macrophage cytotoxic activity (22). In 1991, the enzyme converting arginine to NO was purified, cloned, and was named NOS (now known as neuronal NOS, nNOS) (51). Shortly after this, two additional NOS isoforms were cloned: iNOS from macrophages (52, 53) and endothelial NOS (eNOS) (54).

The three NOS isoforms differ in structure, distribution, regulation, and synthetic capacity, but they catalyze the same reaction: the incorporation of molecular oxygen and the release of NO from the terminal guanidino nitrogen group of arginine and generation of citrulline as a byproduct (22). All NOS enzymes are large homodimeric proteins with two functional domains: (i) an N-terminal oxygenase and catalytic domain, which binds an iron–protoporphyrin IX (heme) prosthetic group and the cofactor tetrahydrobiopterin (BH₄) and (ii) a C-terminal reductase domain with binding sites for flavin mononucleotide (FMN) and flavin-adenine dinucleotide (FAD). The catalytic reaction involves two monooxygenation steps: (i) arginine is hydroxylated by O₂ and NADPH to form N^ω-hydroxy-L-arginine, which is then (ii) oxidized to yield NO and citrulline. All three NOS isoforms can also synthesize superoxide in the absence of arginine and BH₄. This NOS-derived superoxide can react with NO to form peroxynitrite. Both nNOS and eNOS are constitutively expressed enzymes and calcium-dependent in their activity. In contrast, iNOS is regulated via inducible transcription and synthesizes NO independent of calcium since the essential subunit calmodulin is bound to iNOS even at low intracellular calcium concentrations. Most prominently known as microbicidal and inflammatory effector pathway in macrophages (22), iNOS activity has also been demonstrated in a variety of other cell types, e.g., hepatocytes (55), pulmonary

epithelium (56), and colon epithelium (57). A variety of pro-inflammatory cytokines (e.g., IL-1 β , IFN- γ , or TNF- α), microbial products (e.g., LPS), and hypoxia can induce macrophage iNOS transcription, whereas other cytokines (e.g., IL-4, IL-10, TGF- β) suppress iNOS gene transcription (58). Additive or synergistic activities of combinations of multiple cytokines are most efficient in inducing or suppressing iNOS gene expression. NO synthesis can also be limited by arginine availability and/or on the level of iNOS protein expression (59). NOS can further be inhibited by endogenously produced asymmetric dimethylarginine (ADMA) or pharmacologically by synthetic arginine analogs with substitutions at the terminal guanidino group, e.g., N $^{\omega}$ -monomethyl-L-arginine (L-NMMA), N $^{\omega}$ -nitro-L-arginine (L-NNA), or N $^{\omega}$ -nitro-L-arginine methyl ester (L-NAME). Once iNOS has been translated, a prolonged production of potentially large amounts of NO is detectable. NO can act via stimulation of soluble guanylate cyclase to generate cyclic GMP within the target cell. Besides its physiological role as guanylate cyclase stimulator, NO is also a radical with a very short half-life of approximately 3–5 s and it reacts with a variety of molecules leading to (i) further reactive nitrogen species (RNS) like N₂O₃, peroxynitrite (ONOO $^-$), or nitronium ion (NO $_2^+$) in the presence of oxygen radicals (60) and (ii) nitrosylated proteins with potentially altered or impaired function.

ARGINASE: ARGININE – ORNITHINE PATHWAY

The enzyme arginase drives the second or alternative pathway of arginine metabolism in macrophages, catalyzing the hydrolysis of arginine to ornithine and urea. While arginase was known as an enzyme of the hepatic urea cycle since the discovery of the latter in 1932 by Krebs and Henseleit, it is also expressed in many non-hepatic cells. There are two isoforms of arginase (arginase I and arginase II), which catalyze the same biochemical reaction but differ in cellular expression, cell-type-specific regulation, and subcellular localization (17, 61). Hepatic urea cycle arginase I is expressed as a cytosolic enzyme, while human granulocyte arginase I is found in the granular compartment (41) and arginase II is a mitochondrial enzyme (61). It was initially demonstrated that murine macrophage arginase is inducible by PGE₂ (62), Th2 cytokines, and cAMP, both alone (62) and synergistically acting together (63). The molecular details of this gene regulation were then clarified: the transcription factors STAT-6 and CEBP β assemble at an enhancer element 3 kb upstream from the basal promoter of arginase I and Th2 cytokine-mediated murine arginase I mRNA induction is controlled by this mechanism (64). Meanwhile, human macrophage arginase I expression was demonstrated by synergistic induction with cAMP increasing treatments (PDE4 inhibition) in combination with IL-4 or TGF- β (65). The molecular details of this induction were also clarified recently in the murine RAW264.7 macrophage cell line, involving STAT-6 and CEBP β binding to an IL-4 response element in the arginase I promoter (66). Another layer of complexity comes into play by the demonstration of pathogen-induced toll-like receptor (TLR)-mediated induction of arginase I in murine macrophages (67, 68).

Ornithine serves as a substrate for ornithine decarboxylase (ODC), a rate-limiting enzyme in the synthesis of polyamines. Polyamines are small, polycationic molecules, which regulate a

multitude of cellular processes like DNA replication, protein translation, cell growth, and differentiation (69, 70). Much less is known about an involvement of polyamines in immune reactions: the polyamine spermine, e.g., inhibits pro-inflammatory cytokine synthesis of human, LPS-stimulated PBMC (71), arginine transport (72), and NO synthesis in rat (72) and murine macrophages (73). Ornithine, via the enzyme OAT, is also a precursor amino acid for the synthesis of proline, which itself is essential for the synthesis of collagen. Accordingly, arginase-derived ornithine might be important in tissue (re-)modeling processes. This hypothesis was supported by studies that demonstrated an increase in arginase levels in fibrotic lung disease (74) or allergic asthma (75, 76).

What are the biological functions of arginase-expressing macrophages? While there is ample evidence, at least in the murine system, of the fundamental role of NO-producing macrophages for infection control (22), a multitude of pathophysiological scenarios have also been described in which arginase-expressing macrophages are key players (17). In the initial groundbreaking analysis on macrophage phenotypes during wound healing, Mills and co-workers (77, 78) showed that arginase-expressing and ornithine-producing macrophages were crucial for wound healing as opposed to NO-producing macrophages, which dominated the initial phase of antibacterial inflammation. The same two macrophage phenotypes were then also correlated with tumor growth (arginase/ornithine) or tumor killing (NOS/NO) (79). Based on these dramatic differences in function, the two different macrophage populations were then named as M1 and M2, based on their route of arginine metabolism (Figure 2) (5). In general, M2 type macrophages act as anti-inflammatory cells (“healing” mode) via diversion of arginine away from NOS or via the synthesis of downstream products derived from the ornithine that is generated via arginase (see above). For illustration, we want to list just a few, more recently published examples for the role of macrophage or, in this context, also DC arginase in (i) infection-induced inflammation, (ii) immune evasion in tumor and infection, (iii) regulation of fibrosis, and (iv) direct control of parasite growth. (i) The anti-inflammatory property of macrophage arginase during infection was shown in murine schistosomiasis where excessive tissue injury is prevented by arginase-expressing macrophages due to suppression of pro-inflammatory cytokines IL-12 and IL-23 and the maintenance of the Treg/Th17 balance (80). (ii) IL-6-induced arginase I in DCs leads to downmodulation of MHC-II and this is subsequently responsible for suppression of CD4 $^+$ T cell-mediated antitumoral immunity (81). Immune evasion of chronic *Helicobacter pylori* infection is mediated by arginase II induction in gastric macrophages, due to inhibition of NOS-mediated bacterial killing and suppression of pro-inflammatory cytokine production (82). (iii) Macrophage arginase I restricts Th2 cytokine driven inflammation and fibrosis in murine schistosomiasis (83) although macrophage arginase itself can be profibrogenic via direct production of proline as collagen precursor in schistosomiasis (84). (iv) Macrophage arginase-mediated synthesis of polyamines enhances growth of intracellular *Leishmania* parasites in murine macrophages (85) and arginase-expressing granulocytes and the levels of arginase activities are markers of disease severity in human visceral leishmaniasis and in HIV infections (86–88).

One further important consequence of macrophage arginase expression is reduction of extracellular arginine. This is most likely more apparent in the immediate microenvironment of M2 macrophages due to the continuous flux of nutrients and the arginine synthetic capacity of the whole organism. Suppression of T cell activation via arginine depletion has been studied quite extensively *in vitro* (47, 89–91) and is known as one of the prime immunosuppressive mechanisms of arginase-expressing myeloid-derived suppressor cells (MDSC) (18). In contrast, its role in macrophage-driven pathophysiology *in vivo* is still not really clear in most relevant disease entities and needs to be analyzed in the future. We speculate that M2/arginase macrophages might be more efficient in the induction of extracellular arginine depletion since there is no intracellular reconstitutive mechanism for arginine recycling for M2 macrophages as opposed to M1 macrophages, which can use the citrulline–NO rescue pathway. Suppression of T cell activation, proliferation, and/or differentiation by macrophage arginase I was shown *in vivo* in murine disease models dominated by M2 macrophages, like schistosomiasis (80, 83) or leishmaniasis (92). Interestingly, extracellular arginine depletion has also been shown to inhibit ERK1/2 activation and subsequently pro-inflammatory cytokine production in LPS-stimulated macrophages (93). It will be interesting to study a potential influence of intracellular arginine depletion on potential pro-inflammatory signaling pathways within macrophages and to analyze if there is regulation of innate immune responses and macrophage polarization already at such basic level. Clearly, arginine depletion does not inhibit immune responses broadly and indiscriminately: important activation aspects of T cells (91, 94) and granulocytes (95) are preserved in an arginine-depleted milieu and other cellular responses, e.g., induction of arginine transport protein CAT-1 (33), are even enhanced in eukaryotic cells under arginine nutrient deprivation.

INTERACTIONS BETWEEN NOS AND ARGINASE PATHWAYS

Nitric oxide synthase and arginase can both antagonize or synergize in the generation of oxidative and nitrosative stress: inadequate supply of arginine (or the cofactor tetrahydrobiopterin) leads to the production of superoxide (O_2^-) instead of NO, increasing oxidative stress and also the production of peroxynitrite (22). In general, though, mutually exclusive expression of iNOS and arginase I in individual macrophages prevails (96) and there are multiple cross-inhibitory interactions between the two arginine metabolic pathways in macrophages: NOHA, the intermediate product in NO synthesis, is a potent inhibitor of both arginase isoforms (97). In non-macrophage cell types, it was also demonstrated that NO is an effective inhibitor of ODC via nitrosylation of the enzyme with consecutively reduced polyamine synthesis (98). Arginase can limit arginine availability for NO synthesis, as demonstrated by pharmacological arginase inhibition in different types of macrophages (99). The expression of iNOS is translationally controlled by the availability of arginine (100) and in murine M2 macrophages, induced by the cytokine IL-13, iNOS translation, and NO production are restricted by arginase-mediated arginine depletion (59). Polyamines and/or polyamine aldehyde metabolites can inhibit NO synthesis in murine and rat macrophages (101). Spermine suppresses the induction of both iNOS and

CAT-2B arginine transporter (72) and inhibition of ODC-mediated polyamine synthesis in murine macrophages enhances LPS-induced iNOS expression and NO synthesis (102). Spermine also inhibits *H. pylori*-induced iNOS protein translation in the RAW264.7 macrophage cell line and siRNA-mediated ODC inhibition enhances macrophage NO-mediated bacterial killing (103).

In summary, these biochemical crossregulatory interactions are in line with the concept of two types of polarized macrophages – M1/NOS versus M2/arginase – which are defined not only by the intracellular fate of arginine, but – most importantly – also by its associated functional consequences.

M1/NOS VERSUS M2/ARGINASE MACROPHAGES: NOVEL ASPECTS

The earlier simplified scheme of “proinflammation = M1/Th1” versus “anti-inflammation = M2/Th2” has meanwhile been clarified as one possible scenario of a more broader conceptual framework: M1 versus M2 polarization clearly leads to opposing outcomes of inflammatory reactions, but depending on the inflammatory or infectious context, M1 and M2 macrophages can be central players of both pro- or anti-inflammatory reactions. Notably, M1/M2 macrophage polarization can be driven by microbial infection or innate danger signals without any influence of adaptive immune cells, secondarily driving the Th1/Th2 polarization of the evolving adaptive immune response (104). Microbial stimulation of macrophages via TLRs leads to the activation of certain transcription factors (e.g., NF-κB, AP-1), which upregulate pro-inflammatory cytokines like IFN-γ and TNF-α leading to M1 macrophage polarization with high iNOS expression whereas cytokines like IL-4 or IL-13 lead to STAT6 phosphorylation with consecutive arginase expression and varying further aspects of M2 polarization (mannose receptor, Ym1, Fizz1).

Various novel aspects regarding the evolution of the M1/M2 macrophage polarization in light of the differential expression of NOS versus arginase have been clarified recently and some examples are summarized in the following sections:

NOVEL EXOGENOUS ENVIRONMENTAL FACTORS INFLUENCING THE NOS/ARGINASE BALANCE

Tumor cells are known to metabolize glucose preferentially via aerobic glycolysis, known as “Warburg phenomenon,” and this leads to high concentrations of lactate in the tumor microenvironment. This tumor cell-derived lactic acid is a potent inducer of arginase I expression in tumor-associated macrophages (TAM) and these M2/arginase macrophages then foster tumor growth (105). Since activated T cells also use aerobic glycolysis with consecutive production of lactic acid, it will be interesting to study if the same mechanism of arginase I induction is also operative in macrophage-T cell interactions. Gliadin, a major component of cereal gluten, and therefore omnipresent in our daily food, was shown to induce arginase I in human monocytes (106). A parallel stimulation with IFN-γ leads to a reduction of cellular arginine efflux via downregulation of the arginine export protein γ^+ LAT-2 (SLC7A6), thereby increasing intracellular availability of arginine for gliadin-induced arginase (106). Finally, growth factors have also entered the M1/M2 macrophage world: in a hamster model of visceral leishmaniasis, macrophage arginase I expression

is driven by fibroblast growth factor-2 (FGF-2) and insulin-like growth factor-1 (IGF-1), which both signal via STAT6 and are amplified by co-stimulation with IL-4 (107).

INTRACELLULAR SIGNALING MODULES INVOLVED IN M1(NOS)/M2(ARGINASE) POLARIZATION

The tyrosine phosphatase Shp2 restricts M2 macrophage polarization as demonstrated by the preferential polarization of Shp2^{-/-} macrophages into an M2 direction with enhanced arginase expression, associated with a better protection against schistosomiasis (108). Also, PI3K/PTEN activity is involved in regulating arginase expression in murine macrophages since deletion of PTEN leads to M2 polarization via C/EBP β and STAT3 (109). These results are in line with earlier reports demonstrating that SHIP phosphatase (which – like PTEN – also downregulates the PI3K pathway) dampens M2 polarization and arginase I expression in different types of tissue macrophages (110). Deacetylation of C/EBP β is required for its binding to a DNA enhancer element and its role in IL-4-mediated arginase I induction in bone marrow-derived murine macrophages (111).

AUTO- OR PARACRINE M1/M2 POLARIZATION

In murine macrophages, induction of arginase I by mycobacteria is driven by an autocrine–paracrine signaling loop: TLR-MyD88 mediate induction of the cytokines IL-6, G-CSF, and IL-10, which then induce arginase I, involving the transcription factors STAT3 and C/EBP β (112). Respiratory syncytial virus (RSV) infection induces production of IL-4 and IL-13 of macrophages themselves and this leads to auto- and paracrine induction of arginase I expression in macrophages (68). It has long been known that murine DCs share the fundamental arginase/iNOS polarization with macrophages (113). A novel aspect here is that DCs produce RA, which then induces arginase I and the arginine transporter CAT-2B in the DCs themselves (34). This RA-mediated autocrine–paracrine induction of arginase I in DCs is induced by binding of RA to an RA-responsive element in the 5' non-coding region of the arginase I gene and is enhanced by known exogenous arginase inducers like IL-4 or GM-CSF (34).

M1/M2 POLARIZATION IN VIVO

In human tuberculosis, the distribution of M1 (iNOS) and M2 (arginase) macrophages is spatially organized within granulomas: M1 macrophages can preferentially be found in the inner region closer to viable mycobacteria, whereas a higher frequency of M2 macrophages is detectable on the outer “healing” margins. This clearly forms an organized microenvironment in which antibacterial (M1) responses are physically separated by M2-based anti-inflammation and fibrosis from uninvolved tissue (96). In a murine tuberculosis model, overexpression of IL-13 precipitates expansion of the M2 arginase-expressing macrophage response to the pathogens recapitulating human pathology of post-primary tuberculosis, while the endogenous inhibition of arginase I expression via NOHA restrains arginase expression and pathology (114). This is in line with an earlier study showing a disease-exacerbating role of macrophage arginase (67). In contrast to the latter two reports, a recently published study analyzed the role of macrophage arginase in a hypoxic model

of *Mycobacterium tuberculosis* granuloma formation, in which iNOS-based synthesis of RNS is impaired and which likely reflects *in vivo* reality. Here, it was shown that arginase I expression in granuloma-associated macrophages restricts immune pathology since macrophage-specific deletion of arginase I led to larger granulomas and bacterial burden load (115). These discrepant study results clearly demonstrate the fundamental importance of the microenvironment and the multitude of potential factors that act on macrophages *in vivo* and which can simply not be mimicked *in vitro*.

SUMMARY AND OUTLOOK

The concept of macrophage M1/M2 dichotomy, based on differential usage of arginine via NOS or arginase was born 25 years ago based on analyses of healing wounds (5, 104). Since then, we have witnessed a bewildering explosion of knowledge regarding macrophage surface markers, activation requirements, signal transduction elements and gene regulation. Within this universe of complexity the simple discriminator arginase versus NOS expression has not only remained valid to explain how a mammalian organism deals with wounding (which is important enough in itself) but also has demonstrated enormous power to better explain and understand such diverse problems as cancer control versus cancer-induced immunosuppression, autoimmune pathology versus preservation of tolerance to self, infection control versus chronicity or death due to infection as well as tissue healing versus exaggerated fibrosis. Despite this scientific progress during the last quarter-century, we have by far not reached the summit of the Everest, but rather a solid base camp I, in which to plan and prepare the next steps. The arginase/iNOS dichotomy of macrophage amino acid metabolism has counterparts in the other major components of cellular metabolism: (a) genes of fatty acid oxidation are preferentially expressed in M2 macrophages and inhibition of fatty acid oxidation leads to an abrogation of M2 activation (116); (b) M1 macrophages preferentially use glycolysis and glutamine anaplerosis while M2 macrophages preferentially use oxidative metabolism (117). The emerging interconnections between macrophage metabolism and M1/M2 polarization (118) have recently been reviewed (119, 120) and we anticipate exciting progress in this field in the upcoming years. Another crucial aspect of the M1/M2 dichotomy that eagerly awaits more progress and clarification is the current discrepancy between murine and human macrophage biology in terms of iNOS/arginase expression and regulation (17, 42, 121). We definitely need more carefully executed *in vivo* and *ex vivo* analyses of human macrophage activation phenotypes in diverse disease settings. This will then also lay the foundation for targeted therapeutic intervention to harness the enormous power of the arginine metabolic phenotypes of M1/M2 macrophage polarization.

ACKNOWLEDGMENTS

Supported by the Deutsche Forschungsgemeinschaft (MU 1547/4-1 to Markus Munder and Cl100/6-1 to Ellen I. Closs).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 24 September 2014; **paper pending published:** 07 October 2014; **accepted:** 09 October 2014; **published online:** 27 October 2014.

Citation: Rath M, Müller I, Kropf P, Closs EI and Munder M (2014) Metabolism via arginase or nitric oxide synthase: two competing arginine pathways in macrophages. *Front. Immunol.* **5**:532. doi: 10.3389/fimmu.2014.00532

This article was submitted to Inflammation, a section of the journal *Frontiers in Immunology*.

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Evolution of innate immunity: clues from invertebrates via fish to mammals

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Host responses against invading pathogens are basic physiological reactions of all living organisms. Since the appearance of the first eukaryotic cells, a series of defense mechanisms have evolved in order to secure cellular integrity, homeostasis, and survival of the host. Invertebrates, ranging from protozoans to metazoans, possess cellular receptors, which bind to foreign elements and differentiate self from non-self. This ability is in multicellular animals associated with presence of phagocytes, bearing different names (amebocytes, hemocytes, coelomocytes) in various groups including animal sponges, worms, cnidarians, mollusks, crustaceans, chelicerates, insects, and echinoderms (sea stars and urchins). Basically, these cells have a macrophage-like appearance and function and the repair and/or fight functions associated with these cells are prominent even at the earliest evolutionary stage. The cells possess pathogen recognition receptors recognizing pathogen-associated molecular patterns, which are well-conserved molecular structures expressed by various pathogens (virus, bacteria, fungi, protozoans, helminths). Scavenger receptors, Toll-like receptors, and Nod-like receptors (NLRs) are prominent representatives within this group of host receptors. Following receptor-ligand binding, signal transduction initiates a complex cascade of cellular reactions, which lead to production of one or more of a wide array of effector molecules. Cytokines take part in this orchestration of responses even in lower invertebrates, which eventually may result in elimination or inactivation of the intruder. Important innate effector molecules are oxygen and nitrogen species, antimicrobial peptides, lectins, fibrinogen-related peptides, leucine rich repeats (LRRs), pentraxins, and complement-related proteins. Echinoderms represent the most developed invertebrates and the bridge leading to the primitive chordates, cephalochordates, and urochordates, in which many autologous genes and functions from their ancestors can be found. They exhibit numerous variants of innate recognition and effector molecules, which allow fast and innate responses toward diverse pathogens despite lack of adaptive responses. The primitive vertebrates (agnathans also termed jawless fish) were the first to supplement innate responses with adaptive elements. Thus hagfish and lampreys use LRRs as variable lymphocyte receptors, whereas higher vertebrates [cartilaginous and bony fishes (jawed fish), amphibians, reptiles, birds, and mammals] developed the major histocompatibility complex, T-cell receptors, and B-cell receptors (immunoglobulins) as additional adaptive weaponry to assist innate responses. Extensive cytokine networks are recognized in fish, but related signal molecules can be traced among invertebrates. The high specificity, antibody maturation, immunological memory, and secondary responses of adaptive immunity were so successful that it allowed higher vertebrates to reduce the number of variants of the innate molecules originating from both invertebrates and lower vertebrates. Nonetheless, vertebrates combine the two arms in an intricate inter-dependent network. Organisms at all developmental stages have, in order to survive, applied available genes and functions of which some may have been lost or may have changed function through evolution. The molecular mechanisms involved in evolution of immune molecules, might apart from simple base substitutions be as diverse as gene duplication, deletions, alternative splicing, gene recombination, domain shuffling, retrotransposition, and gene conversion. Further, variable regulation of gene expression may have played a role.

Keywords: evolution, immunity, innate immunity, adaptive immunity, invertebrates, vertebrates

INTRODUCTION

Host responses against invading pathogens are basic physiological reactions of all living organisms. Even prokaryotes protect themselves by use of restriction enzymes and clustered regularly interspaced palindromic repeats (CRISPRs), being able to degrade invading foreign pathogens (1). Since the appearance of the first eukaryotic cells, a series of additional defense mechanisms have evolved in order to secure cellular integrity, homeostasis, and survival of the host. Unicellular amebae developed the ability to phagocytose foreign material as a part of their food uptake mechanisms (2) and this basic phagocyte function is conserved in higher invertebrates and vertebrates in which the immunological function is more evident. Discrimination between self and non-self is also crucial for sexual functions securing genetic variation by exchange of genes between members of the same species. Recognition of non-self in both unicellular and multicellular organisms is based on cellular receptors allowing the host organism to bind, engulf, and/or kill potential invaders and offenders (3). Among the invertebrates, important groups such as protozoans (amebae, flagellates, and ciliates), sponges (such as bath sponges), cnidarians (e.g., jellyfish), worms (e.g., platyhelminths, annelids, and nematodes), mollusks (snails and bivalves), crustaceans (e.g., crabs and prawns), chelicerates (spiders, mites), insects (e.g., flies), and echinoderms (sea stars and sea urchins), are known to possess cells with receptors, which bind to foreign elements and allow differentiation of self and non-self (4). This ability is associated with presence of phagocytes bearing different names in various groups (amebocytes, hemocytes, coelomocytes, granulocytes, monocytes, macrophages), but basically they have a macrophage-like appearance and have, to a certain extent, comparable functions (5–7). Chordate evolution was based on the usage of existing genomes from ancestors and although deletions of significant parts of these have occurred, it is possible to trace some main lines from early and primitive organisms to highly developed mammals. The most primitive chordates comprising acranians (*Amphioxus*) (8–10) and tunicates (ascidians) (11) display a wide array of innate immune functions. In the primitive vertebrates comprising jawless fish (agnathans such as hagfish and lampreys), these functions became combined with an extensive use of leucine rich repeats (LRRs) as lymphocyte receptors (12, 13). With the advent of cartilaginous and bony fish, the adaptive armament [major histocompatibility complex (MHC), immunoglobulins, T-cell receptors, extensive cytokine networks] appeared, and these new tools were further developed to a high level of sophistication through amphibians, reptiles, and birds to mammals (14). This allowed a reduction of the copy number of many innate immune genes, but still the innate effector molecules have been taken into a complex network combining the obvious talents of fast acting ancient molecules with the highly developed specific recognition with memory seen in adaptive immunity. The main outlines of these aspects, which are presented below, highlight how innate immune responses evolved from ancient precursors and still play a vital and basic role even in higher vertebrates where adaptive elements are so prominent.

THE TIME SCALE – IN BRIEF

Evolution of the animal immune system, in its broadest sense, can be viewed over a time span of at least 1000 million years (Figure 1).

The age of the Earth has been estimated to more than 4.6 billion years, but the first traces of life appeared later with the appearance of primitive prokaryotes. The initial relatively inactive period is called the Precambrian period (or Proterozoic era), and it exhibited a series of primitive single celled organisms, which could exist in colonies, toward its end (one billion years ago). However, even these primitive organisms may have developed defense mechanisms to preserve their integrity. The environmental conditions prevailing then and at later stages during the Earth's life may have placed a strong selective pressure on the organisms. Major extinctions of existing organisms (seen several times during evolution) may be due to harsh environmental and physiochemical changes, which probably have played an active role in creation of mutations, gene and chromosome deletions, duplications, and gene shuffling. The Paleozoic era spanning the period 542–240 million years ago (mya) was initiated by a new period called Cambrian 542 mya. At this stage, more complex organisms such as cnidarians (including jellyfish) were prevalent but an impressive diversification, called the Cambrian explosion or radiation, was put in action, which resulted in appearance of some major animal groups. Then over a relatively short time span, the ancestors of both invertebrates and vertebrates known today appeared. The diversification of all the multicellular animals continued. During the following, millions of years called the Ordovician, Silurian, and Devonian periods more advanced invertebrates (echinoderms), chordates (ascidians and acranians), and vertebrates (jawless and jawed vertebrates) came into play. Thus, in this last period, jawed fishes (and thereby the adaptive immune system) were seen for the first time around 450 mya and they were soon followed by amphibians. In the Carboniferous period (from around 350 mya), the reptiles appeared and diversified in the Permian period (from about 300 mya). By the end of this period, a major extinction affecting parts of all animal groups occurred probably due to some major climate changes. With the advent of the Mesozoic era initiated with the Triassic period (250–200 mya), the first dinosaurs and mammals were seen. In the Jurassic period 200–140 mya, dinosaurs radiated and birds appeared as one lineage in this group. In the following Cretaceous period (140–65 mya), the first primates developed, but again a major extinction process occurred, which primarily known as the end of the dinosaur time span. This event was followed by the Cenozoic era including the Paleogene and Neogene periods where further mammalian diversification took place and finally, in the Quaternary period, humans arose around 60,000–120,000 years ago. When dealing with innate immune mechanisms, it is thus likely that some genes involved in the defense of the early invertebrate ancestors 5–600 mya are still playing a role in the innate and even adaptive immune reactions of mammals. As will be suggested from the report below, invertebrate genes (immune-related or not) may have been used as bricks directly or modified for later and alternative use when appropriate.

DISCRIMINATION OF SELF FROM NON-SELF

Even the most primitive unicellular organism needs to discriminate self from non-self. This applies for a basic nutrition and feeding process in which the ameba or flagellate select food items and subsequently exert phagocytosis or pinocytosis. In addition, genetic exchange and sexual reproduction is dependent on this type of discrimination. It may have arisen several times during

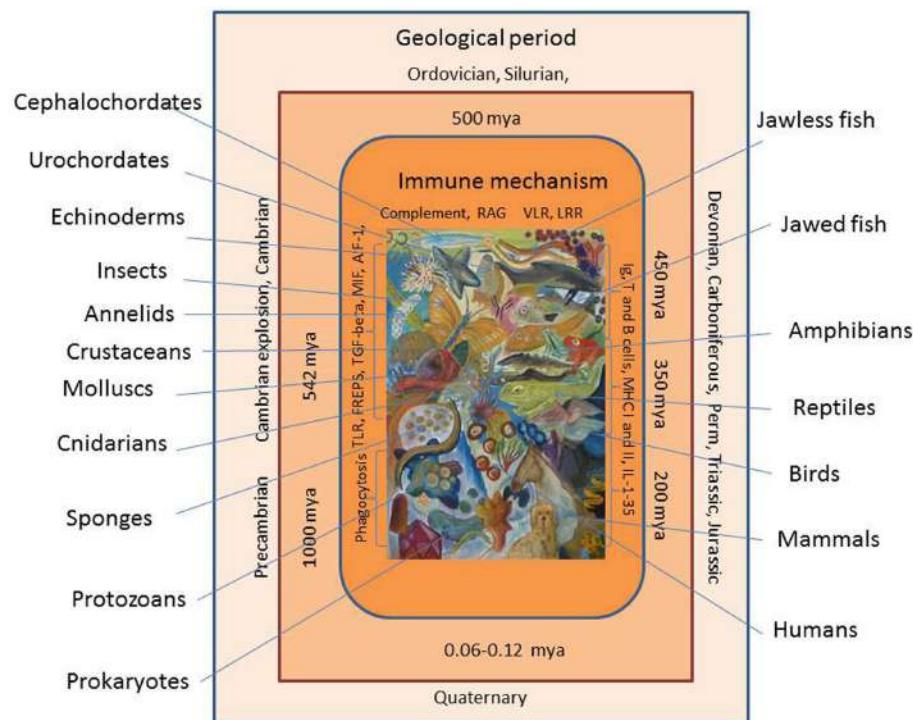


FIGURE 1 | Evolution of the immune system is shown. Immune cells and molecules from early invertebrates to vertebrates are shown. Geological periods and time periods (million years ago, mya) are shown with extant representatives of animal groups appearing at different time during evolution.

evolution but genetic evidence points to a conservation of several genes encoding molecules active in cell to cell interaction. The primitive cnidarian *Hydractinia* has at least two innate histocompatibility loci alr1 and alr2 (15). Allorecognition and rejection has been well studied for the colonial tunicate *Botryllus schlosseri* applying a locus called FuHC (fusion/histocompatibility) associated with putative receptor proteins named fester and Uncle fester, which are very polymorphic (16) and it was recently reported that a polymorphic HSP40-like protein is encoded within the FuHC locus (17). The MHC, a central element in adaptive responses, is well established in fish but its origin in invertebrates is still enigmatic. A common ancestral region traced in the early chordates (urochordates and cephalochordates) is referred to as the proto-MHC. It is likely to be the first building block for the MHC, which probably was established later in evolution by the process of chromosome duplications (18).

EFFECTOR CELLS

The basic phagocytic ability of unicellular organisms (e.g., amoebae) is also found in the most primitive multicellular animals belonging to the group Porifera (sponges) and cnidarians (the group including jellyfish and sea-anemones). These animals apply phagocytic amoebocytes for nutrition and recognition of foreign elements in the environment. Similar cell types have been conserved through evolution as they are recognized in all groups from invertebrates (annelids, arthropods, mollusks, echinoderms) to vertebrates (4). Several terms have been assigned to these cells in various groups and it must be expected that future investigations

will sub-divide groups further. Sponges carry amoebocytes in their mesoglea, cnidarians possess interstitial cells with a phagocytic function, hemocytes are found in the vascular system, and coelomocytes occur in coelomate animals. Thus, earthworms possess several subtypes of coelomocytes including eleocytes, and granular amoebocytes (5) and in arthropods, comprising both crustaceans and insects, several effector cell types have been characterized (19). The evolutionary importance of corresponding phagocytes/macrophages is reflected in the range of subsets described from invertebrates and primitive chordates. Various cell types within this theme are found in advanced invertebrates (represented by echinoderms such as sea stars and sea urchins) and in the cephalochordate *Branchiostoma* (*Amphioxus*) and in urochordates (tunicates, ascidians) where both granulocyte-like cells and macrophages occur (20, 21). An even more diverse array of cell types and subsets occur in jawless vertebrates (hagfish and lampreys), cartilaginous fish (sharks and rays), and in bony fish. Besides phagocytes, jawless fish possess different subsets of lymphocytes with special membrane receptors. These primitive vertebrates without jaws have evolved an alternative antigen recognition system, which are composed of LRRs. These molecules provide agnathans a basis for establishing various lymphocyte lines corresponding to B and T lymphocytes. However, in cartilaginous and bony fish, the lymphocyte receptors are immunoglobulin (B-cell receptors) or T-cell receptors whereas agnathans apply at least two forms of variable lymphocyte receptor (VLR) based on LRR (13).

In bony fish, the cellular armament might include lymphocytes, macrophages, monocytes, dendritic cells, neutrophils, granulocytes,

eosinophils, basophils, mast cells, and NK-cells and an even higher specialization is known in mammals (6, 7, 22). Leukocytes have traditionally been divided into the myeloid and lymphoid line based on their development from certain stem cells. However, B-lymphocytes in rainbow trout have been shown to exert phagocytosis (23), which suggests that the border between these developmental cell lines is less rigid at least in fish. In this context, it is interesting that the Ikaros multigene family, which take part in vertebrate hemopoietic stem cell differentiation and production of B, T and NK cell lineages, seems to find an early version in the most primitive vertebrates (the agnathan hagfish *Myxine*) and the even earlier urochordates (the tunicate *Oikopleura*) (24). The ancient origin of genes, which are central in cellular adaptive immunity in higher vertebrates, is also reflected by the finding of a Nuclear Factor of Activated T-cells (NFAT)-like gene in the primitive chordate *Branchiostoma belcheri* (*Amphioxus* group). In this chordate, this gene seems to play a role in innate recognition of lipopolysaccharide (LPS).

RECEPTORS

In order to respond to non-self and potential pathogens and initiate phagocytosis or production of killing mechanisms, the phagocytic cells must possess receptors, which can bind relevant ligands. The primitive multicellular sponges possess LPS binding receptors, which can interact with structural polysaccharides (beta-glucan) from fungi (25). This group has also been reported to express intracellular receptors nucleotide-binding domain and LRR (NLR) (26) (also termed the nucleotide-binding oligomerization domain receptors, Nod-like receptors), which bind bacterial or viral RNA, flagellin, and peptidoglycan leaving the host cell with an ability to fight pathogens or pathogen-related molecules, which have managed to enter the cytosol (26). RIG-like receptors (RLR) are able to bind viral RNA and establish innate defense reactions and their ancestral form seems to occur shortly before the first vertebrates evolved (27). These are all examples of pathogen-recognition receptors (PRRs) recognizing pathogen-associated molecular patterns (PAMPs), which are well-conserved molecular structures expressed by various pathogens (virus, bacteria, fungi, protozoans, helminths). PAMPs may among others be LPS, peptidoglycans, flagellin, double-strand RNA (dsRNA), and structural carbohydrates. The term damage-associated molecular patterns (DAMPs) are being used to signify the danger reflected by presence of cell constituents released to the extracellular milieu following tissue injury. Toll-like receptors (TLRs) play a major role within this group of host receptors. They are composed of an extracellular domain bearing LRRs and a cytoplasmic domain (interleukin-1 receptor like). Following receptor-ligand binding, signal transduction initiates a complex cascade of reactions, which leads to production of one or more of a wide array of effector molecules eventually resulting in elimination or inactivation of the intruder. A large number of TLRs are known with individual affinities to various PAMPs (28). TLRs have been traced to the most ancient multicellular invertebrates such as sponges, cnidarians (29), oligochaetes (earthworms) (30), mollusks (snails and mussels) (31), crustaceans (e.g., shrimps), and insects (32). The echinoderms, representing the most developed invertebrates, exhibit a complex and rich array of innate recognition molecules

where among TLRs are present in numerous copies (33). The most primitive fish, the agnathans, have at least 7 identified TLRs, bony fish at least 18, amphibians 14, birds 10, and mammals 13 (28). One major receptor group comprises the scavenger receptors binding bacteria and a range of antigens including lipoproteins, which are polyanionic (34). They are ancient receptors occurring on most cells in sponges, the most primitive multicellular animals. They have a cysteine rich domain (SRCR), which can be traced through insects, echinoderms, early chordates, and fish (35).

EFFECTOR MOLECULES

Invertebrates exhibit a rich variation of innate immune molecules allowing recognition, pathogen binding, and pathogen killing (16). Sponges apply oxidative killing processes based on production of reactive O- (ROS) and N-(NOS) species. Gastropods (snails) exemplified by *Biomphalaria glabrata* are able to produce ROS when exposed to one or more carbohydrate ligands (36) and NOS when infected by sporocysts of the digenetic trematode *Schistosoma mansoni* (37). It is not clear if these animals possess preformed molecules (or enzymes), which are released immediately upon stimulation in order to exert their function instantly. Agglutination, clotting, and coagulation are other effective methods used to inactivate, and combat intruders and mollusks apply fibrinogen-related peptides (FREPS) as central players in the process. Melanization is another innate response mechanism in which pathogens are encapsulated and inactivated by reaction products including cytotoxic quinones and reactive O- and N-species. Melanin itself may protect against light and ionization and the prophenoloxidase system is an enzyme complex associated with these reactions (3). Many similar mechanisms have been extensively studied in fish in which inducible NO synthase is readily expressed following parasite infection (38). Other innate factors produced by fish include antimicrobial peptides (AMP), lysozyme, hemolysins, transferrins, lectins (MBL), SAA, SAP, CRP, and complement factors (39). The complement system, which is linking innate and adaptive responses in vertebrates, can be traced even in primitive invertebrates such as cnidarians (40) but exhibit the most complex cascade reactions in vertebrates. The function and interactions between the individual complement factors in lower chordates and invertebrates are unexplored and probably differ from the cascade reactions known from higher vertebrates (41). With the advent of cartilaginous and bony fishes, the adaptive immune system found its basic form including the ability to produce various classes of functional immunoglobulins. Although immunoglobulin-like sequences have been found in invertebrates, the high specificity and re-arrangement of V, D, and J domains associated with antibodies was first seen in these fish groups. The recombination activating genes RAG1 and RAG2 (RAGs) play a central role in this process and it is noteworthy that RAG-like sequence genes have been recognized in the early chordate *Amphioxus* (10). This adds to the notion that some immune-related genes in invertebrates and early chordates have had other functions before the adaptive immune system evolved.

SIGNAL MOLECULES

Coordination of cellular processes must be an integrated function even in the most primitive multicellular animals in order to

maintain shape, structure, and function. Orchestration of complex reactions is carried out by various cytokines. Such molecules have been described in primitive invertebrates and although many of these may not be homologous to vertebrate cytokines, several studies have shown effects on the immune reactivity in invertebrates following stimulation with recombinant vertebrate cytokines. Thus, TNF-alpha, IFN- γ , and IL-8, have been demonstrated to induce reactions in worms, mollusks, and insects suggesting that these animals apply interleukin-like signal molecules (42–44). Earthworm coelomocytes responded to recombinant human IL-12 and IFN- γ by increasing phagocytosis (43) and Blue mussel hemocytes responded to TNF- α stimulation by increased stress reaction and decreased phagocytosis (42). Likewise, insect (fruit-fly) cells were stimulated by recombinant human IL-8, which is associated with increase of phagocytic cells and subsequent expression of insect cytokines upd-3 and dhf (44). However, based on the fact that corresponding genes have not yet been described in these invertebrates it must be framed that these results should be observed with some caution.

However, some cytokines have been found encoded in the genome of certain invertebrates. A central regulating molecule is TGF- β , which may secure moderation of inflammation and initiate and sustain repair functions. It belongs to a family with numerous members in mollusks, nematodes, insects, echinoderms, and tunicates. Even the genome of cnidarians represented by the sea anemone *Aiptasia pallida* contains genes encoding TGF- β , and it was demonstrated experimentally that this cytokine depressed immune reactions including nitric oxide production (45). Another central cytokine is the macrophage migration inhibitory factor (MIF), which was released following infection with the digenetic trematode parasite *Schistosoma mansoni* (46). MIF has also been described from the Pacific white shrimp *Litopenaeus vannamei* in which it functions as a prominent pro-inflammatory cytokine, which is up-regulated following viral infections (47) and predominantly expressed in blood cells, heart, and hepatopancreas. The Pacific oyster genome encodes an IL-17 like cytokine, which is highly expressed following injection with pathogenic bacteria (48). The cytokine allograft inflammatory factor-1 (AIF-1) has been described from the same host (49). It was found to stimulate phagocytic activity of oyster granulocytes. Crustaceans such as the freshwater crayfish produce a series of astakine cytokines (50–52), which have impact on hematopoiesis. The Chinese mitten crab produces suppressors of cytokine signaling (SOCS2) in various cells and organs following challenge with pathogenic bacteria (53). Fruitflies produce various cytokines including helical cytokines (44). In more developed invertebrates (echinoderms) (33) and primitive chordates (8), corresponding signal molecules have been described. The LPS-induced TNF- α factor (LITAF) gene was recently detected in *Amphioxus* (8) where it functions not only as a transcription factor for expression of TNF- α but also may be regulating innate responses in general. In lampreys, one of the most primitive vertebrates, a tumor protein homolog has been found to regulate cytokine secretion from various leukocytes (54). Our knowledge within cytokine evolution has recently been expanded particularly with regard to fish. Thus, IL-1, IL-2, IL4/13, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, IL-21, IL-22, IL-25, and IL-35 have been recognized in bony fish (55) and corresponding

arrays might be expected to occur in cartilaginous fish (sharks and rays) (22). It should be framed that although sequence similarities suggest that lines of development from primitive animals (invertebrates and chordates) to higher vertebrates exist, one should be open for change of function of gene products during evolution. Thus, regulation of cellular communication may apply different cytokines at different stages even for corresponding functions.

EVOLUTION OF MACROPHAGE FUNCTION

Macrophage function in a higher vertebrate host organism may be directed along different pathways characterized as M1 and M2 functions (56). These lines are specialized in “Fight” or “Repair” systems, respectively, related at least partly to cellular use of Arginine. This amino acid can be converted to nitric oxide (NO) by inducible nitric oxide synthase (iNOS) or to Ornithine and Urea by Arginase. The former reaction (previously termed classical activation) makes the macrophage capable of fighting and killing invading microbes by use of the reactive N-species whereas the latter (alternative activation) can be characterized as the repairing pathway. In higher vertebrates, M1 and M2 cells are associated with expression of different cytokine profiles but it cannot be excluded that these two phagocyte functions are regulated in a special way in invertebrates. Evidence has been produced that this division of macrophage function may occur in fish. In rainbow trout putative macrophages (MHCII positive cells) are found widespread in various tissues even in the early yolk sac larva (57). Infections with *Ichthyophthirus multifiliis* (a ciliated skin and gill parasite) elicit expression of iNOS in rainbow trout (38, 39) and *Myxobolus cerebralis* infection lead to iNOS or Arginase-2 expression dependent on the susceptibility of the rainbow trout strain used (58). In addition, salmon louse *Lepeophtheirus salmonis* infections of Atlantic salmon skin was associated with an upregulation of the arginase gene (59). A related switch from a Th1 to a Th2-like reaction in rainbow trout skin infected with flagellates was recently described (60). So although M1 and M2 differentiation has not yet been detected in invertebrates, at least fish seems to have developed arginase, which makes M1 and M2 differentiation possible. Thus, Arginase is found in only one form in micro-organisms and invertebrates, a form which is not related to the ornithine–urea cycle, whereas fish may possess the necessary enzymes (61).

CONNECTING INNATE AND ADAPTIVE RESPONSES

It was with the appearance of the vertebrates that a higher degree of immunological sophistication (adaptive immunity) was evolved. Vertebrates developed the MHC, T-cell receptors, and immunoglobulins as an additional weapon and regulatory system. The most primitive fish (agnathans such as hagfish and lampreys) possess special lymphocyte receptors composed of leucine reach repeats suggesting that this group followed a divergent line of development. With the cartilaginous fish (sharks and rays) and bony fish, immunoglobulins appeared. Some modern fish today carry at least three classes of immunoglobulins [IgM, IgT (Z), IgD], an array, which has been further developed in amphibians (IgM, IgX, IgY, IgD, IgF), reptiles (IgM, IgY, IgA, IgD), birds (IgM, IgY, IgA, IgD), and mammals (IgM, IgG, IgA, IgD, IgE) (62). Despite the lack of these specialized proteins (immunoglobulins) in lower vertebrates and invertebrates, this does not mean that

immunity is less well developed in primitive animals. In fact, a rich array of innate immune genes and high variability of innate effector molecules provide animals such as earthworms, snails, mussels, shrimps, and insects with a capability to combat continuous attacks from microbes in their environment. Although central parts of these innate immune mechanisms present in invertebrates are conserved in higher vertebrates, it seems that the variability and diversity is much higher among invertebrates whereas higher vertebrates by fine-tuning the adaptive components (Igs, TCR, MHC) reach the same goal of clearing pathogens from the host organism. It may be hypothesized that the efficacy of the adaptive weaponry has allowed vertebrates to reduce the often impressive variety of innate effector molecules, which was available in earlier lineages.

COEVOLUTION OF PARASITE AND HOST AS AND ADDITIONAL DRIVER OF INNATE IMMUNITY VARIATION

It is evident that ancestors of existing pathogens have been able to evade innate and adaptive host immune mechanisms. Thus, immune reactions against viral, bacterial, and parasitic pathogens are in most cases only partly effective with regard to elimination of the intruding or established parasite in the vertebrate host (63, 64). It is a characteristic trait of both protozoan and metazoan parasites that the pathogens are able to deal with extensive cellular and humoral elements of the host immune system, a trait, which is securing parasite survival for extended periods (65). Coevolution of hosts and parasites has resulted in a tight interaction between innate and adaptive immune elements in the host and a rich but, to a certain extent, unexplored array of immune evasion mechanisms in the parasites. Also bacteria and virus apply an intricate system of immune evading mechanisms during invasion in order to survive host defenses (66, 67). Consequently, hosts may only survive, reproduce, and contribute to evolution by exhibiting new and more efficient immune molecules. This never ending arms race may be speculated to be at least partly responsible for the presence in modern times of an immense number of both hosts and parasites (68). However, in order to understand the principles of parasite immune evasion in higher vertebrates, including humans, it may be speculated that the basis for evasion will be found primarily in primitive invertebrates (16). Secondarily, we may trace it in the oldest and most original hosts possessing an adaptive immune system (12).

CONCLUSION

Immune factors and recognition systems involved in differentiation of self from non-self may have been an integrated part of animal physiology since multicellular animals developed more than 600 mya. These innate mechanisms differ from the MHC system arising with the vertebrate lineage. Receptors, ligands, and signal molecules may initiate relevant actions by use of a series of effector molecules, which lead to elimination of pathogens or re-establishment of the injured tissue in the individual. These basic elements have been found even in sponges and cnidarians, two ancient invertebrate groups. The immune molecules and cellular products involved in these reactions are encoded by genes, which have similarities with elements even in higher vertebrates. Mollusks, crustaceans, insects, and echinoderms make use

of cytokine like molecules resembling TGF, MIF, TNF, and interleukins. In addition, receptor molecules (TLRs), complement, and immunoglobulin-like sequences are being used by these invertebrates for various purposes. However, it is likely that although many immune genes and effector molecules can be found in the early invertebrates, their mode of action may differ considerably from the corresponding reactions in vertebrates. It is even likely that genes encoding factors with non-immunological roles in invertebrates may be used for immunological purposes in higher vertebrates, and vice versa. The dramatic environmental events on the geological time scale, with several periods of climate changes and extinction of major animal groups, have created a basis for selection of a multitude of new variants. Interactions with pathogens, which continuously are developing immune evasion mechanisms in their encounter with the host immune system, may further stimulate the never ending evolution of the immune system. The phagocyte function, taken by macrophages in vertebrates, has also been present in the earliest invertebrates. Corresponding cells have reached increasingly sophisticated levels during invertebrate evolution, and in vertebrates they exhibit high diversity. These cells have, in vertebrates, been equipped with MHC II molecules, which make them indispensable partners for B- and T-lymphocytes. They have obtained the ability to produce and communicate through an extensive cytokine network and they seem to be able to take a fight or repair function on their own reactions, which were seen also in the early invertebrates. In brief, immune reaction building blocks are ancient and appeared at various stages during evolution. Some were lost, some were moderated, and some even obtained another function during evolution. When adaptive immunity evolved with the vertebrate lineage, the old and still existing elements were further incorporated in the new hosts for optimization of immunity under the new conditions.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 01 July 2014; paper pending published: 06 August 2014; accepted: 08 September 2014; published online: 23 September 2014.

Citation: Buchmann K (2014) Evolution of innate immunity: clues from invertebrates via fish to mammals. Front. Immunol. 5:459. doi: 10.3389/fimmu.2014.00459

This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology.

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Evolutionary roots of arginase expression and regulation

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Two main types of macrophage functions are known: classical (M1), producing nitric oxide, NO, and M2, in which arginase activity is primarily expressed. Ornithine, the product of arginase, is a substrate for synthesis of polyamines and collagen, important for growth and ontogeny of animals. M2 macrophages, expressing high level of mitochondrial arginase, have been implicated in promoting cell division and deposition of collagen during ontogeny and wound repair. Arginase expression is the default mode of tissue macrophages, but can also be amplified by signals, such as IL-4/13 or transforming growth factor- β (TGF- β) that accelerates wound healing and tissue repair. In worms, the induction of collagen gene is coupled with induction of immune response genes, both depending on the same TGF- β -like pathway. This suggests that the main function of M2 “heal” type macrophages is originally connected with the TGF- β superfamily of proteins, which are involved in regulation of tissue and organ differentiation in embryogenesis. Excretory-secretory products of metazoan parasites are able to induce M2-type of macrophage responses promoting wound healing without participation of Th2 cytokines IL-4/IL-13. The expression of arginase in lower animals can be induced by the presence of parasite antigens and TGF- β signals leading to collagen synthesis. This also means that the main proteins, which, in primitive metazoans, are involved in regulation of tissue and organ differentiation in embryogenesis are produced by innate immunity. The signaling function of NO is known already from the sponge stage of animal evolution. The cytotoxic role of NO molecule appeared later, as documented in immunity of marine mollusks and some insects. This implies that the M2-wound healing promoting function predates the defensive role of NO, a characteristic of M1 macrophages. Understanding when and how the M1 and M2 activities came to be in animals is useful for understanding how macrophage immunity, and immune responses operate.

Keywords: arginase, evolution, hemocytes, M1/M2 macrophages, nitric oxide synthase, parasites, TGF- β , wound healing

Vertebrate macrophages play an innate defense role against various pathogens. They perform phagocytosis, bacterial killing, defend against protozoan and metazoan parasites and take part in wound healing. To fulfill such protective functions, “resting” macrophages must be activated. Two main types of macrophage functions have been identified: classical (M1), producing nitric oxide (NO), and M2-type, in which arginase (producing the healing molecule, ornithine) is expressed (1, 2). These responses from macrophages demand different cascades of biochemical reactions, which are regulated by specific sets of cytokines. M1 type can be stimulated by pathogen associated molecular patterns (PAMP) or amplified by T cell cytokines, such as IFN- γ . In contrast, M2 activity is the resident tissue type, and can be amplified by molecules such as IL-4, IL-13, and transforming growth factor- β (TGF- β). Local signals polarize macrophages to an appropriate response. These immune functions are indispensable for both life of an individual and lasting of a species. It is apparent that macrophages, as well as other cells of innate immune response acting in vertebrates, have their deep evolutionary roots in cells serving similar function in ancestral invertebrates. Various names have been used to define such cells in different invertebrate groups, i.e., hemocyte, coelomocyte, amebocyte, or plasmacyte, collectively named immunocytes (3).

However, regardless of the terminology, they perform the same immune functions, and are of similar morphology.

Macrophages, the professional phagocytic cells in humans, derive from circulating monocytes or by self renewal in the tissues, and acquire new morphological and physiological characteristics according to the organs and microenvironments, in which they settle. However, this unitarian origin is uncertain for circulating and tissue phagocytes (immunocytes) in invertebrates (3). Immunocytes and macrophages share ability to be activated by non-self material and to react through the release a variety of biologically active molecules such as cytokines, NO, reactive oxygen species, hydrolytic enzymes, and neuroendocrine mediators. In vertebrate immunity, various organs and immune cells are involved, while all the molecules determining invertebrate immune response are harbored in the immunocytes. From the perspective of this review, the multifunctional role of invertebrate cells seems instructive in respect to its inheritance by vertebrates.

In search for selection pressure for macrophage differentiation into M1 and M2 phenotypes, it is tempting to look back to recognize which of the functions of M2 macrophages is aligned straight with invertebrate immunocytes and is found possibly in the most primitive invertebrates. Accumulated evidences

strongly suggest that a primary function of M2 macrophages is tissue repair and wound healing (4, 5). This process demands polyamines and collagen synthesis what strongly depend on arginase activity (6).

ANIMAL ARGINASES

Arginase (amidinohydrolase, EC 3.5.3.1) is an ubiquitous enzyme found in bacteria, yeasts, plants, invertebrates, and vertebrates. Some bacteria possess a related enzyme, agmatinase (*Escherichia coli* or *Methanobacterium*, also *Methanococcus*), which belong to Archaeabacteria. Agmatinase produce putrescine and urea, arginase ornithine and urea. Plant arginases are closer to the agmatinase clade than to the animal one (7, 8). Agmatinases origin predates that of arginases and the latter would have appeared in the Bacteria by recruitment of a wide specificity agmatinase and then its transfer to an eukaryotic cell (according to Sekowska et al. perhaps through mitochondria) (8).

Most microorganisms and invertebrates studied to date have only one type of arginase (9). Arginases from plants and ammoniotelic animals are localized in mitochondria (10). In ureotelic animals, arginase is involved in ammonia detoxication in the ornithine-urea cycle and is localized in cytosol. The cytosolic and mitochondrial arginases are isoenzymes named A-I and A-II, respectively (11). They are encoded by two separate genes. The arginase gene duplication is relatively recent, and occurred after separation of vertebrates and invertebrates (9). It has been suggested that the mitochondrial A-II is a surviving form of the ancestral arginase, because the cytosolic A-I is restricted to a subset of more recently evolved species (11).

The pattern of occurrence of the arginase isoenzymes implies that the primordial function of the enzyme is regulation of cellular arginine and ornithine metabolism, unrelated to the urea cycle. Ornithine, the product of arginase-catalyzed reaction, is a substrate for synthesis of proline and polyamines. It is important to know that ornithine is formed from glutamate *via* the pathway leading to arginine synthesis as well. This pathway occurs in bacteria, plants and animals (10). Both in plants and animals, polyamines (putrescine, spermidine, and spermine) are involved in a variety of growth and developmental processes, and they bind directly to DNA and RNA (12). It has been also shown that polyamines play a pivotal role in wound healing. Proline (as hydroxyproline) is indispensable for the collagen biosynthesis in animals and for the synthesis of cell wall proteins in plants. Participation of ornithine in such vital processes suggests that arginase could be regulated by factors influencing development and growth of organisms. TGF- β belongs to a superfamily of ancient proteins, known in all bilaterians, members of which play important signaling roles in embryogenesis (13).

TGF- β SUPERFAMILY OF PROTEINS

Transforming growth factor- β was originally discovered as a secreted factor that induced malignant transformation *in vitro*. It is a prototype member of a superfamily of secreted, homodimeric polypeptides. These factors affect a variety of biological processes in both transformed and normal cells, including regulation of embryogenesis, adult cell differentiation, inflammation, and wound repair (14, 15).

The TGF- β superfamily may be divided into subfamilies according to sequence homology. One subfamily consists of the closely related TGF- β 1, -2, and -3. TGF- β 2 and - β 3 take part in development signaling, while TGF- β 1 signals act in inflammatory responses and tissue necrosis (15). TGF- β 1 cDNAs from different animal species (together with chicken and *Xenopus*) show an extremely high degree of conservation (14). Fish TGF- β homologs cluster with their mammalian TGF- β 1, - β 2, and - β 3 counterparts (15).

Bone morphogenetic proteins were initially characterized as factors that induce bone and cartilage formation (16, 17). Bone morphogenetic proteins (BMPs) are critical in development, hematopoiesis, as well as cellular chemotaxis, and cellular differentiation (18, 19). In the BMPs subfamily, BMPs-2 and -4 bear closest homology to the decapentaplegic complex protein, a *Drosophila* protein mediating dorsal/ventral axis specification (14). BMPs-5, -6, -7, and -8 most closely resemble *Drosophila* protein 60A, which is required for the growth of imaginal tissues and for patterning of the adult wing (20). Genes encoding members of the bone morphogenetic factor (BMP) protein family have been identified in a sea anemone and an echinoderm (21).

Genes of TGF- β superfamily members cluster in two major clades: TGF- β sensu stricto/TGF- β related (e.g., Activins, Leftys, and GDF8s) ligands and BMP related (e.g., BMPs and Nodals) (22). TGF- β sensu stricto ligands have been identified only in deuterostomes (Echinodermata, Hemichordata, and Chordata) and are not present in genomic screens of *Caenorhabditis*, *Drosophila*, or *Nematostella* (23).

Receptors of TGF- β pathway are serine threonine kinases categorized as type I and type II (24, 25). Vertebrates have seven distinct type I receptors, each of which can mix and match with one of five type II receptors to mediate signals for the TGF- β family ligands (26). Ligand binding to the constitutively phosphorylated type II receptors stimulates recruitment of type I receptors and formation of a heterodimeric receptor complex. In the complex, type I receptors are transphosphorylated by type II receptors (13). A signal from type I receptor to the nucleus is channeled into one of two intracellular pathways *via* Smad family of proteins. Three of the receptors phosphorylate the R-Smads (receptor-regulated Smads); Smad2 and Smad3 and thereby transduce TGF- β -like signals, whereas the other four receptors activate the R-Smads; Smad1, Smad5, and Smad8 to mediate signals characteristic of those initiated by BMPs (26–28). These R-Smads form multi-subunit complexes with a common partner Smads (Co-Smads; Smad4) before entering the nucleus to affect a response (29). Both R-Smads and Co-Smads are found in all metazoans (30). I-Smads; Smad6/7 play inhibitory function, stimulating receptor degradation, or competing with R-Smads in formation of complexes with Smad4 (29, 31). The regulatory activity of I-Smads evolved after divergence of the poriferan lineage (32).

Binding of Smads to DNA is not especially specific; they play a role of comodulators, which act together with transcription factors (pan-metazoan Fos/Jun and Myc), transcription coactivators (pan-metazoan CBP and CBF- β), and transcription corepressors (Ski/Sno) to recruit basal transcription machinery (32). The formation of Smad complexes gives a wide range of cooperative interactions, thus enables TGF- β signaling to evoke multiple responses

ranging from embryonic development to wound repair. AR-Smads (activin/TGF- β -specific R-Smads) transactivate various target genes through interaction with various DNA-binding partners, including plasminogen activator inhibitor-1 (PAI-1), type I collagen, junB, Smad7, and Mix.2. For inhibition of cell growth by TGF- β , AR-Smads induce the transcription of cyclin-dependent kinase (CDK) inhibitors p21 and p15. In addition, Smad3 binds directly to the promoter region of c-myc and represses the transcription of c-myc. In contrast, only a few target genes for BMPs have been identified, including Id (inhibitor of differentiation or inhibitor of DNA-binding) 1/2/3, Smad6, Vent-2, and Tlx-2. Id proteins, however, play important roles in multiple biological activities of BMPs (27). Id proteins act as negative regulators of cell differentiation and positive regulators of cell proliferation (33).

Ligands, receptors of the TGF- β pathway, and Smads are ancient proteins. They emerged already in the metazoan stem lineage. I-Smads, multiple ligand traps, and SARA have been added to the signaling pathway after the divergence of sponges (32).

Transforming growth factor- β and BMP signaling pathway is evolutionary conserved, as it was shown for worms, flies, and vertebrates [Ref. (26)].

TGF- β FUNCTION IN EMBRYOGENESIS AND WOUND HEALING OF INVERTEBRATES

Caenorhabditis elegans is a free-living nematode, a member of the lineage, which appeared more than 470 million years ago (34). There are at least three distinct TGF- β -like pathways in this worm (35). One of them controls the body size and morphology of the male tail, but five genes of this pathway (*dbl-1*, *sma-2/-3/-4/-6*) contribute to resistance against *Pseudomonas aeruginosa* infection (36). This pathway controls induction of some genes induced after *Serratia marcescens* infection, including *lys-8* (lysozyme) (37). Moreover, *dpp*, a *dbl-1* homolog in *Drosophila*, is up-regulated upon immune challenge (38, 39). This pathway shows a clear homology to the mammalian TGF- β pathway (40), which plays an important role in immune responses (41). Some targets of this pathway in *Caenorhabditis* are known: *mab-21*, involved in male ray pattern formation, and *lon-1* and *lon-3*, involved in regulation of the body size. The latter two encode a cysteine-rich secretory protein and collagen, respectively. The *lon-1* and *lon-3* are mainly expressed in the hypodermis, as they are essential for the body size regulation (42, 43).

This finding implies that the ancestral pathway of TGF- β signaling in embryogenesis is bound with immune reactions in the Protostomia, suggesting that TGF- β pathway in immunity has been conserved generally across their evolution. Induction of collagen gene in *Caenorhabditis* during bacterial infection links developmental processes with the tissue repair induced by pathogens. Collagen is needed for extracellular matrix deposition during both embryogenesis and wound repair. Hemocytes must migrate to wounded area for the synthesis of collagen fibrilles. In lesioned leeches (Annelida), immunocytes are the first cells that are also involved in closing the wound by using pseudopodia to bridge the epithelial edges. Subsequently, additional immunocytes complete the obstruction together with granulocytes and NK-like cells (3). Throughout embryogenesis, hemocytes carry out important developmental functions within the embryo, such as the

engulfment and removal apoptic cells and the laying down of many extracellular matrix molecules, including collagen IV and laminin, which compose the basement membrane surrounding internal organs [Ref. (44)]. *Drosophila* hemocytes are similar to leukocytes in respect of activation and migration toward wounds. A requirement for phosphoinositide 3-kinase (PI3K) for the polarization and active hemotaxis of hemocytes toward an epithelial wound shows a striking analogy with the mechanism of cell chemotaxis used by *Dictyostelium discoideum*, mammalian neutrophils (45), as well as fish macrophages (46). Migration of *Drosophila* hemocytes toward wounds depends on CDC42 and Rho signal transmission (47) and PI3K signaling (45). The migratory pattern of hemocytes in *Drosophila* embryos is independent of PI3K signaling, but depends on chemotactic signals from the PDGF/VEGF ligands (45).

During wound healing in the snails *Lymnaea stagnalis* (48) and *Limax maximus* (49), hemocytes exhibit fibroblast activity while secreting the extracellular matrix. Similar transformation of a cell type was observed in fibroblasts transformed into myofibroblasts at injured sites when acting in wound contraction (50). It points to the role of hemocytes in invertebrate tissue repair, similarly to the mammalian wound models, in which the macrophages are essential cells participating in wound healing (51). PDGF-AB (heterodimer) and TGF- β 1 stimulate chemotaxis of different cell types, especially hemocytes (49). The increased number of hemocytes contribute to earlier wound closure at the injured site. The removal of damaged tissue residues is also accelerated by stimulation of the phagocytic activity of recruited hemocytes. TGF- β 1 regulates expression of genes of collagen type I and III, and fibronectin (52, 53). This may mean that the mechanism of wound healing is conserved from invertebrates to mammals what's more, arginase gene expression has been documented in hemocytes (54).

Tissue injury in humans triggers migration of macrophages, platelets, fibroblasts, myofibroblasts, and eosinophils releasing TGF- β . TGF- β stimulates fibroblasts and other reparative cells to proliferate and synthesize extracellular matrix components (elastin fibers, collagen fibrils, protein-polysaccharides, and glycoproteins). This leads to a provisional repair, followed by fibrosis and ultimately scarring. Fibrosis of many organs (liver, heart, kidney, pancreas, and skin) is mediated by TGF- β [Ref. (55)]. In experimental murine *Schistosoma mansoni* infection, gene expression of type I and type III interstitial collagens, basement membrane collagen, and TGF- β 1 show increased levels of expression after primary infection (56). Transcription of type I procollagen chains *proα1* and *proα2* is TGF- β -regulated through two different pathways during tissue fibrosis. Expression of *proα1* depends on the TGF- β activator protein and expression of *proα2* depends on Smad signaling of TGF- β pathway. In addition, there are other cellular factors and DNA-binding elements required for the transcription of these type I procollagen genes. New synthesized procollagen molecules are processed by enzymes outside the cell.

In the evolution of nematodes, they changed their original free-living habitus to commensal one and finally to parasitize tissues of animals (57). The evolution of platelmintns was different. They changed ectoparasitic mode of life of monogenean trematodes to endoparasitic one of digeneans and tapeworms. Invertebrates that are parasitized by these worms, heal damages to the tissue due

to activation of polyamine and collagen synthesis. Thus arginase induction required for wound healing in animals without acquired immunity could be based on the TGF- β signaling pathway.

Aside being a substrate for proline synthesis pathway, ornithine is a substrate for polyamine production in all eukaryotic cells. As polyamines are required for high rates of protein synthesis and cell proliferation (58), they play a pivotal role in repair processes. Stimulation of putrescine synthesis was observed during regeneration of earthworms and planarians (59). Regenerating tissues produce spermine, and injured or dying cells release spermine into the extracellular milieu, so that tissue levels of this compound increase significantly at inflammatory sites of infection or injury (60). In snails resistant to the *Schistosoma mansoni* infection (61), increased gene expression of ornithine decarboxylase in hemocytes points to the enhancement of arginase activity, which results in ornithine production (62, 63). Ornithine decarboxylase produces putrescine used for the synthesis of other polyamines involved in DNA protection during cell proliferation. Polyamines assist in wound healing following miracidial penetration.

Mollusks can be infected with viruses, bacteria, fungi, protists, digenetic trematodes, polychetes, and copepods (64). The infective stage of the protozoan (haplosporidian) *Haplosporidium nelsoni* invades the bivalve tissues through gills and palps spreading then through the body. Infection of the bivalve *Crassostrea virginica* with this protist leads to an increase in the number of circulating hemocytes and their infiltration into tissues (65). It is suggested that these cells are involved in limiting parasite damage by plugging lesions, removing debris, and repairing damaged tissue.

In hemocytes of the snail *Biophalaria glabrata* infected with the digenetic trematode *Schistosoma mansoni*, for which a definitive host is human, the expression of TGF- β receptor gene was slightly lowered in comparison with those of resistant strains. Early gene expression was measured only 2 h post exposure to miracidia (61).

Transforming growth factor- β signaling is essential for extra-cellular matrix development in cold-blooded animals (66). As a result of infection of salmonid fish with the ectoparasitic caligid crustacean *Lepeophtheirus salmonis* insufficient expression of several regulatory proteins, among them TGF- β , brought up delayed expression of collagen 2a and delayed wound healing. Arginase gene expression was markedly increased in intact skin of infected fish (67). Support for the hypothesis that arginase expression is related to collagen expression comes from observations that arginase transcripts are down-regulated in concert with collagen a in resistant oysters five days after challenge with the gram-negative bacterium *Roseovarius crassostreae* (68). This extracellular pathogen colonizes the oyster's inner shell surface and causes lesions in the epithelial mantle.

Efficient wound healing in invertebrates based on induction of genes for arginase and collagen biosynthesis (68) mediated by TGF β (49, 65), but without cytokines of Th2 cluster being involved, may mean that also in vertebrates such mechanism of healing is possible. An innate response to injury may occur in absence of any adaptive response and can be triggered solely by tissue injury (69). Although IL-4/IL-13 mediated responses may be important in tissue repair, they do not appear to be essential, as the incision is effectively healed in the mice that lack IL-4 or IL-4 receptor. Nonetheless, the importance of type 2 cytokines in damage tissue

remodeling and fibrosis is well documented (70). Possibly IL-4 and/or IL-13 mediate a more rapid form of tissue repair that it is necessary just to maintain tissue integrity. According to Allen and Wynn (71), Th2 immunity in vertebrates evolved as a means to rapid tissue damage repair caused by metazoan invaders rather than just to control parasite numbers.

M2-TYPE OF MACROPHAGE RESPONSE WITHOUT HELP OF TH2 CYTOKINES

Transforming growth factor- β , IL-4, and IL-13 are key cytokines skewing macrophages to the M2-type response that is typical for allergy and metazoan parasite infection. Arginase induction is the hallmark of this response. This raises the question whether M2-type of macrophage response could be induced solely by multicellular parasites without help of Th2 cytokines.

A strong wound healing response would occur in helminth infection, as tissue migratory or tissue invasive parasites often lead to physical trauma. A Th2-type protective immune response develops following infection with many tissue-dwelling intestinal nematode parasites (*Heligmosomoides polygyrus*, *Trichuris muris*, or *Trichinella spiralis*) or trematodes and is characterized by elevations in IL-4 and IL-13 and increased numbers of CD4 $^{+}$ T cells, granulocytes, and macrophages. These cells accumulate at the site of infection and may mediate resistance to worms (72, 73). This is a kind of defense strategy of the host, but it eventually favors a survival of parasite.

Excretory-secretory (ES) products or parasite enzymes activate and regulate host-immune response at the macrophage level through inhibition of pro-inflammatory cytokines production and induction of macrophages toward the M2-type of activation.

Trichinella spiralis is the parasitic nematode of higher vertebrates, which causes pathological changes in various tissues of the host. Binding of TGF- β with specific antibodies abrogated effect of infection on arginase activity in guinea pig alveolar macrophages (74). ES products from *Trichinella spiralis* raise the expression of interleukin-10, TGF- β , and arginase-1 in J774 A.1 macrophages in the absence of Th2 cytokines (75). In addition, ES products significantly inhibit translocation of NF- κ B into the nucleus and the phosphorylation of both ERK1/2- and p38MAP-kinases in J774A.1 macrophages stimulated with lipopolysaccharide (LPS) (an antigen of Gram-negative bacteria). Treatment of peritoneal macrophages with a recombinant of 53-kDa protein derived from *T. spiralis* brought about expression of mannose receptor, a novel mammalian lectin (Ym1), arginase-1, and IL-10, hallmarks of M2 phenotype. This effect was independent of IL-4R α , but dependent on STAT6 (76).

Infections with the trematodes *Fasciola hepatica* or *Schistosoma mansoni* cause destruction of the host liver tissues, damage to bile ducts, atrophy of the portal vessels, and secondary pathological conditions. Secreted peroxiredoxins may induce alternative activation of macrophages. They stimulate Ym1 expression *in vitro*, which shows their action independent of IL-4/IL-13 signaling (77). As expected, administration of recombinant peroxiredoxins from these trematodes to the wild type and IL-4 $^{-/-}$ and IL-13 $^{-/-}$ mice induces alternatively activated macrophages. Also eggs of *S. mansoni* laid in the smallest blood vessels cause tissue reaction in the form of inflammation, necrosis, connective tissue encapsulation,

and eventual scar formation during their migration through the tissue to the colon. The eggs trapped in the liver induce fibrosis and are associated with production of proline (78). The immunomodulatory pentasaccharide LNFPIII, which contains the Lewis X trisaccharide, is a component of schistosome soluble egg antigen. It up-regulates expression and activity of arginase-1, as well as expression of Ym1 in macrophages (79) but does not induce expression of FIZZ-1, MGL-1, or MMR. Upregulation of arginase I and Ym1 is independent of IL-4 and IL-13. Binding of LNFPIII to C-type lectins on the surface of macrophages leads to alternative nuclear factor (NF)- κ B activation (80) and may induce arginase-1 and Ym1 directly without IL-4 and IL-13. An injection of LNFPIII initiates alternative activation, do not mimicking complete infection because it does not cause FIZZ-1 expression, besides upregulation of Ym1. Interestingly, Loke et al. (69) have found that surgical trauma leads to elevation of markers of alternative activation without presence of T cells. However, the innate expression of Ym1, FIZZ-1, and arginase-1 requires either IL-4 or IL-13. Expression of arginase-1 occurred early in response to surgery. It increases with growing up to third day post surgery and then returns to baseline by 1 week, but is sustained only in the parasite-implanted animals.

Protozoan parasite *Toxoplasma* type I and type III strains may induce the M2 phenotype, while the type II strain induces M1 phenotype (81). The alternative activation of macrophages is dependent in large part on the *Toxoplasma* polymorphic protein kinase ROP16, while the classical activation of macrophages by the type II strain is due to unique ability of its GRA15 protein to activate NF- κ B pathway and elicit pro-inflammatory cytokines. Both enzymes seem act in a way specific to the host. According to authors, parasite effectors from different *Toxoplasma* strains evolved to work optimally in hosts predisposed to certain types of immune responses, such as those along the Th1/Th2/Th17 or M1/M2 axes. Ending up to the wrong host might lead to severe disease and failure to establish chronic infection.

MACROPHAGE POLARIZATION BY TGF- β SUPERFAMILY PROTEINS

In the adult *Drosophila* immune response, *Dpp* (*decapentaplegic*), a BMP-type signal, is rapidly activated by wounds and represses the production of antimicrobial peptides. The activin/TGF- β -like signal *dawdle* (*daw*), in contrast, is activated by Gram-positive bacterial infection but repressed by Gram-negative infection or wounding; its role is to limit infection-induced melanization. Genes *dpp* and *daw* are expressed in hemocytes but also in other tissues. The hemocyte population in the adult fly is comprised of subsets of cell that can be defined through distinct gene expression profile. According to Clark et al. (82), it is likely that expression of *dpp* and *daw* by a subset or subsets of phagocytes indicates distinct immunomodulatory functions by these cells. Both *dpp* and *daw* inhibit immune responses. This makes the fly similar to mammals, in which both activin and TGF- β -like and BMP-like signals are largely anti-inflammatory (83, 84), in contrast with the nematode *Caenorhabditis*, where the TGF- β superfamily member *dbl-1* analog of *dpp* promotes a variety of antimicrobial responses (85).

Both anti- and pro-inflammatory response due to activation of TGF- β superfamily receptors by their ligands, TGF- β ,

bone morphogenetic protein-7 (BMP-7), BMP-6 have been found in macrophages of rodents. Surprisingly few studies have evaluated the effect of TGF- β signaling on macrophages. Mouse macrophages lacking *T β RII* (transforming growth factor- β receptor II) are defective in expression of genes that characterize the M2-type of activation, suggesting that TGF- β signaling is needed for the alternative activation of macrophages. Lack of *T β RII*^{-/-} is associated with basal expression of arginase-1 (protein and mRNA) significantly decreased in comparison with the wild type both in naïve peritoneal macrophages and bone marrow-derived macrophages, BMDM, (86). Moreover, when *T β RII*^{-/-} BMD macrophages are polarized toward an M2 phenotype with IL-4, induction of Arg-1 is very low. Expression of Arg-1 is increased in WT macrophages stimulated with TGF- β 1. As transcription of other M2 markers including *ym1* ceases in *T β RII*^{-/-} BMDMs, apparently signals through *T β RII* modulate the M2 transcription program. TGF- β contributes to M2 polarization of macrophages with IL-4 through co-signaling to Akt, which is one of the TGF- β 1 non-Smad-associated signal transduction pathways in other cell types (87).

Bone morphogenetic protein-7 activates receptor BMPR2 in monocytes, which results in phosphorylation of R-SMAD1/5/8 and activation of down-stream mediators in the Smad pathway. It plays a role in polarization also in M2 macrophages, as manifested by increased expression of anti-inflammatory cytokines (88). In bone marrow-derived M2 macrophages, increased polarization results from activation of PI3K pathway (89). Activation of the PI3K pathway controls production of transcription factors. They regulate key inflammatory cytokines resulting in increased expression of anti-inflammatory markers (90). Arginase-1 and IL-10 level is significantly increased following treatment of monocytes with BMP-7 (88). In addition to the canonical Smad-dependent pathway for TGF- β signaling, a Smad-independent pathway, namely the mitogen-activated protein kinase (MAPK) pathway (p38MAPK and JNK) may act (91). Activation of the NF- κ B pathway via the X-linked inhibitor of apoptosis (XIAP) transduces BMP signaling (92). Different to BMP-7, BMP-6 may induce pro-inflammatory inducible NOS (iNOS) and TNF- α in peritoneal macrophages (93). The general phenotype of macrophages in response to BMP-6 is similar to that of macrophages exposed to LPS (94). BMP-6 in macrophages appears to counteract TGF- β . It is likely that the BMP-6 induction of expression of inducible NO synthase occurs through IL-1 β via Smad and NF-kappaB signaling pathways (95). IL-1 β , in turn, up-regulates iNOS expression via the NF- κ B pathway. However, the possibility that BMP-6 may directly activate NF- κ B signaling could not be excluded because TGF- β activates kinase 1 (TAK1), which is a component of the BMP signaling pathway in *Xenopus* and mouse embryonic development (96, 97). TAK1 and its regulators (TAB1 and TAB2) form complexes and activate the IKK complex (98). The latter possibility suggest that pathway used in embryonic signaling could be used in innate immunity response to induce NO production. Interestingly, NO and ornithine (the product of arginase described earlier), both originate from the same amino acid, arginine, via different enzymatic reactions, which were called figuratively “The arginine fork in the road” (99).

EVOLUTION OF NITRIC OXIDE SYNTHASE

In macrophages, NO is a crucial mediator of cytotoxicity. It has been shown to have microbicidal, antiviral, antiparasitic, and antitumor effects. NO production is usually mediated by nitric oxide synthase (NOS) (EC 1.14.13.39). To date, three isoforms of NOS have been characterized: iNOS, neuronal NOS (nNOS), and endothelial NOS (eNOS). In macrophages, iNOS is transcriptionally induced in response to LPS, TNF- α , interferon- γ (IFN- γ), and interleukin-1 β (IL-1 β) (100). The signaling pathway for iNOS expression in macrophages involves NF- κ B and signal transducer and activator of transcription (STAT).

Three NOS isoforms originally described in mammalian tissues (100, 101) are encoded by distinct genes: for eNOS, nNOS, and iNOS. All they share much of their sequence with cytochrome P450 reductase in their C-terminal reductase domains and have a common oxygenase domain.

The interdomain linker between the oxygenase and reductase domains contains a calmodulin-binding sequence. In eNOS and nNOS, physiological concentrations of Ca^{2+} in cells regulate the binding of calmodulin to the linker, thereby initiating electron transfer from the flavins to the heme moieties. In contrast, calmodulin remains tightly bound to the iNOS (Ca^{2+} -insensitive isoform). Expression of iNOS is strongly activated in the presence of LPSs or in response to potentially damaging stimuli, resulting in a high and long-term NO yield. iNOS is primarily involved in defense reactions and cytotoxicity.

Nitric oxide synthase ancestry goes back to early bacteria from before a couple billion years ago. In all prokaryotic enzymes only the oxygenase domain is found (102, 103). In NOS evolution, multiple events of gene loss and gain in various lineages occurred.

Nitric oxide synthase occurs probably in almost all invertebrates ranging from jellyfish (104) and hydra (105) to fly (106) and parasitic worms (107), as well as mollusks and arthropods (108). NOS enzymes from insects (109–111) and mollusks [(112), Ref. (113)] have greater overall sequence similarity to a neuronal-like NOS than to iNOS or eNOS in vertebrates. However, cnidarian (*Discosoma*) and slime mold (*Physarum*) NOSs (113, 114) lack the distinct structural element that is present as an insertion in the reductase domains of constitutive NOSs but is absent in iNOSs of vertebrates. This insert is thought to be an autoinhibitory loop, which impedes binding of Ca^{2+} to calmodulin and enzymatic activation. This insert reduces potentially toxic NO yields following the activation of iNOS. Since the *Discosoma* NOS is structurally similar both to the only known non-animal conventional NOS and to vertebrate iNOS isoforms, the inducible type of the enzyme may be ancestral for animal NOSs. In contrast to vertebrate species, which have three NOS genes, only one type of NOS isoform has been found in the genomes from insects and tunicates. Mollusks, sea urchins, and cephalochordates have at least two NOS genes but no NOS genes have been identified in *Caenorhabditis elegans*. These findings imply that more than one NOS co-existed in the common ancestor of all animals and it was lost in some animal lineages in the course of evolution. On the other hand, in some groups, such as mollusks (with at least two different types of NOS) and chordates (2–3 NOS genes), duplication events for NOS genes may have occurred more than once. Moreover, duplications happened independently in the evolution of inducible type NOS,

since some fishes have more than one iNOS-like gene (113). The diversification of vertebrate NOSs occurred in parallel in many lineages, which cluster into three distinct groups corresponding to the mammalian iNOS, eNOS, and nNOS, iNOS probably being most basal. eNOS apparently originated as the last, within the mammalian clade.

The primary and evolutionary conservative role of NO is NO-cGMP signaling, acting in many different invertebrates from sponges, insects, and mollusks to cephalopods [Ref. (115)]. A defense function of NO was observed in the crustacean (116), and mollusk hemocytes [Ref. (117)]. In general, defense functions in invertebrates are accomplished by superoxide produced by phagocyte NADPH oxidase (which appeared before the divergence of the Choanoflagellata and metazoans), antimicrobial peptides, lysozymes, hemolymph clotting, and melanization [Ref. (115)]. This suggests that the function of arginase as the key enzyme producing ornithine in metazoans, indispensable for tissue repair, is more ancient than the cytotoxic activity of free radical NO, the product of NOS. One may conclude that the wound healing function of M2 macrophages is more deeply rooted in history of life than the cytotoxic activity of M1 macrophages.

REGULATION OF NITRIC OXIDE PRODUCTION IN INSECTS AND MICE

Most of research on the NOS refers to three animal species: fruit flies *Drosophila melanogaster*, mice *Mus musculus*, and humans *Homo sapiens*. Presumably, the insects are the least advanced in its evolution. Mice, as rodents, are relatively primitive mammals, closest relatives of the order Primates, to which humans belong. From evolutionary point of view, the rodent macrophages, commonly used as a model for immunological investigations, better suit to studies on innate immunity reactions in vertebrates than human ones.

The model for studying arthropod immunity is the antimicrobial defense in *Drosophila*. IMD pathway performs a signaling function by inducing host defenses in response to Gram-negative bacteria. Activation the IMD signaling pathway leads to the activation of NF- κ B homolog Relish and production of antimicrobial peptides (118, 119). The cells in *Drosophila* gut detect the pathogen and activate hemocytes via an NO-dependent signal. The hemocytes act in turn to activate immune-inducible gene expression in the fat body (the insect liver analog) by an as-yet-unknown signal (118). In *Anopheles stephensi*, expression of immune responsive genes, including NOS, is up-regulated in response to the presence of *Plasmodium* parasites in the midgut (120).

It has been found that *A. stephensi* NOS possesses a putative LPS- and cytokine-responsive transcription factor binding site (121). Invertebrates have cytokine-like proteins similar to the interleukins and tumor necrosis factors of vertebrates (122). Transcription factor binding sites in the 5'-flanking sequence demonstrate a bipartite distribution of LPS- and inflammatory cytokine-responsive elements that are strikingly similar to that described for murine iNOS gene promoters (123, 124). Studies of *Drosophila* NOS regulation have shown (125) that insect NOS activity is solely dependent on Ca^{2+} and calmodulin, like the constitutive vertebrate NOSs. Although the activity of *Drosophila* NOS

is very low compared to other NOSs, low amount of NO produced may be sufficient for functioning as a signaling molecule (126).

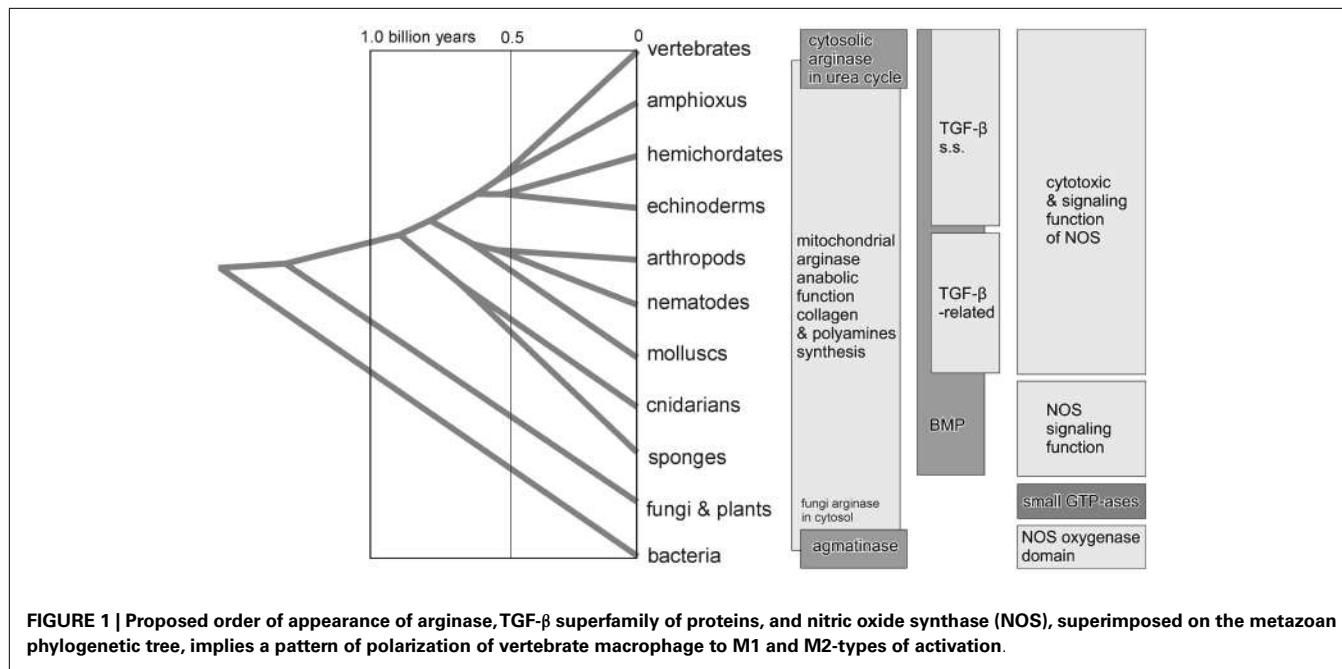
As already commented, NO is rather a signaling than cytotoxic molecule in invertebrates. In vertebrates, as exemplified by rodent macrophages, high amounts of NO are produced as a result of activation of iNOS with LPS and IFN γ . This is a part of defense armor, which otherwise acts as a double edge sword. However, stimulation of macrophages only with LPS results in a low NO production and less than 15% of cells is iNOS positive. IFN γ enhanced LPS-induced secretion of NO by recruiting increasingly greater numbers of macrophages into the production of iNOS (127). The gene of iNOS is synergistically activated by LPS and IFN γ (123, 124). The iNOS promotor contains two important regions termed RI and RII. The effects of LPS stimulation are mediated by elements in both RI and RII, whereas IFN γ functions through RII only (123, 124). In addition, IFN γ alone is not able to activate through RII, acting solely to augment the effect of LPS on RI (123). A variety of LPS response elements, including NF- κ B, occurs and the NF- κ B site in LPS-mediated transcriptional activation of iNOS is required (128). Perhaps the increase of frequency of LPS responsive cells in effect of IFN γ action, and consequent NO production is the key factor to enhance NO formation. It leads to M1 type of response.

TGF- β 1 seems to be the most potent regulator of iNOS. In natural killer cells, neutrophils and macrophages, TGF- β 1 diminishes iNOS activity, influencing gene expression, mRNA stability and translation, and NOS protein stability (129). For suppression of LPS-stimulated iNOS in bone marrow-derived mouse macrophages, both Smad2 and Smad3 are required. Down regulation of iNOS mRNA undergoes by suppressing the IRF3- IFN β -STAT1 pathway (130). Mutual feedback regulation between iNOS and TGF- β 1 is also possible, as latent TGF- β 1 can be activated by exogenous NO (131). TGF- β appears to be important endogenous

mediator that keeps resident/wound healing macrophages in M2 dominant mode. A decrease in TGF- β production in macrophages brought about “activation” of these cells. Similarly, removing of TGF- β from cell culture (coming from serum added), caused much more NO produced, and less synergy between LPS and IFN- γ in stimulation of NO production (1).

CONCLUDING REMARKS

Sophisticated ways of signaling observed in contemporary vertebrates show how complex are results of molecular evolution. Although specialization of macrophage responses is based on two ancient mechanisms: cytotoxic activity of iNOS and anabolic function of arginase (Figure 1). It is suggested here that not defense against infection but rather the TGF- β -signaling was at the origin of the M1/M2 macrophage specialized functions. Such signaling is known to operate already in primitive invertebrates, both in their embryonic development and wound healing. A prototypic inducer of M1 response bacterial LPS, alone activates production of only a small amount of NO by iNOS-type enzyme and generates signal propagation through cGMP cyclase in invertebrates. It remains unknown, to what degree invertebrate analogs of IFN γ would be able to enhance LPS-induced NO production, as no experimental data about enhancement of NO production by IFN γ -like cytokine in *Drosophila* are available. Presumably, the ability of M1 macrophages to produce large amounts of NO in response to microbial infection is a vertebrate evolutionary invention, known to be present already at the fish grade (132). At this stage the arginase function in M2 macrophages, inherited after invertebrate ancestors, was to deliver ornithine for processes of extracellular matrix synthesis, of importance in organogenesis and wound healing. The latter serves also as a protection against metazoan parasites. Thus, the main function of M2 macrophages is originally connected with the TGF- β



superfamily of proteins, which in primitive metazoans are involved in regulation of tissue and organ differentiation in embryogenesis. Looking back in evolution also indicates that both NOS/NO and arginases/ornithine are primitive innate responses in macrophages that long preceded the development of T and B cells (adaptive immunity).

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 21 August 2014; paper pending published: 23 September 2014; accepted: 13 October 2014; published online: 07 November 2014.

Citation: Dzik JM (2014) Evolutionary roots of arginase expression and regulation. *Front. Immunol.* **5**:544. doi: 10.3389/fimmu.2014.00544

This article was submitted to Molecular Innate Immunity, a section of the journal *Frontiers in Immunology*.

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Macrophage: SHIP of Immunity

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Keywords: macrophage, M1, M2, nitric oxide, iNOS, arginase, wound, cancer

Immunology. Why does it exist? Two words. Cure disease. People get diseases. “Test tubes” do not. People fund immunologists for solutions to their health problems. But, immunologists often study leukocytes in test tubes – the laboratory – away from diseases. Why? Because much can be learned from analyzing cellular biochemistry and behaviors *in vitro* that cannot be ascertained when leukocytes are in animals. At the same time, isolated leukocyte reactions often do not reflect how the immune system operates as a unit. So, it is critical to verify *in vitro* observations *in vivo*. Among leukocytes, macrophages are the central initiating and directing element in immune systems, and serve this role through four basic “SHIP” functions *in vivo*: Sample; Heal; Inhibit; and Present (antigen) (1–4). The polar-opposite functions of Heal (M2-type) and Inhibit (M1-type) can have profoundly different effects on host survival, and require unique and major changes in macrophage metabolism and physiology. In turn, macrophage populations are necessarily heterogeneous as they adapt to protect hosts in different ways: they exhibit “plasticity.” Some have focused on measuring ever-expanding lists of cell surface or various other “markers” (mostly *in vitro*) to try and sub-type macrophages. But, the “heterogeneity” created by such studies can be “illusory” because there are many more markers than there are functions (e.g., M1/inhibit and M2/heal). Thus, it is important to focus on classifying macrophages by functions, such as SHIP, to navigate through a “sea of plasticity.” And, thereby realize the enormous potential of macrophages/innate immunity for improving health.

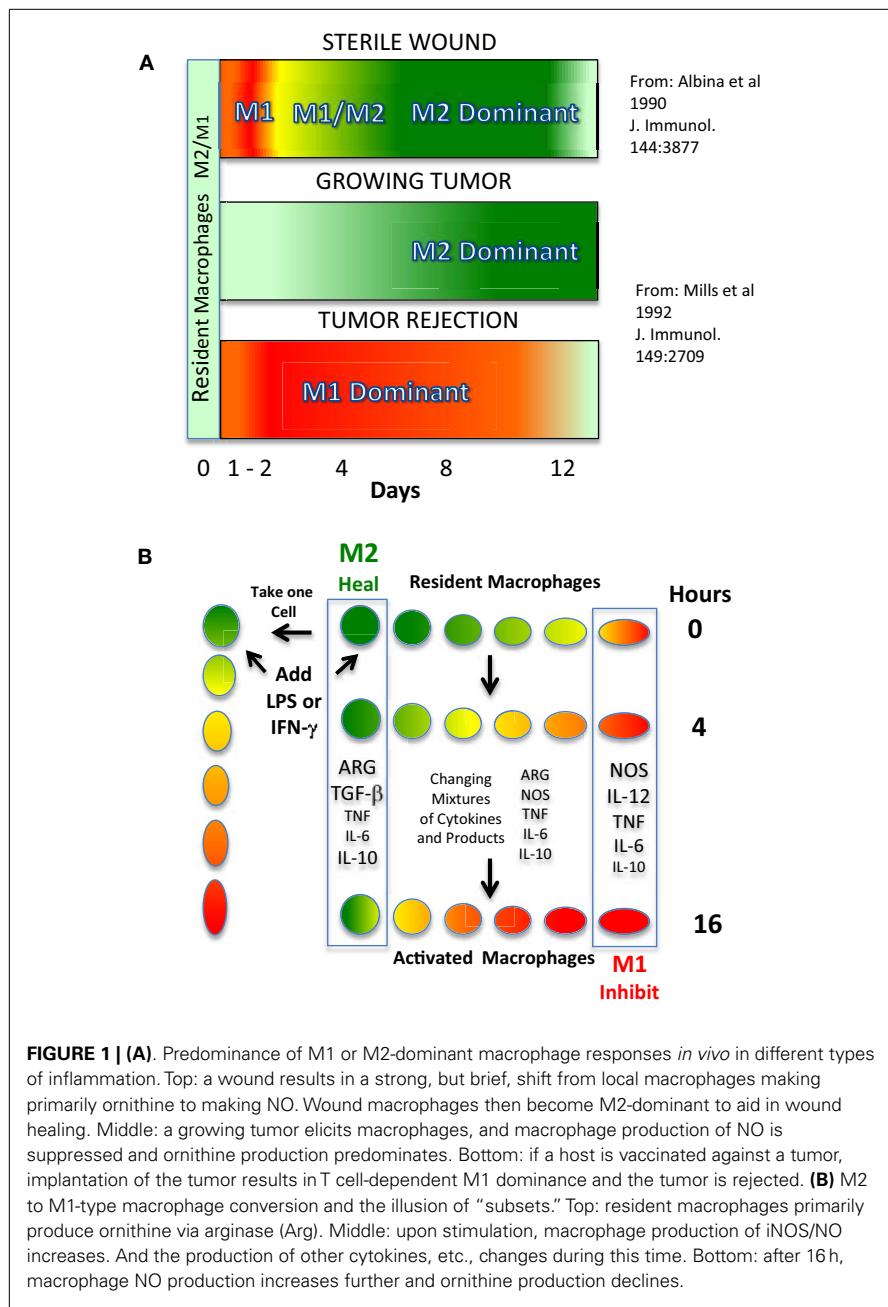
BASIC MACROPHAGE FUNCTIONS *IN VIVO*

The earliest *in vivo* SHIP function observed in macrophages was their ability to “sample” by ingesting items in their surroundings (5, 6). Through sampling, macrophages routinely receive “self” signals that instruct them to repair or replace lost or effete cells and intercellular matrices. The heal-type function of macrophages is now called M2 [(7), reviewed in Ref. (2)]. Following infection (or trauma), M2/heal-type macrophages can rapidly switch to become M1/inhibit-type, to promote host defense (1). M2/heal responses are mediated by ornithine, and other growth-promoting molecules (8, 9). M1/inhibit is mediated by nitric oxide (NO) and other molecules that promote cellular killing activity (10, 11). Fascinatingly, both ornithine and NO arise from one amino acid: arginine (12).

The biochemical basis for the M2/heal function of macrophages was discovered before the M1/inhibit function (8, 12). As illustrated in Figure 1A (top), in sterile wounds, macrophages produce ornithine (a precursor of polyamines and collagen for repair) as healing proceeds (Green – M2 dominance) (13). Around the same time, it was observed that macrophages in growing tumors were also the M2/ornithine-producing type (Figure 1A middle). This latter finding provided a biological explanation for the association of intratumor macrophages with tumor growth (14). M2/heal-type macrophages have since been shown to also dominate in human tumors, and are associated with poor survival (15–18).

The biochemical basis of how M1/inhibit-type macrophages kill pathogens

(or, abnormal “self”) also came from the study of wounds and cancer described above, as well as other studies [(19–26), reviewed in Ref. (3, 12)]. As mentioned, macrophages have a unique ability to switch from making the heal molecule, ornithine, *in vivo* to making the killer molecule, NO (1). Such a switch is shown in Figure 1A (top). For a brief period following wounding, a high concentration of NO is present (M1 activity, Red), which can protect the wound if infectious agents have been introduced (13). It is not clear exactly what stimuli cause this injury-induced NO production, though neutrophils are also involved (27, 28). If the wound is sterile, macrophage NO production stops, neutrophil emigration ends, and wound macrophages make ornithine (M2 activity) as mentioned. Another example of the key protective function of macrophages making NO is shown in Figure 1A (bottom). It can be seen that if a host is vaccinated against the tumor shown in Figure 1A (middle), implantation of the tumor causes intratumor macrophages to make a large quantity of NO that helps cause tumor rejection (12). Macrophage NO is also an important defense against a variety of infectious diseases (29). That M2/ornithine or M1/NO are important effector molecules are supported by studies showing that interference with these activities *in vivo* alters healing or host protection [reviewed in Ref. (12)]. Conversely, overexpression of M1/inhibit responses is associated with conditions such as atherosclerosis and arthritis, while M2-type contribute to chronic infections, promotion of tumor growth, and allergies (3, 29). Together, these results demonstrated



two core functions that result from macrophages sampling their environment, and that affect health in very important, and opposite, ways: the M1/inhibit response and the M2/heal response.

Inhibit-type macrophages and heal-type macrophages were specifically renamed M1 and M2 because these macrophage responses [or dendritic cells¹

(30–32)] were also found to stimulate T cells to make Th1-type (cellular-based), or Th2-type (antibody-based) cytokines (7), respectively. This fourth SHIP function of present (antigen) is only expressed in vertebrates (1). Although T cells can produce molecules that "activate" or "alternatively activate" macrophages (33, 34), macrophages evolved first and respond

first. They directly sense Pathogen or Damage – Associated Molecular Patterns (PAMP or DAMP) that can initiate M1 or M2-type responses (35–39). Subsequently, macrophages can stimulate T cells (that cannot recognize antigens directly), and thereby further amplify M1 or M2 responses. This "secondary" type of T cell-driven response (macrophage "activation") was discovered by Mackaness and colleagues using mice preimmunized to *Listeria* and other pathogens (40). It was not known at this time that macrophages were actually responsible for initially activating T cells (41, 42). The recent revelation about the central role of macrophages in immune responses caused a sea change in understanding how immune responses occur and are regulated *in vivo* (1, 7).

BIOCHEMICAL AND PHYSIOLOGIC HOST ELEMENTS THAT INFLUENCE HOW MACROPHAGES PROTECT HOSTS

Macrophage SHIP functions (sample, heal, inhibit, and present) are regulated by integration of a variety of endogenous (e.g., host-derived and resident microbiota) and exogenous signals (1, 43). For example, in the absence of infection or injury, TGF-β helps maintain macrophages in the routine M2/heal mode (7). Other host-derived molecules, such as oxidized LDL, can stimulate M1-type responses that contribute to atherosclerosis (4, 44). Following infection or injury, certain PAMPs and DAMPs stimulate macrophages to switch from M2/heal to M1/inhibit mode (35–37). IFN-γ was shown to be the primary T cell product that further amplifies M1/inhibit activity (45). Later, macrophage IL-12 was found to be a key cytokine (along with increased Class II MHC expression) that stimulates IFN-γ production by T cells (41, 42, 46). Macrophages have also been reported to secrete IFN-γ upon stimulation via IL-12 and IL-18 (47) or via CD40 (48), which might further enhance M1 polarization through auto- or paracrine activity. Not all pathogens stimulate macrophages to switch from M2/heal to M1/inhibit, and some seem to suppress such a switch. In this circumstance, M2-type macrophages can stimulate T cells to make very different cytokines (such as IL-4, IL-13, and

¹Both macrophages and dendritic cells can direct the type of T cell response. So, the word "macrophage" will be used here to refer to both. Readers are directed elsewhere for discussions of macrophages and dendritic cells.

TGF- β) that cause B cells to become antibody-producing plasma cells (7, 29). These same cytokines also inhibit the M2 to M1 switch, and thus can amplify M2/heal activity (1). Of course, because there are many different pathogens invading different locales of hosts, there are always mixtures of M1/Th1 or M2/Th2-type responses as disease regression or progression occurs. In this connection, it is now known that tissue macrophages can arise from local renewal or from the blood (1, 4). The ontogeny of M1 and M2-type macrophages is not yet clear, and is beyond the scope of this article. Recent advances in metabolomics, defining resident microbiota, other areas, are opening up new horizons for understanding the myriad signals that regulate “immunity” (43). Though more is to be known, the aforementioned results have established important biochemical and physiologic elements that influence how macrophages serve to protect (or fail to protect) against infectious or other threats to host homeostasis.

IN VITRO VERSUS IN VIVO MACROPHAGE CONUNDRUM

In addition to the basic macrophage functions necessary for life (such as SHIP), some investigators (primarily working *in vitro*) have employed ever-expanding lists of “markers” for macrophage “activation.” These include: cell surface antigens; expressed gene products; and other factors, and have created the notion that there are many different “varieties” of macrophages such as “M2 a, b, c,” “regulatory,” and “alternatively activated” macrophages (49–53). Unlike classifying macrophages by functions (e.g., M1/inhibit or M2/heal), the use of markers has created subsets without clear functional roles *in vivo*. Likewise, defining macrophage populations based on cytokine production patterns has caveats that are often overlooked. For example, macrophage cytokines such as IL-6 are “inflammatory,” yet they can be found in almost any site where macrophages are present (1). Indeed, the very presence of macrophages is inflammatory that raises questions about what “anti-inflammatory” macrophages are (47–49). Efforts to define macrophage “subsets” based on which cytokine (or agonist) has been used to stimulate them *in vitro* (such as IL-4 or IFN- γ)

also leads to confusion since macrophages do not encounter isolated cytokines *in vivo*. Rather, they are constantly receiving hundreds of signals, the integration of which ultimately defines a cell’s behavior. Furthermore, because a selected cytokine can elicit a given macrophage reaction *in vitro* does not mean it has the same effect *in vivo*. For example, adding IL-4 to macrophages *in vitro* does increase M2-type activity (50). And IL-4 from T cells or innate cells can upregulate M2-type antibody responses (7, 29): what has been termed “alternative activation”). However, it is hard to ascribe M2-type responses in circumstances such as sterile wounds or tumors to “alternative activation” because little or no IL-4 is present (54, 55). Using T cell-derived cytokines to stimulate macrophages *in vitro* has also propagated the long-held notion that T cells are necessary to “activate” macrophages (23, 24). This perception runs counter to the observations that macrophages initiate and direct innate or adaptive responses (1). Another potential artifact of *in vitro* cultures is that macrophages can exhaust critical media components, and thus behave in ways (including dying) that are not observed *in vivo* where nutrients/other products are replenished (24).

Finally, the source of the “macrophages” being studied *in vitro* varies and has created confusion. Specifically, people studying humans have primarily used monocytes from blood because of convenience. And doing so has caused some to conclude there are major species differences in “macrophages,” including that humans seem less able (or unable) to produce iNOS/NO or arginase/ornithine (3, 56, 57). However, comparing monocyte-derived macrophages to tissue macrophages is an apples and oranges-type comparison. When human tissue macrophages have been examined, they do not appear fundamentally different from those of other vertebrate species (58).

Thus, a variety of pitfalls can make it difficult to translate results from *in vitro* cultures to understanding how macrophages function *in vivo*. In turn, rather than relying on “markers” or selected culture stimuli to try and define different macrophage “activation” states (59), it seems prudent to focus on characterizing macrophages

by their known *in vivo* functions, such as SHIP (1).

SHIP FUNCTIONS TO NAVIGATE A SEA OF PLASTICITY

Macrophage SHIP functions are associated with major differences in their metabolism and physiology (1). And hence, at the population level, macrophages must display considerable heterogeneity. “Plasticity” usefully describes the unique adaptability of macrophages as they change from, for example, producing a growth-promoting molecule (ornithine) to producing a growth-inhibiting molecule (NO) (12, 60). However, for some the concept of plasticity has morphed into a notion that macrophages are a fluid cell type that are always *only* changing (47–51). Like they say, “change is good” (humor intended). But, like changing clothes, it is not the changing that matters: it is the result. Perhaps, the clothes help one get a job, or, get a date, etc. And so it is with macrophages. As macrophages make major switches in their metabolism, they are “changing.” But, the changes in functional properties of macrophages can create illusory heterogeneity as illustrated in

Figure 1B. Specifically, if a population of resting/resident macrophages (or a single macrophage, left) receives appropriate signals (e.g., LPS and/or IFN- γ) and commits to switching from M2/heal to M1/inhibit dominant activity, it takes the cell(s) several hours to accomplish this major change in metabolism. In turn, at any given time there will be a variety of different macrophages expressing different M2 and (increasingly in this example) M1-type activity. In turn, if one examines macrophages (or a single macrophage) at any given time there will be intermediate phenotypes in terms of marker or cytokine expression. Also often overlooked is that M1-type macrophages produce non-specific killer molecules (like NO) that inhibit or kill macrophages too (24). In turn, analysis of whole populations can create the additional illusion that M1-type have converted back to M2-type, when actually, they are dead/missing (1) In turn, examining macrophage populations (particularly *in vitro*) can create impressions of reversible plasticity or heterogeneity, but which are not based on what functions the macrophages have (e.g., M1/inhibit or M2/heal) (49). Thus,

heterogeneity (or plasticity) is a means to an end. The “end” immunologists should strive for is identifying macrophages by their health-impacting functions (1).

SUMMARY

Immunology has and will continue to cure important diseases. And, the ability to culture macrophages *in vitro*, the expanding power of “transcriptome” analysis to examine thousands of genes, the capability of analyzing single macrophages, and other new technologies are providing necessary new information about the cellular biochemistry and physiology of leukocytes. But, as demonstrated here with macrophages, overemphasis on ambiguous “markers,” or analyzing whole populations of macrophages that are changing their functions, can create an illusion – a “sea of plasticity.” Therefore, to navigate this sea, it is critical to focus on SHIP functions (e.g., sample, heal, inhibit, and present) that importantly affect health. Doing so will help unleash the tremendous potential for usefully modulating innate immunity/macrophages against a variety of conditions ranging from cancer to atherosclerosis. To cure disease.

ACKNOWLEDGMENTS

We appreciate the excellent and unselfish input we have received from researchers in forming this paper. Namely, because we are all on the “same team”: the goal being better health.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 29 October 2014; accepted: 20 November 2014; published online: 04 December 2014.

Citation: Mills CD, Thomas AC, Lenz LL and Munder M (2014) Macrophage: SHIP of Immunity. *Front. Immunol.* **5**:620. doi: 10.3389/fimmu.2014.00620

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From monocytes to M1/M2 macrophages: phenotypical vs. functional differentiation

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Studies on monocyte and macrophage biology and differentiation have revealed the pleiotropic activities of these cells. Macrophages are tissue sentinels that maintain tissue integrity by eliminating/repairing damaged cells and matrices. In this M2-like mode, they can also promote tumor growth. Conversely, M1-like macrophages are key effector cells for the elimination of pathogens, virally infected, and cancer cells. Macrophage differentiation from monocytes occurs in the tissue in concomitance with the acquisition of a functional phenotype that depends on microenvironmental signals, thereby accounting for the many and apparently opposed macrophage functions. Many questions arise. When monocytes differentiate into macrophages in a tissue (concomitantly adopting a specific functional program, M1 or M2), do they all die during the inflammatory reaction, or do some of them survive? Do those that survive become quiescent tissue macrophages, able to react as naïve cells to a new challenge? Or, do monocyte-derived tissue macrophages conserve a "memory" of their past inflammatory activation? This review will address some of these important questions under the general framework of the role of monocytes and macrophages in the initiation, development, resolution, and chronicization of inflammation.

Keywords: monocytes, monocyte-derived macrophages, tissue-resident macrophages, functional phenotypes, inflammation

INTRODUCTION

In the healthy organism, the innate immune system provides the first line of defense against external or internal danger signals, by initiating a protective inflammatory response that develops during time through different phases, from initiation and full inflammation, to resolution and re-establishment of tissue integrity. The first phase of an inflammatory response is aimed at destroying pathogens, and is followed by a phase in which dead and dying cells, damaged extracellular matrix material, and cellular debris are removed, to end up with a recovery phase in which the tissue is repaired and restored to a healthy fully functional condition. In fact, if the defense against harmful threats is a priority for avoiding tissue damage, maintaining homeostasis (i.e., maintaining tissue morphology and tissue function) is the ultimate goal of a tissue in multicellular organisms (1). In this perspective, inflammation presumably evolved as an adaptive response to tissue malfunction or homeostatic imbalance (2). Thus, while the disease state is a displacement from homeostasis, inflammation is the tissue response for restoring homeostasis. However, since the inflammatory activities are potentially harmful to the host, these need to be tightly controlled to avoid excessive tissue damage (3).

The mononuclear phagocyte system (MPS) plays major roles in development, scavenging, inflammation, and anti-pathogen defenses, both by the direct elimination of foreign agents and in organizing each different phase of the inflammatory process (4). Under the term, MPS are grouped lineage-committed bone marrow precursors, circulating monocytes, resident macrophages, and dendritic cells (DC) (5). The development, homeostatic maintenance, proliferation, differentiation, and function of the

MPS are regulated by the growth factors colony-stimulating factor (CSF)-1 and interleukin (IL)-34, the second ligand for the CSF-1R (6, 7).

The issue of heterogeneity in the MPS still leads to a confusion and debate about DC as truly distinct cells from macrophages, with separate lineage and functions (8). In fact, macrophages and myeloid DC possibly represent alternative differentiation options of bone marrow progenitors and blood monocytes (9), with overlapping functions and marker expression. Reviewing this issue is beyond the scope of this essay [we refer the reader to recent excellent reviews on the topic; (9–11)], and will only focus on monocytes and their relationship with macrophages.

The traditional view of the MPS suggests that recruited monocytes (that become macrophages in tissues) are key players during inflammation and pathogen challenge, whereas tissue-resident macrophages have important roles in development, tissue homeostasis, and the resolution of inflammation. A basic concept of the MPS is that blood monocytes are precursors that replace tissue macrophages within a single developmental lineage (4). This dogma needs now to be revised in the light of new evidence that macrophages are endowed with self-renewal capacity and can populate tissues before birth, deriving from early hematopoiesis in the yolk sac (12, 13). The discovery of new macrophage progenitors of embryonic origin forces us to reassess definitions, functions, and cell-cell relationships within the MPS. We can synthesize it in three key new questions:

1. Are monocytes more than circulating precursors and can they have effector functions?

2. Is there a functional difference between the monocyte-derived macrophages and the yolk sac-derived self-renewing resident macrophages?
3. What is the relationship between monocytes and macrophages and which are their mutual roles in different phases of inflammatory reactions?

Another new perspective arises from the description of macrophage polarization, i.e., the ability of macrophages to acquire different functional phenotypes, enabling them to steer adaptive immunity in different directions. This highlights the central role of macrophages in immune defense, overturning the long-held notion that macrophages need to be activated by T-cells (14).

This review will summarize what has been so far investigated and established on monocyte/macrophage biology, highlighting what remains outstanding, and which questions are still unanswered. We will consider key studies that have been carried out in mice, with reference to the human situation when data are available. We will review the various aspects (monocyte recruitment, monocyte functions, macrophage polarization) before (homeostatic conditions), during (inflammatory reaction), and after a damaging event (resolution/repair).

MONOCYTES

MONOCYTE DEVELOPMENT AND HETEROGENEITY

Monocytes are a group of cells circulating in the blood, bone marrow, and spleen, and constituting ~10% of the total leukocytes in human beings and only 2–4% in mice. They have typical morphological features, such as irregular cell shape, oval- or kidney-shaped nucleus, cytoplasmic vesicles, and high cytoplasm-to-nucleus ratio. Monocytes can remain in the circulation for up to 1–2 days, after which time, if they have not been recruited into a tissue for facing a danger, they die and are removed. Monocytes originate in the bone marrow from hematopoietic stem cells (HSCs) and develop through a series of sequential differentiation stages: the common myeloid progenitor (CMP) (15), the granulocyte-macrophage progenitor (GMP) (15), the common macrophage and DC precursor (MDP) (16), and finally the committed monocyte progenitor (cMoP), a recently identified bone marrow precursor that differs from MDP as it lacks CD135 expression (17). MDP gives rise also to common DC progenitors (CDP), whose differentiation potential is restricted to the DC lineage (18). Monocytes have been considered as the systemic reservoir of myeloid precursors for renewal of tissue macrophages and DC. However, many DC and macrophage subpopulations [for example, lymphoid organ DC, plasmacytoid DC, skin Langerhans cells (LC), and brain microglia] originate from the MDP independent of monocytes (11, 18), and in some cases, they can even develop directly from the bone marrow (19).

Homeostatic control of monocyte/macrophage development is mostly influenced by CSF-1 (also known as M-CSF), produced by stromal cells within the blood and in tissues (20). Mature mononuclear phagocytes in turn express CSF-1 receptors (CSF-1R) and remove circulating CSF-1, allowing a feedback loop responsible for monocyte proliferation decrease (21, 22). Recently, the cytokine IL-34 has been identified as able to bind and signal

through the CSF-1R (6, 23). Unlike broadly expressed CSF-1, IL-34 expression is restricted to the epidermis and central nervous system (24), where it supports the steady-state proliferation of macrophages (LC and microglia, respectively). Granulocyte-macrophage colony-stimulating factor (GM-CSF) is another factor involved in the development of mononuclear phagocytes but only during the inflammatory state and not under homeostatic conditions (25, 26).

Abundant experimental evidence indicates that recruited monocytes are innate effectors of the inflammatory response to microbes, and they kill pathogens via phagocytosis, production of reactive oxygen species (ROS), nitric oxide (NO), myeloperoxidase, and inflammatory cytokines (27). In some circumstances, monocytes can trigger and polarize T-cell responses (27, 28) and may also contribute to angiogenesis and atherogenesis (29).

Human peripheral blood monocytes are not a homogeneous population. Monocyte heterogeneity was first reported with the identification of a minor population of CD16 (FcγRIII)-expressing cells within circulating human monocytes (30). In recent years, investigators have identified three functional subsets of human monocytes, the characterization of which is still in its infancy. Likewise, it is still unclear which are the specific roles that they exert in homeostasis and inflammation *in vivo*, in comparison with those of the previously described classically and alternatively activated macrophages (see below). The new nomenclature that groups monocytes into three subsets, based on the expression of the surface markers CD14 and CD16, has recently been approved by the Nomenclature Committee of the International Union of Immunologic Societies (31). Based on this nomenclature, the major population of human monocytes (90%) with high CD14 but no CD16 expression (CD14⁺⁺CD16⁻ or CD14⁺CD16⁻) are termed classical monocytes, whereas the minor population of human monocytes (10%) is further subdivided into the intermediate subset, with low CD16 and high CD14 (CD14⁺⁺CD16⁺ or CD14⁺CD16⁺), and the non-classical subset, with high CD16 but with relatively lower CD14 expression (CD14⁺CD16⁺⁺ or CD14^{dim}CD16⁺) (31). In this review, we refer only to the main difference, terming classical monocytes simply as CD14⁺, and non-classical as CD16⁺.

Over the recent years, an increasing amount of knowledge has been gained in the field of monocyte subpopulations. Many authors demonstrated that the three subsets express different transcriptomes (32–38), although discrepancies between studies were evident. These discrepancies may be due to differences in cell isolation methodology and in the purity of the cell populations isolated, and the microarray methodologies, which use different amounts of total RNA for the hybridization, different probes to identify the genes, and even distinct solid supports for the probes (39). However, there is stronger agreement for the proximity of relationship between the intermediate and non-classical monocyte subsets, while the classical subset is the most distant subset (36). The close relationship between intermediate and non-classical monocytes suggests a direct developmental relationship between them, although this has yet to be formally proven. Also, it needs to be clarified how the characteristics previously ascribed to CD16⁺ monocytes are distributed between intermediate and non-classical subsets (36). Recent data suggested a sequential developmental

relationship between the two subsets based on the observation that, in time course studies in inflammatory diseases, an increase in the intermediate monocytes is followed by an increase of non-classical monocytes (40).

The physiological role of the monocyte subsets *in vivo* is not fully defined. They might have different roles during the homeostasis, immune defense/inflammation, and tissue repair, in terms of their capacity to become activated and secrete inflammatory cytokines in response to different stimuli, antigen processing and presentation, pro-angiogenic and patrolling behavior. The phenotypic and functional differences between the monocyte subsets were recently discussed in an exhaustive review (41). The authors of this review report a complete and referenced list of studies on bacterial and viral infections, autoimmune diseases, and inflammatory conditions, in which an expansion of CD16⁺ cells in respect to other subsets has been observed. In general terms, both human classical and intermediate monocytes have inflammatory properties reminiscent of the murine Ly6C⁺ monocytes (also termed “inflammatory” monocytes) (42), while non-classical monocytes display patrolling properties similar to those of murine Ly6C⁻ monocytes (also termed “alternative” or “patrolling” monocytes) (43). Both human and mouse inflammatory monocytes express high levels of the chemokine receptor CCR2 and low levels of the chemokine receptor CX3CR1, whereas patrolling monocytes show a reverse pattern. Accordingly, inflammatory monocytes respond to the chemokine CCL2 that mediates Ly6C⁺/CD14⁺ monocyte recruitment to inflammatory sites (44), while patrolling monocytes respond to CX3C-chemokine ligand 1 [CX3CL1, the human fractalkine and mouse neurotactin; (45)], a chemokine present both as soluble protein and as membrane-bound chemokine form that is expressed on endothelial cells and in tissues. Overall, it is clear that the subsets between human being and mouse are similar but not identical (42, 46). **Table 1** summarizes the main features of monocytes in human beings and mice. Of note, there is a clear difference in the proportion of the two

monocyte subsets, as Ly6C⁻ cells represent about half of the circulating monocytes in mice, whereas CD16⁺ monocytes account for less than 15% in human beings (30). However, Ziegler-Heitbrock hypothesized that the higher proportion of the Ly6C⁻ in mouse blood could be due to stressful blood drawing (cardiac puncture under terminal anesthesia) that mobilizes these monocytes from the marginal pool (46). This hypothesis still needs experimental proof.

To date, a relevant question that is still open concerns the origin of the various monocyte subpopulations. It should be kept in mind that the majority of current knowledge derives from mouse studies. It is unknown if the monocyte subpopulations are end stages of different differentiation paths of a common precursor, or whether they represent subsequent maturation stages in a common path of differentiation, where the intermediate subset could be a phenotypical and/or developmental intermediate between the classical and non-classical subsets. The latter hypothesis seems to be the most reliable. While initial studies suggested that Ly6C⁺ cells were recruited under inflammatory conditions and did not serve as precursors to Ly6C⁻ cells [which in turn were originally considered the immediate precursors of resident macrophages; (43)], recent evidence suggests that, in steady state, Ly6C⁺ monocytes are precursors of Ly6C⁻ monocytes (48, 49), as shown in experiments in which grafted Ly6C⁺ monocytes spontaneously differentiated into Ly6C⁻ in the blood of recipient mice (48). This conversion can also occur in the bone marrow, where Ly6C⁺ monocytes apparently return in the absence of inflammation (47–49). More recently, it has been suggested that CSF-1R signaling was required for the maturation of monocytes from Ly6C⁺ to Ly6C⁻, as blockade of this receptor leads to decrease in the number of Ly6C⁻ cells, shortens their lifespan (48), and concomitantly increases the number of Ly6C⁺ monocytes (50).

It has also been observed that development of the Ly6C⁻ population depends on the transcription factor NR4A1 (Nurr77) (51). NR4A1 deletion alters the number of Ly6C⁻ monocytes in the

Table 1 | Human and murine monocyte subsets.

Species	Subset ^a	% In WB	% In blood monocytes	Half-life	Markers	Chemokine receptors	Other surface markers	Main functions
Human being	Classical	~10%	85%	1–2 days	CD14 ⁺⁺ CD16 ⁻	CCR2 ⁺ CX3CR1 ⁻	CD62L ⁺ , CD64 ⁻ , MHC class II ⁺ , CD163 ⁺	Phagocytosis, inflammatory effectors
	Intermediate		5%	–	CD14 ⁺⁺ CD16 ⁺	CCR2 ⁻ CX3CR1 ⁺	CD62L ⁺ , CD64 ⁻ , MHC class II ⁺⁺ , CD163 ⁺	Inflammatory effectors
	Non-classical		10%	–	CD14 ⁺ CD16 ⁺⁺	CCR2 ⁻ CX3CR1 ⁺	CD62L ⁻ , CD64 ⁺ , MHC class II ⁺⁺ , CD163 ⁻	Patrolling, antiviral role
Mouse	Ly6C ^{low}	4%	~60%	18–20 h	CD11b ⁺ CD115 ⁺ Ly6C ⁺	CCR2 ⁺ CX3CR1 ⁻	F4/80 ⁺ , CD62L ⁻ , MHC class II ^b , CD43 ⁺	Phagocytosis, inflammatory effectors
	Ly6C ^{high}		~40%	5–7 days	CD11b ⁺ CD115 ⁺ Ly6C ⁻	CCR2 ⁻ CX3CR1 ⁺	F4/80 ⁺ , CD62L ⁺ , MHC class II ^b , CD43 ⁻	Patrolling, tissue repair

^aWork by Sunderkötter et al. (47) characterized a population of Ly6C^{med} monocytes with intermediate features between Ly6C⁺ and Ly6C⁻. These are not included in the table, because this population remains poorly characterized in terms of both phenotype and function.

^bInducible.

WB, whole blood; Ly6C, lymphocyte antigen 6 complex; CCR2, chemokine (C-C motif) receptor 2; CX3CR1, CX3C-chemokine receptor 1.

bone marrow but not in blood or spleen (52), but does not alter the number of macrophages within tissues (53). This suggests that either Ly6C⁻ monocytes can develop from MDP within the bone marrow, or that Ly6C⁻ monocytes are a functional end stage. In this regard, given that Ly6C⁻ monocytes exhibit a long steady-state half-life of 5–7 days [which in the absence of their renewal from Ly6C⁺ monocytes can extend to 2 weeks; (48)] compared to ~8 h for Ly6C⁺ cells, Ly6C⁻ monocytes might be considered as terminally differentiated blood-resident macrophages or “vasculature macrophages,” rather than *bona fide* monocytes (48). Indeed, the primary function of these cells seems to be that of patrolling the vascular endothelium and monitoring its integrity (45, 51). Conversely, and in parallel with human CD14⁺ cells, Ly6C⁺ monocytes because of their short half-life are unlikely to have other functions, and thus are more likely to be the direct precursors of the tissue macrophages/peripheral mononuclear phagocytes described in the original MPS model.

FUNCTION OF MONOCYTE SUBSETS DURING HOMEOSTASIS (CLASSICAL VS. ANTIGEN-PRESENTING TISSUE MONOCYTES VS. PATROLLING MONOCYTES)

The original concept of MPS implicated that classical monocytes are recruited in the tissue to become tissue-resident macrophages in homeostatic conditions, and inflammatory activated macrophages during an infection (27, 54). We will examine more in detail the role of recruited cells during the inflammatory response later, while here we will focus on the recruitment of monocytes in homeostasis and their contribution to maintaining the pool of tissue macrophages. In order to avoid misunderstandings, it is important to agree on the definition of monocyte. In our view, *bona fide* monocytes are restricted to the blood compartment, and to the bone marrow and spleen (55), where they wait to be released in the blood. For obvious reasons, in both these compartments, monocytes should not initiate any inflammatory reaction, but they must be ready to be recruited into the blood first and subsequently to all organs and tissues. A phenomenon was recently reported, termed “anticipatory inflammation,” whereby Ly6C⁺ classical monocytes are released from the bone marrow in diurnal rhythmic waves under the control of circadian gene *Bmal1* (or *Arntl*) (56) to provide an adequate innate response to environmental challenges that are expected to occur with a evolutionarily predicted frequency. Despite new evidence supports the view that Ly6C⁺ classical monocytes are not precursors of resident macrophages in all tissues and during certain types of inflammation (see below), it is clear that circulating monocytes contribute to the repopulation of tissue-resident macrophages under homeostatic conditions in tissues like the *lamina propria* of the small intestine and healthy skin. Studies based on functional and lineage tracing and adoptive transfer have revealed that Ly6C⁺ monocytes are precursors of intestinal macrophages that have a short half-life of only 3 weeks (57–59). Conversely, in the dermis are present both resident dermal macrophages and monocyte-derived macrophages (60, 61). A recent work suggests that the number of macrophages is partially replenished by monocytes also in the heart (62) and in the lung (63). It is unknown why some tissue macrophages are constantly maintained by circulating monocytes, whereas other populations

are independent on circulating monocytes (see below). The notion that monocyte-derived macrophages derive from Ly6C⁺ cells suggests that the repopulation/maintenance of resident macrophages in steady-state conditions follows the same mechanism as that occurring during inflammation.

The function of Ly6C⁺ monocytes in circulation remains poorly defined. In the attempt to identify an effective role of monocytes in the blood in homeostatic conditions (besides being precursor cells), a recent work has suggested a distinct surveillance phenotype for Ly6C⁺ monocytes (64). These monocytes can enter non-lymphoid organs without obligatory differentiation into macrophages or DC. The authors propose that these monocytes can upregulate MHC class II expression and subsequently recirculate to lymph nodes, where they are able to present antigens to T-cells. Considering that these cells retain a monocyte-like gene expression profile, the authors term them “tissue monocytes” (64). This study contributes to revising the role of circulating monocytes, suggesting that they are not only precursors of macrophages but also effector cells.

Regarding the role of the Ly6C⁻ subset in the blood in steady-state conditions, intravital microscopy studies have established that these cells display a “patrolling” phenotype, being able to crawl on the luminal surface of the vascular endothelium (45, 51). This patrolling behavior, along with the ability to phagocytose endothelial-associated particles, suggests that a primary role of these monocytes is sensing and scanning the endothelial surface for damage and/or the presence of pathogens (51). The patrolling monocytes mainly respond via Toll-like receptor 7 (TLR7) to local danger signals (while they are poorly responsive to bacterial products such as LPS) by producing inflammatory mediators (51). They are able to induce the intravascular recruitment of neutrophils, which trigger endothelial necrosis, and subsequently they clear the resulting debris (51). A similar patrolling feature and TLR7/TLR8-dependent reactivity were also detected in human CD14⁺CD16⁺⁺ monocytes (35).

Consistent with their functional role of surveillance of the endothelium integrity and with the fact that they are terminally differentiated cells, we agree with the view that Ly6C⁻/CD16⁺ can be considered as the tissue-resident macrophages of the blood. Regarding their ability to produce inflammatory factors, we speculate that the patrolling monocytes have a higher activation threshold than Ly6C⁺ monocytes; therefore, they should be able to produce an amount of inflammatory cytokines and chemokines sufficient for coordinating the repair of a damaged endothelium, but not enough to initiate a strong inflammatory reaction.

A summary of the roles of monocyte subsets in steady state vs. inflammatory conditions is reported in Table 2.

MACROPHAGES

TISSUE-RESIDENT MACROPHAGE DEVELOPMENT IN STEADY STATE: EMBRYONIC ORIGIN VS. MONOCYTE DERIVATION

Resident macrophages are heterogeneous and versatile cells found in virtually all tissues of adult mammals, where they can represent up to 10–15% of the total cell number in quiescent conditions. This number can increase further in response to inflammatory stimuli. The specialization of macrophages in particular microenvironments explains their heterogeneity. Macrophages

take different names according to their tissue location, such as osteoclasts (bone) (see **Box 1**), alveolar macrophages (lung), microglial cells (CNS), histiocytes (connective tissue), Kupffer cells (liver), and LC (skin). These populations have such highly different transcriptional profiles that they could be considered as many different and unique classes of macrophages (74). On the other hand, the functions of macrophages are the same in all tissues. They are key players in tissue development (by shaping the tissue architecture), in immune response to pathogens (by generating and resolving the inflammatory reaction), in surveillance and monitoring of tissue changes (by acting as sentinel and effector cells), and especially in maintenance of tissue homeostasis (by clearing apoptotic or senescent cells, and by remodeling and repairing tissues).

Table 2 | Functions of monocyte subsets in steady state and inflammatory conditions.

Subset	Function	
	Steady-state conditions	Inflammation
Ly6C ⁺	Replenishment of monocyte-derived macrophages in the tissue (gut, skin, heart, and lung)	Differentiation in M1-like functional phenotype and initiation of the inflammatory response
	Differentiation in Ly6C ⁻ cells in the blood and in the bone marrow	Antigen uptake in the tissue, recirculation to lymph nodes, antigen presentation in lymph nodes ("tissue monocytes")
Ly6C ⁻	Patrolling and surveillance of the luminal surface of the endothelium	Promotion of healing in ischemic myocardium, and tissue repair during infection
	Sensing viral nucleic acids	with <i>Listeria monocytogenes</i>

Box 1 | A hint on osteoclasts.

Osteoclasts are multinuclear giant cells with a hematopoietic origin, commonly known as bone macrophages. They function in bone resorption and are involved in a normal skeletal development, growth, and modeling, for the maintenance of its integrity throughout life, and for remodeling through calcium metabolism (65). Moreover, osteoclasts are able to interact with the hematopoietic system and the adaptive immune system (66). Excessive bone loss mediated by osteoclasts plays a major role in certain pathologic conditions, such as rheumatoid arthritis (RA) and osteoporosis (67, 68). On the other hand, insufficient bone resorption due to the lack of functional osteoclasts (as in CSF-1R knock-out mice) leads to excessive bone apposition and osteopetrosis (69).

Osteoclasts really seem a class of macrophages on their own. They are generated from mononuclear phagocyte lineage progenitors in the bone marrow, and their differentiation from an osteoclast precursor (PreOC) depends on CSF-1 and the engagement of receptor activator of nuclear factor- κ B (RANK) and its ligand (RANKL), a specific osteoclast differentiation factor (70). Recently, it has been shown that also IL-34 is involved in the osteoclast development (71).

Osteoclasts can differentiate *in vitro* from a cell population named monocyte-derived multipotential cells (MOMCs), which seem to originate from circulating CD14⁺ monocytes (72). *In vitro* induction of MOMCs from circulating CD14⁺ monocytes apparently requires their binding to fibronectin, and exposure to soluble factor(s) derived from peripheral blood CD14^{dim} monocytes (72).

Thus, culture of unfractionated peripheral blood monocytes with M-CSF and RANKL is sufficient to induce their differentiation into osteoclasts, and it has been assumed that osteoclast precursors are monocytes, although this has not been shown *in vivo*.

The question arises as to why osteoclasts, unlike other macrophages, have their own lineage of commitment and differentiation. Possibly, the reason may lie in the fact that they are phylogenically closely linked to the presence of bone, a tissue that develops late as compared to other organs and tissues during embryonic/fetal development, as in fact vertebrates are the most recent phylogenetic step in the evolution (73).

The view that tissue macrophages originate from circulating peripheral blood monocytes that migrate into tissues under a variety of stimuli, proposed and strongly supported by van Furth in the 1970s (4, 75, 76), needs to be reconsidered. In addition to a wealth of old data (77, 78), two new pieces of evidence have further weakened the view that monocytes are the precursors of tissue macrophages in steady-state conditions: (1) the finding of the macrophage origin from embryonic progenitors that seed developing tissues before birth and give rise to fetal tissue macrophages (79) and (2) the self-maintaining ability of tissue-resident macrophages through local proliferation in adulthood (13). The latter finding will be discussed hereafter.

Two main phases of embryonic hematopoiesis have been described in the mouse: primitive hematopoiesis and definitive hematopoiesis. The former takes place in the ectoderm of the yolk sac and gives rise to macrophages without going through a monocytic progenitor. The latter takes place in the fetal liver, which is initially seeded by hematopoietic progenitors from the yolk sac and subsequently by HSCs from endothelium of the aorta-gonads-mesonephros (80, 81). The fetal liver subsequently becomes the source of definitive hematopoiesis that generates circulating monocytes during embryogenesis. Spleen and bone marrow are also colonized via the circulatory system by hematopoietic progenitors that will ultimately differentiate there. After birth, upon bone formation, hematopoiesis passes from the fetal liver to the bone marrow. The definitive bone marrow hematopoiesis is the source of both Ly6C⁺ and Ly6C⁻ circulating monocytes, from which resident tissue macrophages were thought to derive (10).

The human embryonic hematopoietic system is organized roughly in the same way as in the mouse (82), and early studies propose that macrophages could arise in the embryo independent of bone marrow progenitors in human beings [for more extensive reading, see Ref. (83, 84)]. In summary, macrophages in fetal and adult tissues derive from at least three sources: yolk sac (giving rise to some tissue-resident yolk sac-derived macrophages), fetal liver

(giving rise to fetal liver-derived macrophages), and bone marrow (giving rise to tissue-resident bone marrow-derived macrophages and inflammatory bone marrow-derived macrophages, see below) (**Figure 1**). The primitive yolk sac-derived macrophages have two distinct characteristics: (1) their pattern of differentiation does not go through a monocytic intermediate state but they directly become mature macrophages in fetal tissues (85) and (2) unlike macrophages derived from definitive c-Myb-dependent hematopoiesis, they are independent of the transcriptional factor c-Myb during development, while depending on the transcriptional factor PU.1 (12).

Based on different experimental approaches, from lineage tracing (12, 48) to experiments carried out in parabiotic mice (64, 86), it is evident that monocytes do not contribute or contribute only minimally to the maintenance of peripheral tissue-resident macrophages in steady-state conditions in many adult tissues. Fate-mapping experiments have shown that the adult microglial cell population is exclusively derived from yolk sac progenitors (87, 88), whereas for LC in adult skin it was clearly demonstrated a mixed origin, from the yolk sac and from the fetal liver (12, 89). Moreover, using Myb-deficient mice that lack development of HSCs, followed by transplantation with genetically dissimilar bone marrow together with fate mapping, it has been observed that yolk sac macrophages can generate macrophages with a characteristically high expression of the F4/80 marker (F4/80^{bright} macrophages) in brain (microglia), skin (LC), liver (Kupffer cells), pancreas, and spleen (12). In kidney and lung, tissue-resident macrophages have a double origin, encompassing F4/80^{high} macrophages, derived from yolk sac, and F4/80^{low} macrophages, which have a hematopoietic origin and are continuously replaced by bone marrow-derived progenitors (12).

Moreover, F4/80^{high} shares a common gene signature with yolk sac macrophages, unlike F4/80^{low} cells, as shown by global transcriptional analysis (12). Also, for splenic red pulp macrophages, alveolar, and peritoneal macrophages, an embryonic origin has been confirmed, rather than a monocyte origin (48). All these experiments show that early embryonic progenitor-derived macrophages can persist in tissues to adulthood. As mentioned previously, an exception is the gut, which contains a large population of resident macrophages that are all blood monocyte-derived cells, in steady-state conditions (57). How the mutual contribution of yolk sac-derived macrophages and fetal liver-derived monocytes is regulated in each tissue is unknown, and likewise it is not known how these two distinct populations of macrophages are functionally and ontogenically related. Regarding how much yolk sac progenitors contribute to originating adult tissue macrophages vs. fetal liver hematopoiesis, there are different opinions. One hypothesis is that fetal liver-derived monocytes proliferate and differentiate into adult tissue macrophages markedly diluting the population of yolk sac-derived macrophages (e.g., in lung and heart). This hypothesis stems from the observation that generation of yolk sac-derived macrophages does not go through a monocytic intermediate, therefore being in contrast with normal adult hematopoiesis, while a fetal liver origin for tissue macrophages would be reminiscent of the adult scenario in inflammation (90). Conversely, others believe that all tissue macrophages derive from yolk sac during the embryonic development, and circulating monocytes do not seed the majority of the adult tissues in mice (except kidney and lung) (12, 91) (**Figure 2**). This concept is strengthened by findings in human beings, where a complete loss of CD16⁺ monocytes seems to be of little consequence (92), and many tissue macrophage populations appear to be intact in patients with monocytopenia caused

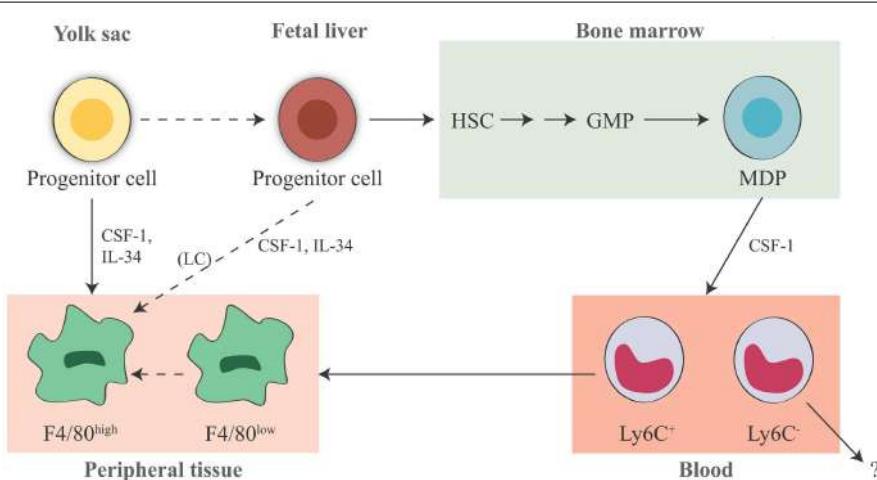
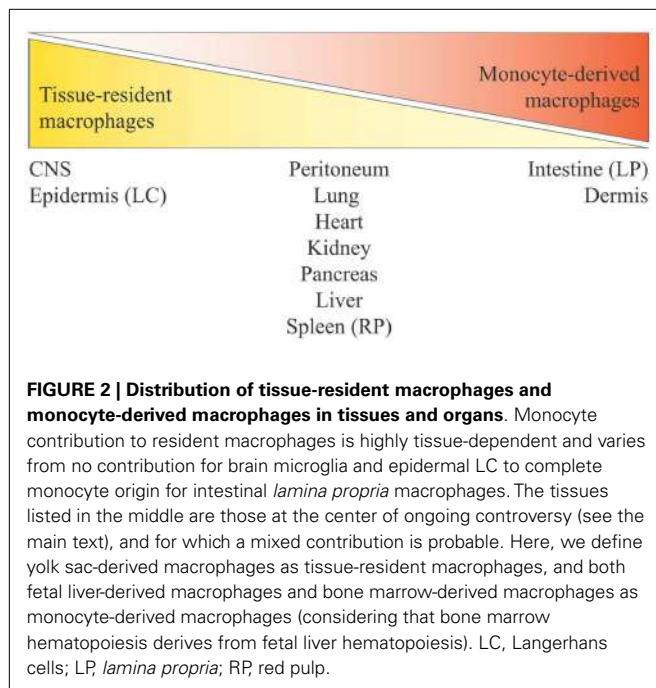


FIGURE 1 | Origin of tissue-resident macrophages in the mouse. In adult tissues, macrophages derive from three sources. The first is the yolk sac in the embryo, where primitive hematopoiesis occurs giving rise to progenitors that seed tissues with F4/80^{high} macrophages. Later during fetal development, hematopoiesis shifts from the yolk sac to the fetal liver (that seems to contribute to the LC pool in the skin, possibly through a yolk sac-derived progenitor). It is unknown whether other resident macrophages in other tissues may also derive from fetal liver hematopoiesis. The third source is the bone marrow, where definitive hematopoiesis occurs in the fetus and in the

adult, giving rise to monocytes and to monocyte-derived F4/80^{low} macrophages. Expression of murine F4/80 (the human EMR1) is an insufficient marker to discriminate between monocyte-derived macrophages and tissue-resident macrophages. It seems that Ly6C⁺ monocytes are the precursors of tissue macrophages, while the exact contribution of Ly6C⁻ monocytes remains unclear. HSC, hematopoietic stem cell; GMP, granulocyte-macrophage progenitor; MDP, macrophage-dendritic cell progenitor; LC, Langerhans cell; CSF-1, colony-stimulating factor 1; IL-34, interleukin 34.



by immune deficiency syndromes (93, 94). In conclusion, to which extent different populations of yolk sac-derived macrophages may be later replaced by fetal liver-derived macrophages or monocytes, and how yolk sac-derived tissue-resident macrophages can proliferate locally through life to maintain their own pool independently of adult monocyte input, these issues remain a matter of debate (90, 91, 95). Thus, three main issues arise from all these findings:

1. The origin of adult macrophages in steady-state conditions can vary considerably between tissues.
2. The exact role of the patrolling Ly6C⁻ monocytes remains unclear, while Ly6C⁺ monocytes are recruited predominantly to sites of infection or injury, or to the organs and tissues that have continuous cyclic recruitment of macrophages (such as the uterus), or that are exposed to microbiota (such as the gut and the skin).
3. Due to some limits and weaknesses of the published studies (whole blood irradiation or other myelo-ablative treatments, parabiotic mice, engrafted bone marrow or monocytes, adoptive transfer of radiolabeled cells, Cre-*loxP*-based fate mapping, CCR2 or CSF-1 inactivation, etc.) (9, 90), none of such studies provides conclusive evidence against a role for monocytes in tissue macrophage homeostasis. Thus, more efficient and specific fate-mapping models of yolk sac-derived macrophages and fetal liver-derived monocytes are needed, along with further investigation, to determine which tissue macrophage populations are constantly replenished by circulating monocytes and which are not.

SELF-RENEWAL/PROLIFERATION CAPACITY OF TISSUE-RESIDENT MACROPHAGES IN HOMEOSTASIS

Given that it is currently not possible to discriminate the two populations of tissue macrophages (yolk sac-derived and

monocyte-derived) during homeostasis, we will report their ability to proliferate without considering them as distinct subpopulations. In any case, we will bear in mind the notion that the tissue macrophages can maintain their number in the absence of monocyte precursors both in steady-state conditions (12, 48, 64, 86) as well as in genetically or experimentally moncytopenic situations (94, 95).

It is important to clarify the difference between self-renewal and proliferative capacity. As stated by Sieweke and Allen (13), in immunology, self-renewal is understood as a replacement of a certain cell population, while in stem cell research as the capacity to generate with a cell division a daughter cell showing the same identity as the parental cell. Local proliferation of tissue macrophages can be considered as self-renewal in both senses [see Ref. (12, 13)], since macrophages can proliferate without change of their differentiated phenotype (96). Having said that, recent evidence demonstrated that macrophages within the adult tissues self-renew via proliferation in homeostatic conditions rather than through an influx of progenitors. This has been shown for LC, which are able to proliferate (97) both in human beings (98) and in mice (99), for brain microglia (19), resident peritoneal macrophages (100), and alveolar macrophages (101). The self-renewal process is regulated by growth factors and cytokines such as CSF-1 and GM-CSF (Box 2).

Resident macrophages can proliferate at low levels in steady-state conditions, but proliferation rates strongly increase after macrophage depletion (86) or under inflammatory challenge (13). Regarding their proliferative ability, Ginhoux and Jung (90) raise the interesting question as to “whether all macrophages within a tissue possess equal self-renewal potential, or whether there are macrophage subpopulations that differ in their capacity of survival and proliferation, which would imply the existence of macrophages subpopulations with stem cell-like features.” The question arises from observations that physiological or experimental depletion of LC leads to a clonal expansion of LC by adjacent proliferative cell clusters with stem cell-like features (120), and that among lung macrophages, different cells can proliferate to maintain the population (87). To solve this issue, there is evidence that a macrophage that had previously divided has the same probability of entering the cell cycle as a cell that had not, suggesting the same proliferative ability for all macrophages (87). This is consistent with the observation that macrophages genetically modified to have an indefinite self-renewal potential can be efficiently cloned (96).

During inflammation, things are quite different, especially because the tissue is enriched with monocyte-derived macrophages. We will discuss later the replenishment of tissue macrophages by monocyte-derived macrophages and their ability to proliferate.

TISSUE MACROPHAGE FUNCTIONS

Table 3 summarizes the functions of resident macrophages in the main body tissues. These functions, mirroring different phenotypes (74, 143), are specific because depending on different tissue microenvironments. Different tissues define different phenotypes of both resident macrophages and monocyte-derived macrophages recruited from the reservoirs of blood, spleen, and

Box 2 | Factors driving monocyte/macrophage self-renewal, proliferation, and functional differentiation.

Macrophage colony-stimulating factor (M-CSF, also known as CSF-1) and granulocyte-macrophage colony-stimulating factor (GM-CSF) drive the monocyte/macrophage development, differentiation, and proliferation along with cytokines such as IL-4 (102) and the recently discovered IL-34 (103). Macrophages and circulating monocytes express the CSF-1 receptor (CSF-1R) (42, 43), and mouse deficient in CSF-1R (osteopetrotic mice, *op/op*) have a decreased number of monocytes in the bone marrow and in circulation, in addition to a decrease in osteoclasts (69, 104). Experimentally blocking CSF-1R with antibodies leads to a reduction in Ly6C⁻ monocytes (69) and to an associated increase in Ly6C⁺ monocytes, suggesting the involvement of CSF-1 in the maturation of monocytes from Ly6C⁺ to Ly6C⁻ (48, 104). CSF-1 is constitutively produced by mesenchymal cells (105) and is detectable in circulation in resting conditions (20). Under homeostatic conditions (106), CSF-1 promotes monocyte development and macrophage proliferation (107), which is controlled in a negative feedback loop. In fact, mature mononuclear phagocytes express high level of CSF-1R and are responsible for the clearance of CSF-1. The decreased CSF-1 levels lead to a decrease in mononuclear cell proliferation, thereby maintaining the cell number to normal levels both systemically and locally [(21, 22); a model of CSF-1-dependent local homeostasis of macrophage density has been described by Jenkins and Hume (9)]. Thus, elevated production of CSF-1 can drive both an increased proliferation of resident macrophages and an increased recruitment of monocytes (103, 108) via macrophage production of CCL2 (109). CSF-1 deficiency in mice affects distinct tissues by different degrees, ranging from marked cell loss in the gut, kidney, peritoneal cavity, and in circulation, as compared to liver (86). CSF-1 is also involved in the proliferation of splenic red pulp macrophages and bone marrow macrophages (110). GM-CSF is also critical for macrophage homeostasis and proliferation, especially in the lung (111) and in the peritoneal cavity *in vivo* (112), but it is less important in hematopoiesis, and, therefore, for monocyte development (113). GM-CSF can support monocyte expansion and differentiation *in vitro* (25, 114), and it seems to be mainly involved in induction of hematopoiesis during inflammation rather than in homeostasis (115, 116). CSF-1 and GM-CSF are also involved in monocyte/macrophage functional differentiation programs: CSF-1 stimulation leads to a homeostatic or anti-inflammatory M2-like phenotype (25, 117, 118), whereas GM-CSF leads to an M1-like inflammatory phenotype (25, 117–119). Thus, CSF-1 stimulation represents a default homeostatic/M2 pathway of monocyte development (119). In summary, CSF-1 is mainly involved in self-renewal of tissue macrophages, consistent with its role in M2 polarization, while GM-CSF is involved in proliferation of monocyte-derived inflammatory macrophages, consistent with its role in M1 polarization.

Table 3 | Macrophage functions and the pathological consequences of their anomalous activation in the main tissues.

Macrophages (MΦ)	Tissue	Functions	Pathology
Microglia	Brain	Brian development (121), immune surveillance, synaptic remodeling (122)	Neurodegeneration (123)
Osteoclasts	Bone	Bone modeling and remodeling, bone resorption (124), support to hematopoiesis (125)	Osteoporosis, osteopetrosis, arthritis (126)
Heart MΦ	Heart and vasculature	Surveillance	Atherosclerosis (127)
Kupffer cells	Liver	Toxin removal, lipid metabolism, iron recycling, erythrocyte clearance, clearance of microbes, and cell debris from blood (128, 129)	Fibrosis (130), impaired erythrocyte clearance (131)
Alveolar MΦ	Lung	Surfactant clearance, surveillance for inhaled pathogens (132)	Alveolar proteinosis (133)
Adipose tissue-associated MΦ	Adipose tissue	Metabolism, adipogenesis, adaptive thermogenesis (134)	Obesity, diabetes, insulin resistance, loss of adaptive thermogenesis (131)
Bone marrow MΦ	Bone marrow	Reservoir of monocytes, waste disposal (131)	Disruption of hematopoiesis (131)
Intestinal MΦ	Gut	Tolerance to microbiota, defense against pathogens, intestinal homeostasis (135)	Inflammatory bowel disease (136)
Langerhans cells	Skin	Immune surveillance (137)	Insufficient healing, fibrosis (138)
Marginal zone MΦ, red pulp MΦ	Spleen	Erythrocyte clearance, iron processing, capture of microbes from blood (139)	Impaired iron recycling and erythrocyte clearance (140)
Inflammatory MΦ ^a	All tissues	Defense against pathogens, protection against dangerous stimuli (141)	Chronic inflammation, tissue damage, autoimmunity (91)
Healing MΦ ^b	All tissues	Branched morphology, angiogenesis (142)	Cancer, fibrosis, epithelial hyperplasia (91)

^aAlso known as inflammatory macrophages or M1 macrophages.

^bAlso known as deactivated or M2 macrophages.

bone marrow (10), phenotypes that are necessary for the tissue-specific needs of defending, maintaining, and regaining homeostasis (144). These homeostatic functions may be altered by chronic insults, which may lead to an anomalous prolongation/amplification of the macrophage attempt to regain homeostasis and to a consequent causal association between macrophages and diseases (**Table 3**). In pathological conditions, the distinction between tissue-resident macrophages and recruited inflammatory macrophages has not yet been possible. For an in-depth analysis of these issues, the reader can refer to recent exhaustive reviews (99, 144, 145). Apart from tissue-specific functions, tissue macrophages share a series of common functions encompassing clearance of cell debris, immune surveillance, wound healing, defense against pathogens, and the initiation and resolution of inflammation. In this review, we will only focus on the role of macrophages in inflammatory responses, considering their capacity to polarize into different functional phenotypes in response to the tissue microenvironmental changes that occur during the different phases of an inflammatory response. This polarization process is based on the M1–M2 paradigm (see below).

PLASTICITY OF MONOCYTES/MACROPHAGES DURING INFLAMMATORY REACTION

MONOCYTE RECRUITMENT DURING THE INFLAMMATORY REACTION: INFLAMMATORY MONOCYTE-DERIVED MACROPHAGES VS.

TISSUE-RESIDENT MACROPHAGES AND THEIR PROLIFERATIVE ABILITY

During the first phases of an inflammatory reaction, there is in the tissue an increase of the effector cell number, necessary for increasing the immune defensive firepower. These cells are

monocyte-derived macrophages. The concomitant drastic loss of resident macrophages, due to tissue adherence, emigration, or death, is a phenomenon termed “the macrophages disappearance reaction” (146), and it is especially evident for peritoneal and alveolar macrophages. To cope with the need of increasing the number of effector cells, two strategies come into play.

First is the recruitment of blood monocytes, driven by resident macrophages alongside with other tissue cells. Recruited blood monocytes are a source of inflammatory macrophages, which take the name of bone marrow-derived or monocyte-derived inflammatory macrophages. The other strategy is the increase of tissue-resident macrophage proliferation by enhancement of their self-renewal ability.

Central to the issue of monocyte recruitment is the difference in monocyte subset trafficking. Such differences have been observed to occur during acute and chronic inflammation in mice, and underline the fact that the monocyte subsets are under the control of distinct trafficking mechanisms, with the classical subset being recruited via CCR2 and the non-classical one utilizing a CXCR1-dependent pathway (see **Box 3**).

In a model of *Listeria monocytogenes* infection, non-classical monocytes (Ly6C⁻) extravasate rapidly within 1 h, invade the surrounding tissues, and develop a very early inflammatory response by producing chemokines responsible for recruiting other effector cells (granulocytes, NK cells, T-cells), and cytokines such as TNF- α (central to macrophage-mediated inflammation and innate responses) (45). This inflammatory response is transient, and 8 h after infection, the main producers of inflammatory cytokines in the tissue are the classical monocytes (Ly6C⁺). As

Box 3 | Chemokines and monocyte recruitment mechanisms.

The two main chemokines and related receptors involved in the inflammation-dependent recruitment of the Ly6C⁺ and Ly6C⁻ monocyte subsets from the blood, bone marrow, and spleen, are CCL2/CCR2 and CX3CL1/CX3CR1, respectively (45, 46). Fibroblasts, epithelial, and endothelial cells produce CCL2 in response to inflammatory cytokines or microbial molecules, and generate a high level of this chemokine in the inflamed tissue (to allow egress of monocytes from the blood and entry in the tissues) and/or in blood (to allow entry of bone marrow monocytes) (44, 147). Recently, it has been proposed that both mesenchymal cells and progenitor cells closely apposed to bone marrow vessels can produce CCL2 in inflammatory situations, to allowing the egress of monocytes from the tissue and their subsequent entry into the blood (148). During a bacterial infection, Ly6C⁺ monocytes require CCR2 for being recruited from the bone marrow into the blood (149). In mice lacking CX3CR1, a reduction of patrolling by Ly6C⁻ was observed (45), and a reduction of their number in infarcted heart (150), suggesting an impaired recruitment from the blood. Genetic destruction of CCR2 reduces the accumulation of both Ly6C⁺ and Ly6C⁻ monocytes in injured skeletal muscle, but it does not alter the recruitment of Ly6C⁻ monocytes in the heart after myocardial infarction (150). A reduction was also observed in skin wounds on the first day from injury, when Ly6C⁺ cells are those principally involved in the early repair phases, but not during the late stage of tissue repair, when Ly6C⁻ cells are dominant (151). These studies underline the importance of monocyte recruitment from blood to the tissue in the injured cardiac or skeletal muscle. Regarding the role of *in situ* differentiation, in addition to the data mentioned above (150), a reduction of Ly6C⁻ monocytes has been observed also in the blood of CCR2-deficient mice, despite the fact that they do not express this receptor (48). Macrophage accumulation in skin wounds is also reduced in mice lacking CX3CR1 (152). CX3CL1 and CX3CR1 provide a survival or anti-apoptotic signal to Ly6C⁻ cells (153). Two models have been proposed for the CCL2-dependent Ly6C⁺ cell recruitment from the bone marrow: CCL2 increases monocyte chemokinesis and contact with blood vessels; CCL2 associates with tissue glycosaminoglycans and forms a gradient driving monocytes to exit the bone marrow for entering into circulation (54). Intravenous administration of CCL2 leads to the mobilization of monocytes into the circulation, which is consistent with a role for peripheral CCL2 production responsible for replenishment of circulating monocytes from bone marrow (154).

Under steady-state conditions, the release of Ly6C⁺ and Ly6C⁻ monocytes from bone marrow depends on two genes, the circadian clock gene *Bmal1* for Ly6C⁺ cells (55), and the G-coupled receptor for sphingosine-1-phosphate S1PR5 for Ly6C⁻ monocytes (155). In mice with myeloid cell-restricted *Bmal1*-deficiency, the rhythmic release of CCL2 was ablated along with monocyte pools. Thus, myeloid cells produce low diurnal levels of CCL2 in a circadian fashion, and CCL2 in turn stimulates the release of CCR2-expressing monocytes from the bone marrow into the blood. On the other hand, in S1PR5-deficient mice, Ly6C⁻ monocytes are retained in the bone marrow and are not released in the blood and spleen.

previously mentioned (45), it has been observed that the two subsets of monocytes differentiate into two distinct cell types. Ly6C⁻ patrolling monocytes initiate a macrophage differentiation program that resembles that of M2 macrophages (see below), while Ly6C⁺ monocytes differentiate into DC-like cells that resemble Tip-DC (45). However, in other systems, this double recruitment of different monocyte subsets has not been observed. Only Ly6C⁺ monocytes were observed to migrate to the injured tissue in a model of skeletal muscle injury and be responsible for early inflammatory responses (156). Generally, classical monocytes infiltrate inflamed tissues more robustly than their non-classical counterparts, and their number is significantly increased in the circulation during systemic or chronic infection (27). After engulfing dying cells in the tissue, the recruited classical monocytes differentiate into cells that resemble Ly6C⁻ monocytes, and become involved in tissue repair mechanisms (156). Likewise, in a mouse model of sterile wound (subcutaneous polyvinyl alcohol sponge implantation), it has been recently demonstrated that Ly6C⁺ monocytes recruited from the circulation into the skin acquired an inflammatory function and, despite time of maturation was long, they matured into Ly6C⁻ macrophages with repair functions (157). Yet, another situation is that of myocardial infarction, during which both monocyte subsets appear to home to the same tissue at different stages of inflammation (150). Specifically, the Ly6C⁺ subset first infiltrates the infarcted heart and exhibits inflammatory functions, while the Ly6C⁻ subset is recruited at a later stage and promotes tissue healing by expressing high amounts of vascular endothelial growth factor, exhibiting angiogenic capacity, and promoting deposition of collagen (150).

In atherosclerosis, as a model of chronic inflammation, both monocyte subsets are recruited at the same time to the activated endothelium/plaques, and healing seems to be correlated with a reduction in total monocyte recruitment (158). However, it was recently demonstrated that the maintenance and accumulation of monocyte-derived macrophages in atherosclerotic plaques mainly depend on local proliferation of bone marrow-derived macrophages rather than on the influx of circulating monocytes (127, 159). In an atopic dermatitis model and in experimental autoimmune encephalomyelitis, a massive proliferation of LC and microglia cells has been observed (160, 161), despite a significant monocyte influx (161). In the peritoneal cavity and in the lung, where the macrophage disappearance phenomenon occurs upon bacterial and virus insults, the few remaining macrophages are responsible for repopulating the tissue (86, 101, 106). Similarly, in the context of Th2-mediated immunity against nematode infection, IL-4 drives tissue-resident macrophage expansion in the pleural cavity in the absence of peripheral monocyte recruitment (102).

Proliferation of macrophages is observed in a variety of human diseases [see Ref. (91)], including tumor-associated macrophages in solid tumors (162), and adipose tissue-associated macrophages in obesity (163).

In this context, a question is still open. Having established that monocytes are recruited into tissues during an inflammatory event, to what extent are they capable to differentiate in tissue macrophages and to proliferate? As proposed by Jenkins

and Hume, the negligible contribution of monocytes to the pool of resident macrophages could be due to the fact that monocyte recruitment is specifically aiming at providing a population of functionally differentiated cells needed for resolving an acute inflammatory event, rather than being triggered by the homeostatic need of maintaining the autonomous pool of resident macrophages (9). This view is supported by another interesting hypothesis, i.e., in inflammatory conditions, monocyte-derived macrophages are mostly end-type killer cells, as the non-specific toxic molecules they produce will also cause their own death (164).

The gastrointestinal tract provides evidence in favor of this hypothesis. In the gut, blood monocytes are constantly recruited to the tissue where they contribute to maintaining the resident macrophage population, but during an inflammatory event they re-program their differentiation plan toward adopting an inflammatory phenotype (57, 165).

Thus, we should consider that monocyte-derived macrophages adopt different and opposing phenotypes based on microenvironmental signals. Adoption of a phenotype or another depends on the time by which the sequential waves of recruited Ly6C⁺ monocytes reach the tissue during the course of the inflammatory reaction, since the incoming monocytes will find a different microenvironment in different phases of the reaction. In this context, it is conceivable that monocytes entering the tissue at later times could find conditions favorable to adopting an M2-like phenotype (see above), thereby becoming tissue macrophages over time.

Inflammatory monocyte-derived macrophages (12, 86) and tissue monocytes (64) can be phenotypically and functionally distinguished from resident macrophages in many tissues. In the central nervous system, inflammatory monocyte-derived macrophages do not contribute to the resident population (161). In contrast, fate-mapping experiments revealed that monocyte-derived macrophages recruited to the peritoneal cavity upon thioglycolate injection differentiate into resident macrophages and persist over time (48). The fraction of monocyte-derived macrophages that do not die upon inflammation and become tissue-resident macrophages share gene profiling with resident macrophages (45, 64, 165), but there is no information as to whether they are functionally different or not.

The accumulation of inflammatory monocytes in an inflamed tissue is due to their influx from blood rather than by their proliferative ability, and in fact inflammatory signals of microbial origin generally prevent their proliferation. An exception to this general paradigm comes from a recent study that has demonstrated that also inflammatory monocyte-derived macrophages can proliferate at certain stages during the resolution of zymosan-induced peritonitis (106).

All these findings are summarized in **Figure 3**.

Finally, two issues should be reminded:

1. The precise nature and extent of the contribution of monocyte-derived macrophages to tissue macrophages could depend on how, and to which extent, inflammation or its cause has affected the tissue-resident macrophages. In this view, as proposed by Ginhoux and Jung (90), tissue-resident macrophages are more involved in tissue macrophage repopulation after mild injury,

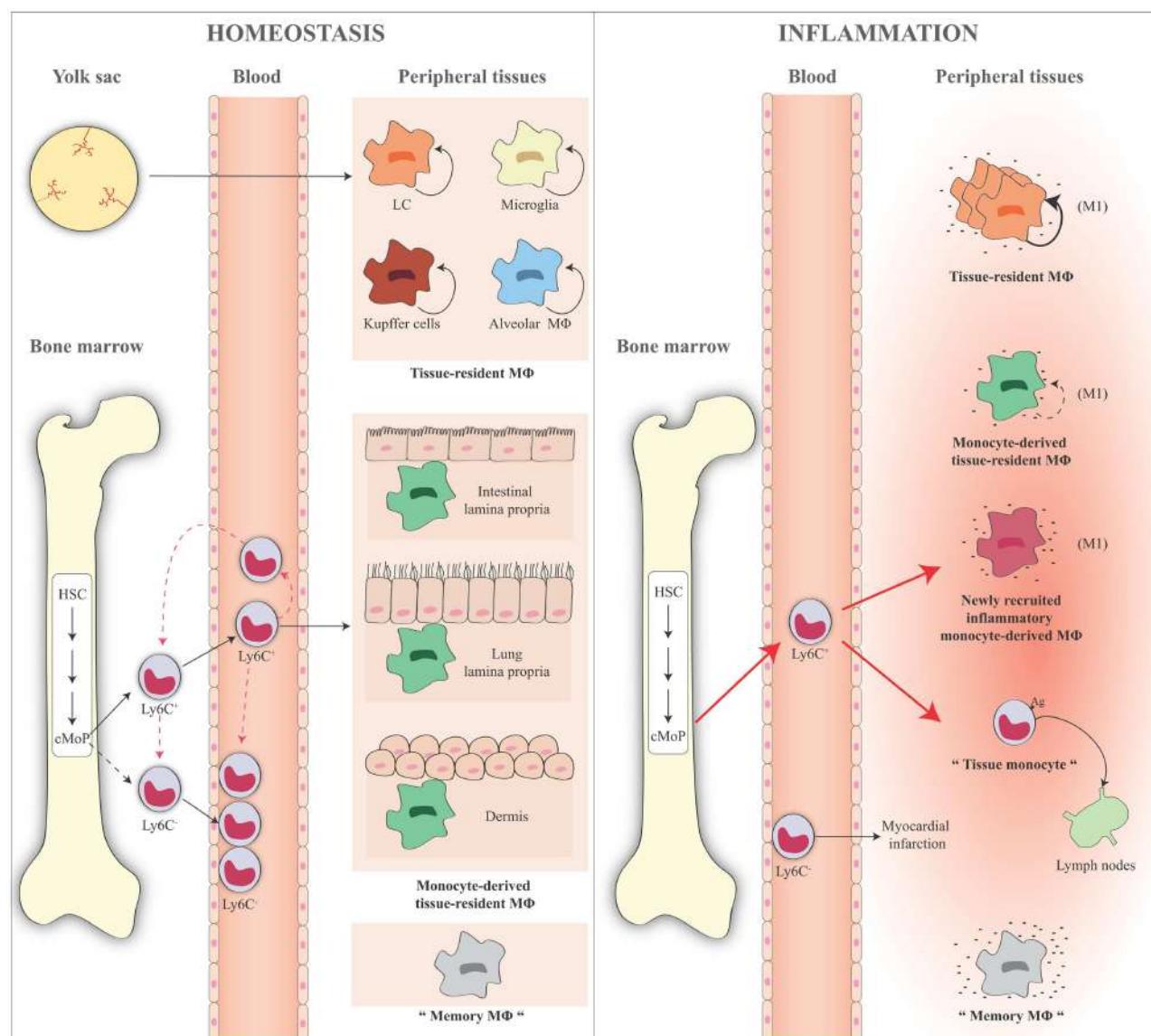


FIGURE 3 | Schematic representation of monocyte and macrophage populations in homeostasis and inflammation. Under homeostatic conditions (left panel), Ly6C⁺ monocytes derive from the bone marrow and circulate via the blood into the tissue. A minor fraction of these cells lose Ly6C expression and become Ly6C⁻ monocytes in the blood or in the bone marrow where some of them might return in the absence of inflammation. Ly6C⁺ blood monocytes enter tissues and become either macrophages, for example, in the gut, lung, and dermis (monocyte-derived macrophages or monocyte-derived tissue-resident macrophages). Some tissue macrophages derive directly from yolk sac during the embryogenesis (e.g., LC, microglia, liver Kupffer cells, and alveolar macrophages), are long lived, and are mainly maintained by self-renewal (tissue-resident macrophages). Ly6C⁻ monocytes act as resident macrophages of the vasculature, patrolling, and monitoring the endothelial surface in the blood vessel lumen. In the figure, the presence of "trained" macrophages is also considered, which we define as "memory macrophages," i.e., the tissue macrophages that retain the memory of a previous inflammation and are in a quiescent state in the tissue. During an inflammatory reaction (right panel), the number of blood Ly6C⁺ monocytes recruited to an inflamed tissue increases considerably. The large majority of these cells gives rise to the inflammatory monocyte-derived macrophages, while some of them do not differentiate

into macrophages and remain monocyte-like cells, are able to take up antigens, and to migrate to the draining lymph nodes (tissue monocytes). These are the antigen-uptaking and -presenting cells of the tissue. During inflammation, all macrophages (tissue-resident macrophages, monocyte-derived tissue macrophages, inflammatory monocyte-derived macrophages) are activated and differentiate into M1-like inflammatory cells following interaction with pathogenic and damaged signals/insults in surrounding microenvironment. These cells produce a series of cytokines and other inflammatory factors. Tissue-resident macrophages increase their capacity of proliferation to compensate the loss of macrophages caused by the inflammatory reaction. Recent evidence demonstrates that also inflammatory monocyte-derived macrophages are able to proliferate in a late phase of the inflammatory reaction. Memory macrophages are important players in the inflammatory reaction, as they can react to inflammatory stimuli with a faster and stronger inflammatory cytokine production. The role of circulating Ly6C⁻ cells during an inflammatory reaction is not fully identified. They probably remain in the blood vessels as sentinels, and in some cases they could enter in the tissue, as it has been reported in the case of myocardial infarction, to take up a repair function. HSC, hematopoietic stem cell; cMoP, common monocyte progenitor; Ly6C, lymphocyte antigen 6 complex; LC, Langerhans cells; MΦ, macrophages.

- while monocyte-derived macrophages are more involved in severe inflammatory injuries.
2. In the mouse it seems that non-classical monocytes contribute to the resident macrophage population. It is possible (although there is little evidence in this respect) that when non-classical monocytes are recruited in the inflamed tissue, they may differentiate into alternatively activated macrophages, while classical monocytes would give rise to classically activated macrophages. In this context, the developmental relationship between the different monocyte subsets and the different macrophage functional phenotypes has yet to be fully and formally proven. No evidence in this sense has been generated yet in human being (see below).

MACROPHAGE DIFFERENTIATION AND FUNCTIONAL PHENOTYPES

Macrophage polarization occurs through different activation programs by which macrophages carry out their defensive functions. In this way, macrophages become able to respond with appropriate functions in distinct contexts, functional diversity becoming the key feature of these cells. Essentially, macrophages can modify their metabolic functions from a heal/growth promoting setting (M2 macrophages), to a killing/inhibitory capacity (M1 macrophages) (145, 166). The main difference between these cells is that in M2 macrophages the arginine metabolism is shifted to ornithine and polyamines, while in M1 cells it is shifted to NO and citrulline (166). M2-produced ornithine can promote cell proliferation and repair through polyamine and collagen synthesis, fibrosis and other tissue remodeling functions (167), while M1-produced NO is an important effector molecule with microbicidal activity and cell proliferation inhibitory capacity (168). Interestingly, polyamine production *per se* has been reported to be a driver of M2 polarization (169), and M2 is the normal “default” program adopted by resident macrophages (170). Moreover, M1 and M2 macrophages have distinct features in terms of chemokine production profiles (171), and iron and glucose metabolism (172, 173).

The description of macrophages polarization is leading immunologists to take a step back and revise their concept on how the immune system works (14, 145). The M1 and M2 definition was formulated by mirroring the Th1/Th2 polarization concept. However, this definition might be misleading in that it may suggest that Th1/Th2 cells do instruct M1/M2 polarization, whereas it is now obvious that the reverse is true, i.e., macrophages are initiating and directing T-cell polarization. Since it has been shown that distinct populations of macrophages promote and control CD4⁺ T-cell-dependent type 1 and type 2 immune inflammatory responses (i.e., those against viruses and bacteria, and against multicellular parasites, respectively), not surprisingly they have been termed M1 and M2 (166). Thus, considering that macrophages recognize pathogens directly (174, 175), while T-cell do not, and considering that T-cells proliferate through interaction with macrophages (176), it is logical to think that macrophages are the cells that initiate and direct T-cell response, and that the adaptive immune response needs the triggering and guidance of innate immunity (14). Notably, M1 and M2 macrophage activities do not need the presence of lymphocytes (145). The fact that T-cell-derived cytokines such

as IFN- γ and IL-4 may amplify macrophage polarization (see below) should not deceive us into believing that macrophage polarization cannot occur without lymphocytes, as this is not the case. *In vitro*, macrophages are activated toward an M1 functional program by infectious microorganism-related molecules (e.g., the gram-negative product LPS) and by inflammation-related cytokines TNF- α or IFN- γ , alone or in combination. M1 macrophages are characterized *in vitro* by an IL-12^{hi}IL-23^{hi}IL-10^{lo} phenotype; are efficient producers of toxic effector molecules (ROS and NO) and inflammatory cytokines (IL-1 β , TNF, IL-6); participate as inducers and effector cells in polarized Th1 responses; and mediate resistance against intracellular parasites and tumors (177). Conversely, M2-like polarization has been observed *in vitro* in response to the Th2-related cytokines IL-4 or IL-13 (178), to the concomitant triggering of Fc γ receptors and Toll-like receptors (TLR), to immune complexes, and to anti-inflammatory molecules such as IL-10, TGF- β , and glucocorticoids (115). The variety of functional programs adopted by macrophages in response to the stimuli listed above has been termed M2a (IL-4 and IL-13; alternative inflammation), M2b (immune complexes, Fc γ R/TLR triggering), and M2c (IL-10, TGF- β , glucocorticoids; deactivation) (178, 179). M2 cells are characterized *in vitro* by an IL-12^{lo}IL-23^{lo}IL-10^{hi}TGF- β ^{hi} phenotype and generally have high levels of scavenger, mannose, and galactose-type receptors. In general, these macrophages take part in polarized Th2 responses, allergy, parasites clearance, dampening of inflammation, tissue remodeling, angiogenesis, immunoregulation, and tumor promotion (180).

Macrophage taxonomy is an attempt to rationally categorize an extended variety of cell functions. Indeed, the M1/M2 paradigm is a limited attempt to define the complexity and plasticity of mononuclear phagocytes. *In vivo*, macrophages can adopt a variety of functional phenotypes depending on subtle and continuous changes in the tissue microenvironment. So, the M1/M2 polarization of macrophage functions may be taken as a simplified conceptual framework describing a continuum of diverse functional states, of which M1 and M2 activation states are not ontogenetically defined subsets but represent the extremes (180–182). In this regard, Mosser and Edwards (181) have suggested a macrophage classification that takes into account the three functions of these cells in maintaining homeostasis: host defense, wound healing, and immune regulation. Classifying macrophages according to these functions provides three basic macrophage populations: classically activated macrophages, wound-healing macrophages, and regulatory macrophages (183, 184). The authors believe that this classification also helps to illustrate how macrophages can evolve to exhibit characteristics that are shared by more than one macrophage population (181).

Without going into details [for which we refer the reader to other reviews; (14, 185)], the M1/M2 classification in different subsets can create the illusory perception of a heterogeneity, which is not proven to exist *in vivo*. Thus, as already mentioned above, it is logical to hypothesize that the subsets are varying mixtures of M1- and M2-type macrophages, as observed in the lung and in the peritoneal cavity, where tissue-specific variations in the balance of M1- and M2-type responses have been revealed (74, 186). This situation has also been observed in pathological

conditions, where macrophages can develop mixed M1 and M2 phenotypes (187, 188). Moreover, it has also been proposed to consider the heterogeneity of macrophage functions as a consequence of interaction with different immunological pathways (e.g., interaction with different growth and survival factors, interaction with lymphoid and myeloid cytokines, interaction with pathogens, resolution), rather than attributing them to distinct macrophages subsets (185).

In summary, the initial inflammatory response is carried out by activated macrophages in classical or alternative modality (depending on the triggering events), aiming at eliminating invading microbes by promoting the inflammatory response. Then, the resolution phase is carried out by macrophages in deactivated modality, unresponsive to inflammatory stimuli, and active in the elimination of the injured cells and tissue components, in promoting angiogenesis, cell proliferation, matrix deposition, and in general in tissue remodeling. The mechanisms that account for macrophage deactivation play a key role in maintaining homeostasis and keeping the immune response under control (189). Both innate and adaptive signals can influence the macrophage functional phenotype, which can have potentially dangerous consequences if not appropriately regulated. For example, classically activated M1 macrophages can cause damage to host tissues, predispose surrounding tissue to neoplastic transformation and influence glucose metabolism by promoting insulin resistance. Macrophages that are normally involved in wound healing can promote fibrosis, exacerbate allergic responses, and be exploited by pathogens for intracellular survival. These M2-type macrophages can contribute to the progression of neoplasia by promoting tumor survival (see **Table 3**).

CURRENT HYPOTHESIS ON MACROPHAGE POLARIZATION

Plasticity and flexibility are key features of macrophages and of their activation states. A controversial issue is whether a phenotypic and functional evolution of macrophages occurs *in vivo*, and how it happens. As mentioned above, it has been observed in mice that the M1 to M2 switch during the progression of the inflammatory response enables macrophages to perform different activities in the different phases of the reaction. The controversy refers to the mechanisms underlying this switch, i.e., whether M1 and M2 macrophages are phenotypically distinct subpopulations that can serve different functions in different phases of an inflammatory reaction (45, 150), or the same cells can shift from one to another functional phenotype in response to microenvironmental signals (156, 157).

Several hypotheses are attempting to explain the issue. A first hypothesis is that different subsets of monocytes or macrophages can adopt a different functional phenotype. Thus, Ly6C⁺ monocytes and/or monocyte-derived macrophages in the tissue become M1 macrophages, and Ly6C⁻ monocytes and/or tissue-resident macrophages become M2 macrophages. It is possible that resident macrophages maintain cytoprotective and reparative functions, whereas macrophages derived from circulating inflammatory monocytes perform mainly M1 type functions. This hypothesis is not fully supported by the studies previously cited, where in different situations it was possible to observe both the differentiation of Ly6C⁺ cells in M1 and of Ly6C⁻ cells in M2 (45, 150) and

the transdifferentiation from Ly6C⁺ M1 cells to Ly6C⁻ M2 cells (156, 157).

A second hypothesis is that there are sequential waves of monocyte recruitment into a tissue throughout the course of an inflammatory reaction. Therefore, monocytes recruited into the tissue at different times encounter different microenvironments with different signals that can polarize them in M1 during early phases and in M2 in late phases (156). In this case, cytokines and other microenvironmental signals in the tissue play a key role in determining the different functional phenotypes of macrophages. Although the role of cytokines in steering the macrophage functional phenotypes has been proven *in vitro* (179), the situation could be very different *in vivo*, where M2 activity is strongly increased in sterile wounds (157) or injured kidney (190) in absence of Th2-like cytokines IL-4 or IL-13 (which in any case do not induce the typical M2 phenotype, i.e., the deactivated healing/repairing functional phenotype). In these cases, M2 macrophages derive largely from M1 macrophages, with monocytes recruited from the circulation first acquiring an inflammatory phenotype, and then persisting in the tissue and maturing into repair macrophages.

Based on the latter data, a third hypothesis is that polarized macrophage populations can switch one to the other in response to different conditions. Data from *in vitro* studies demonstrate that human monocytes can acquire the phenotype of polarized M1 macrophages and then mature into M2 repair macrophages upon exposure in culture to sequential changes in the microenvironmental conditions (191). Other studies demonstrated that M2 macrophages are reprogramed to express M1 genes following exposure to TLR ligands or IFN- γ (192, 193).

A related question is whether both tissue-resident macrophages and monocyte-derived macrophages can polarize in M1 or/and M2 functional phenotypes. We have described above that tissue macrophages have basically an M2-like phenotype, whereas infiltrating recruited monocytes differentiate in M1 or M2 depending on the tissue conditions. For instance, it has been shown that tissue-resident macrophages, rather than recruited monocytes, are alternatively activated in the tissue during infection with *Litomosoides sigmodondis* (102). Also, recruited monocytes can be directly polarized into an anti-inflammatory M2 phenotype by basophil-derived IL-4, in order to alleviate allergic inflammation in the skin (194). Although it is not possible discriminating between tissue-resident and monocyte-derived macrophages in steady-state conditions, it seems that alternatively activated tissue macrophages have a transcriptional profile and phenotype different from that of alternatively activated monocyte-derived macrophages, with the latter having immunoregulatory properties (195).

It should be considered that *in vitro* studies do not fully recapitulate *in vivo* differentiation for two main reasons:

1. These studies are generally based on an heterogeneous population of monocytes, encompassing all the blood subsets (Ly6C⁺ and Ly6C⁻ in the mouse, and CD14⁺ and CD16⁺ in human being), thus it cannot be defined whether upon different stimuli the same cells can pass from a phenotype to another or whether different subsets are activated in response to different stimuli.

2. While M2 macrophages can convert to the M1 phenotype, the reverse generally does not occur, or it may only occur in particular conditions (e.g., in very mild inflammatory responses). In fact, M1 is probably an end-stage killer cell that dies during the inflammatory response, possibly succumbing to its own NO production, as it was demonstrated *in vitro* (196). So, their selective death may give the impression that they convert in M2 cells, which in fact proportionally increase (145). It seems that M1 vs. M2 polarization correlates with the capacity of macrophages to produce NO (166) as opposed to the important M2 driver TGF- β (164, 197, 198), thus the decrease in NO-producing macrophages would increase TGF- β production and amplify M2 polarization.

There are cases in which a phenotypic switch in the macrophage population occurs over time, often associated with pathology (91, 141). Three specific examples of this phenotypic switch are the following:

endotoxin tolerance, an altered state of responsiveness to secondary stimulation with LPS, resulting in a global and sustained switch of the gene expression program from an inflammatory M1 signature to an anti-inflammatory phenotype (199); obesity-induced insulin resistance or type 2 diabetes, and atherosclerosis lesions. These are *metabolic syndromes* that can lead to a switch in the phenotype of adipose tissue macrophages from wound healing (as in healthy non-obese human beings) to classically activated macrophages (200, 201); *cancer*, where the tumor-infiltrating classically activated macrophages have the potential to contribute to the earliest stages of neoplasia (202–204), and then, as the tumor progresses, can progressively differentiate to a regulatory phenotype and eventually become cells that share the characteristics of both regulatory and wound-healing macrophages (181).

Although the pathology provides the proof-of-principle that macrophages can undergo dynamic transitions between different functional states, it is possible that a mixture of M1/M2 phenotypes underlies these conditions (14, 145, 166). In the past few years, gene expression profiling techniques and genetic approaches have been used to cast some light on the plasticity of macrophage activation. The commonly held view is that macrophage polarization is driven by cues in the tissue microenvironment, which can include cytokines, growth factors, and microorganism-associated molecular patterns. These signals are thought to dictate a transcriptional response that shapes the phenotype and function of macrophages based on the physiological or pathological context. Progress has been made in defining the molecular mechanism underlying macrophage polarization, including signaling pathways, miRNA, epigenetic modification, post-transcriptional regulators, and transcriptional factors (189, 205–207). However, the data are still incomplete and far from being systematic, and our knowledge of the mechanistic basis of macrophage diversity in different tissues or in response to changing environment is to a large extent unknown.

POST-INFLAMMATION FATE OF MONOCYTES/MACROPHAGES

ANTIGEN PRESENTATION IN NON-LYMPHOID ORGANS

The capacity of taking up and presenting antigen (i.e., the linking function between innate and adaptive immunity) is one of the most important features of tissue macrophages (208). It has been mentioned above that some monocytes that enter the tissue during inflammation do not differentiate into macrophages, and are able to take up antigen in the tissue and carry it to lymph nodes where they can present it to naïve T-cells (64). In addition to this population of monocyte-like cells, tissue macrophages are also able to present antigen, despite the fact that they do not recirculate to lymph nodes after antigen uptake. That tissue macrophages are highly phagocytic and can take up microorganisms and other matter in the tissue is well known, as this is their major function both in homeostasis and during inflammation. That antigen presentation may occur also in non-lymphoid organs has been suggested by several experimental evidence describing antigen-specific local activation and expansion of primed T-cells, but not of naïve T-cells (209–215). Based on this evidence, the hypothesis proposed by Ley is that initial priming of naïve T-cells occurs in the lymph node (to which antigen-loaded tissue monocytes recirculate), but that the full activation and effector functions of T-cells occur in the tissue where the inflammatory reaction is taking place, upon the productive interaction and formation of immunological synapse between primed T-cells and the antigen-presenting tissue macrophages (the difference between monocyte-derived tissue DC and tissue macrophages is bleared, as they seem to be not much more than slightly different functional differentiation states from a common precursor). Most likely, the inflammatory monocyte-derived cells with an M1-like functional phenotype are the antigen-presenting cells (APC) that induce activation/polarization of effector Th1 and Th17 cells upon production of IL-12 and IL-23, respectively, and in a TNFRSF and TNFSF-dependent fashion (but independent of CD80, CD86, and CD28 co-stimulation). Likewise, M2-like tissue macrophages, which produce TGF- β and express the $\alpha V \beta 8$ integrin are likely involved in the polarization of iTreg cells, whereas their role in Th2 polarization is less clear (208).

FATE OF ACTIVATED RESIDENT MACROPHAGES AND RECRUITED MONOCYTES: PROLIFERATION, REPLACEMENT, AND M2-LIKE POLARIZATION

Based on what described above, the cell populations present in the tissue during the acute phase of an inflammatory reaction are the following:

- Tissue-resident macrophages and monocyte-derived macrophages. These, after initial recognition of microbial or damage-associated molecules, drive the influx of blood-derived monocytes, which will become inflammatory macrophages. Their role in initiating the inflammatory reaction possibly depends on the nature and grade of challenge.
- Monocyte-derived macrophages, newly recruited and rapidly occupying the inflammatory lesion, becoming the majority of the macrophages present in the tissue. These cells induce the

inflammatory response by differentiating in the M1 functional phenotype.

- Tissue monocytes, the recently described cells that can take up antigens in the tissue and move to lymph nodes, where they are able to present antigens to naïve T-cells.
- Memory macrophages, or trained monocytes, cells functionally programmed by a previously stimulus for either enhanced (training) or decreased (tolerance) cytokine production, depending on the type and concentration of the stimulus they encountered [(216); see below]. Here, we consider them as a kind of resident inflammatory monocyte-derived macrophage, able to react in a faster and stronger manner compared to other macrophages.

A summary of the different macrophage types and of their fate after the acute inflammatory phase is given in **Figure 4**.

In general, tissue-resident macrophages are maintained locally by proliferative self-renewal (100, 106), and retain an M2-like phenotype, for example, in the peritoneal cavity, brain, and lung (86, 100, 161). The fate of monocyte-derived resident macrophages is hard to follow, considering that it is not possible to fully discriminate between them. However, we may hypothesize that they have the same fate of tissue-resident macrophages, i.e., they maintain an M2-like phenotype and a low self-renewal capacity. A number of cells of both populations probably die during inflammation, the extent of their survival possibly depending on the nature and magnitude of the insult.

Generally, the inflammatory monocyte-derived macrophages are polarized toward M1, and the majority of them dies, killed by their own NO production (see above). In an experimental acute lung injury model, these cells undergo Fas-mediated death, while the resident alveolar cells persist (217). From that, we can argue that M1 likely is a terminal differentiation phenotype. However, there are reports that they can also undergo *in situ* phenotype conversion to become tissue-resident macrophages either during inflammation or after experimental deletion of tissue macrophages (48, 86). This underlines the notion that macrophage polarization is both transient and plastic.

The survival in the tissue of inflammatory monocyte-derived macrophages raises important questions that need to be answered.

Do monocyte-derived tissue macrophages conserve a “memory” of their past inflammatory activation, thereby becoming memory macrophages? And, do tissue macrophages resume their previous functional phenotype in response to a new inflammatory challenge? Or, do they react as naïve cells?

Memory macrophages (also recently termed “trained monocytes”) have been described, which retain a memory of past challenges (see below). Their fate in the tissue is, however, unknown, since no long-term experiments have been performed in mammals. It is possible that a part of them dies after reacting to a new inflammatory challenge. If some of them survive (again, this possibly depends on the type and magnitude of the new challenge), they would probably behave like inflammatory monocyte-derived macrophages, i.e., they could become M2-like cells, having a low level of self-renewal, and may also form a new population of memory macrophages that retain the memory of multiple challenges.

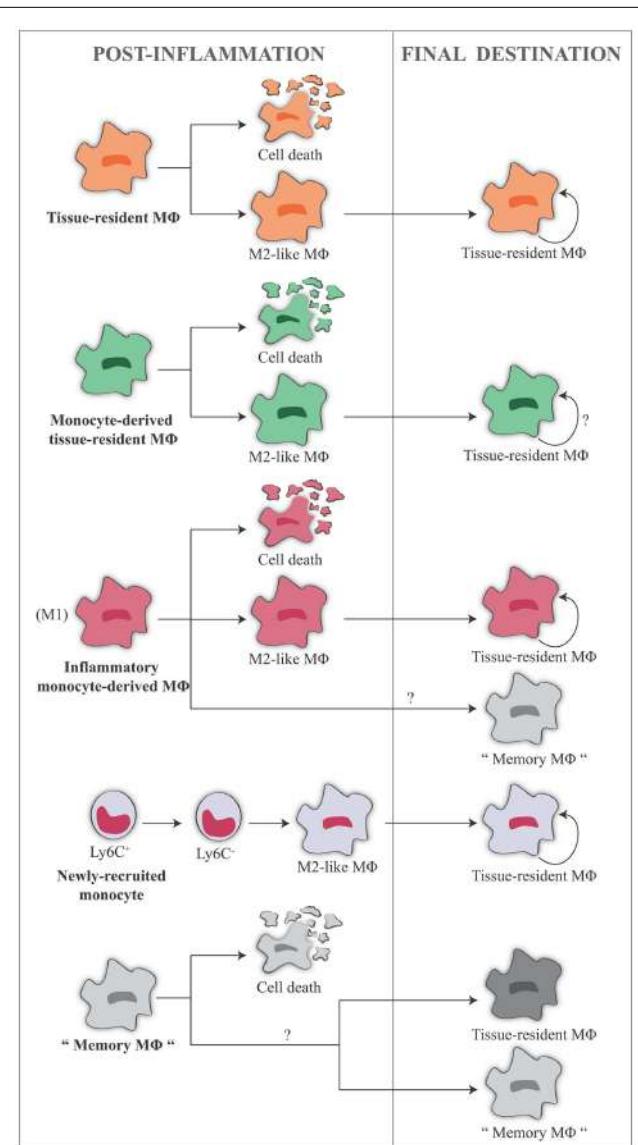


FIGURE 4 | Fate of the different monocyte/macrophage populations in the tissue during the post-inflammatory phase. Tissue-resident macrophages are in general maintained locally by proliferative self-renewal, and retain an M2-like functional phenotype. The same situation is hypothesized for monocyte-derived resident macrophages, since it is not possible to fully discriminate between the two populations. A number of cells of these two populations probably die during the inflammatory reaction. Inflammatory monocyte-derived macrophages can die killed by the NO they have produced, and the surviving cells can undergo *in situ* phenotype conversion and become M2-like tissue-resident macrophages. In addition, a number of these cells can conserve a “memory” of their past inflammatory activation, and become trained monocytes/memory macrophages. Monocytes recruited from the blood during the post-inflammatory phase can lose the expression of Ly6C and become Ly6C- cells, subsequently differentiating in M2 macrophages. They may also become memory macrophages. Memory macrophages that are present in the tissue, reminiscent of previous inflammatory events, would probably behave like naïve macrophages upon a new inflammatory challenge, except for a much quicker reaction, and will, therefore, mostly die or generate M2-like macrophages or again memory macrophages. Their life span in the tissue is presently unknown.

Another population that should be considered is that of monocytes recruited from the blood during the post-inflammatory phase. It is possible that these cells lose Ly6C expression when in the tissue, thereby becoming Ly6C⁻ cells that subsequently differentiate in M2 macrophages.

MEMORY MACROPHAGES

It is long known that innate immune responses are higher to a secondary infection/challenge, and that this higher reactive occurs whether the new challenge is the same or different from the first one (cross-protection). An old example is that of mouse peritoneal macrophages from BCG-infected mice that have little/no activity 7 days after infection, and acquire significant cytolytic activity upon *in vitro* challenge with LPS or with a wealth of other stimuli, while naïve macrophages do not (218). Recently, this phenomenon has been re-named *trained innate immunity* (219). Innate memory plays an important defensive role in organisms lacking adaptive immunity, such as plants and invertebrates, but it is evident also in vertebrates lacking functional T and B lymphocytes (220). In these animals, this innate memory mechanism was shown to involve innate immune cells with low turnover [such as macrophages and NK cells; (221, 222)] that would be responsible for improved pathogen recognition through pathogen recognition receptors, and for an enhanced protective inflammatory response (223, 224). NK cells could generate a memory response to viruses, while macrophages retain memory of both bacterial and viral challenges. A logical possibility is that the microorganisms encountered by the host on a regular basis may serve to differentiating and continually renewing a pool of memory-like macrophages with enhanced reactivity to infectious challenges. The molecular mechanisms responsible for shifting macrophages toward a memory status have not yet been elucidated. Putative mechanisms may involve differences in the monocyte/macrophage population (i.e., CD14⁺ and CD16⁻) or changes in the expression of lectin receptors on cell membrane (221), or in the functional phenotype (e.g., phagocytosis or protein production), but all are probably underlain by epigenetic reprogramming that, through modification of DNA, post-translational modifications of histones (methylation), or microRNA, regulates gene expression by inducing dynamic alterations in the chromatin structure (220). Establishment of macrophage memory, depending on the experienced challenges, is likely to rely on epigenetic changes, as these can be at the basis of a rapid evolution of responsiveness and adaptation to inciting events, thereby allowing to surviving to new environmental threats (220, 225). Efficacy of many vaccines probably implies the induction of non-specific macrophage memory that contributes to the increased resistance to infections. Research in the field of memory macrophages needs a thorough re-assessment of a large body of old evidence accumulated in the past decades in the areas of macrophage activation and of adjuvanticity.

CONCLUSION

An increasing amount of evidence supports four revolutionary concepts/discoveries on monocytes/macrophages that will force the researcher to rewrite the books of immunology:

1. The *embryonic origin of tissue-resident macrophages*, which raises the need to better understand the features/properties

of monocytes (that are no longer simple precursors of tissue macrophages), and those of macrophages, which are capable of self-renewal without loss of their differentiated cellular identity.

2. The *capacity of monocytes/macrophages to polarize* into distinct functional phenotypes able to initiate and direct virtually all immune responses, including adaptive ones.
3. The notion of *innate memory*, an old concept that has been recently revived with the description of the so-called trained innate immunity.
4. The importance of *macrophage-mediated antigen presentation* in tissue responses, with the identification of antigen-up-taking, recirculating, and presenting “tissue monocytes,” and with the notion that tissue macrophages are probably the major APC upon a second challenge at the tissue level, without need of recirculation to the lymph nodes.

The central role of monocytes/macrophages in this new view of immunity implies that innate immunity has a major role in inducing and modulating adaptive immunity (including the induction of polarized T-cell responses), while on the other hand taking advantage of adaptive immune mechanisms (e.g., T-cell-derived cytokines) for modulating its own activity. Thus, new knowledge on macrophage biology and functions will have a direct impact on our understanding of immune responses and on the design of novel therapeutic strategies. For this reason, it is necessary to overcome several experimental obstacles that delay the full understanding of the new dynamics and relationships within the immune system, and that have been identified by the researchers cited in the review.

For example, to date, transcriptome analysis of monocyte subsets has been done at the basal unstimulated level, showing dramatic differences consistent with a different functional repertoire for the three types of human monocytes. Circulating monocytes are most likely “quiescent” (their quiescent status is needed in order to avoid developing a deleterious intravascular inflammation), while their effector functions only develop after relocation and activation in the tissue. Thus, the true role of the different monocyte subsets could be only understood after activation, and the stimulus-induced transcriptome of these cells will be required. Further, the models of inflammation used to test the proliferative capacity of resident macrophages have so far been limited to one or two rounds of tissue repopulation or relatively acute periods of infection/inflammation. This obviously cannot provide reliable information on the long-term capacity of macrophage self-renewal. Moreover, when studying the plasticity and interchangeability of M1 and M2 macrophages, since mixed M1/M2 phenotypes can be found especially in pathological conditions, it is capital to focus not only on populations but also either at the single cell level or by lineage-tracking studies (e.g., with mice expressing Cre recombinase under the iNOS or arginase promoters, to track M1 and M2 lineages, respectively). Precautions need to be taken when drastic experimental procedures such as monocyte depletion or parabiosis are used to study macrophages self-renewal. These treatments can alter the concentration of circulating CSF-1 and CSFR1 signaling, which are important for self-renewal of resident macrophages under homeostatic conditions, and critical for differentiation of monocytes into tissue macrophages.

Likewise, precautions and appropriate controls need to be implemented when using CCR2-deficient mouse for studying monocyte recruitment to the tissue, since the CCL2/CCR2 chemokine system is also responsible of the release of monocytes from bone marrow. Thus, the lack of recruitment of monocytes from the blood to the tissue could be due to lack of release of monocytes from bone marrow to the blood, where circulating monocytes are decreased.

Our final recommendation, therefore, is probably obvious, but it is anyway important to state it again. We need to re-evaluate patiently and critically a huge body of experimental evidence that is already present in the literature. In particular, we need to overcome the lack of consensus in defining and describing the different macrophage phenotypes (226). Many old studies have already generated information that, in light of our present knowledge, can become very important and help us to clarify the general picture. Second recommendation is that of designing experiments very carefully, keeping in mind that the immune system is redundant and that the same factor can have different activities, and that the same activity can be carried out by different factors. Third recommendation: monocytes and macrophages are never isolated in the body, and what they do and what they become are totally influenced by the surrounding cells and tissue. *In vitro* systems may only partially reproduce this complexity. Last recommendation: consider evolution as an incommensurable and most precious source of information that can greatly help us understand the ontology and behavior of monocytes and macrophages. Common mechanisms are many, and also species-specific differences exist, thus we should be able to pick up the relevant common information without, however, forgetting that human being is not a mouse or a mosquito.

AUTHOR CONTRIBUTIONS

Paola Italiani wrote the paper; Diana Boraschi contributed to writing and critically revised the paper.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Charles Mills for helpful discussion and for his continuous assistance in writing this review. They also thank Elfi Töpfer for figure design and realization. This work was supported by HUMUNITY (FP7-PEOPLE-INT-2012 GA no. 316383), BioCog (FP7-HEALTH-2013-INNOVATION-1 GA no. 602461), and by the grant 2011-2114 of Fondazione Cariplo (Milano, Italy).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 22 August 2014; paper pending published: 23 September 2014; accepted: 02 October 2014; published online: 17 October 2014.

Citation: Italiani P and Boraschi D (2014) From monocytes to M1/M2 macrophages: phenotypical vs. functional differentiation. *Front. Immunol.* **5**:514. doi:10.3389/fimmu.2014.00514

This article was submitted to Inflammation, a section of the journal *Frontiers in Immunology*.

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Spatial, temporal, and functional aspects of macrophages during “The Good, the Bad, and the Ugly” phases of inflammation

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Keywords: macrophage activation, M1/M2 macrophages, microenvironment, phenotype, immunotherapy, adoptive

In Sergio Leone’s classic western drama “*The Good, the Bad, and the Ugly*” the final scene depicts the three protagonists, each with their specific personality trait and fast-draw capability, assembled in a graveyard for a shoot-out. The analogy is thus to focal sites of inflammation, with different subpopulations of myeloid cells assembled within a tissue in proximity to each-other, but with different functional phenotypes, associated surface marker expression, and enacting different functions. The basic macrophage functional states are described as *pro-inflammatory*, *anti-inflammatory*, and *wound healing*, respectively. The M1 versus M2 phenotypic paradigm was first coined to distinguish macrophage populations and has been instrumental in increasing our knowledge of myeloid biology (1). Despite more recent suggestions that there is a continuum of activation states between the extremes of M1- and M2-type responses (2–4), this partly reflects an over-emphasis on cell surface phenotypes. We should now have the technologies to be able to assess the relevance of specific cells within specific microenvironments within a given healthy or diseased tissue. The issue of functionality (irrespective of surface phenotype) and the concept of functional diversity within distinct microenvironments within a tissue have been less studied, and is the focus of this commentary.

Let us first consider in simplistic terms three stages of an inflammatory response (Figure 1A): the “Good” non-inflammatory phase in which normal tissue homeostasis is maintained by resident macrophages (green arrows); the “Bad”

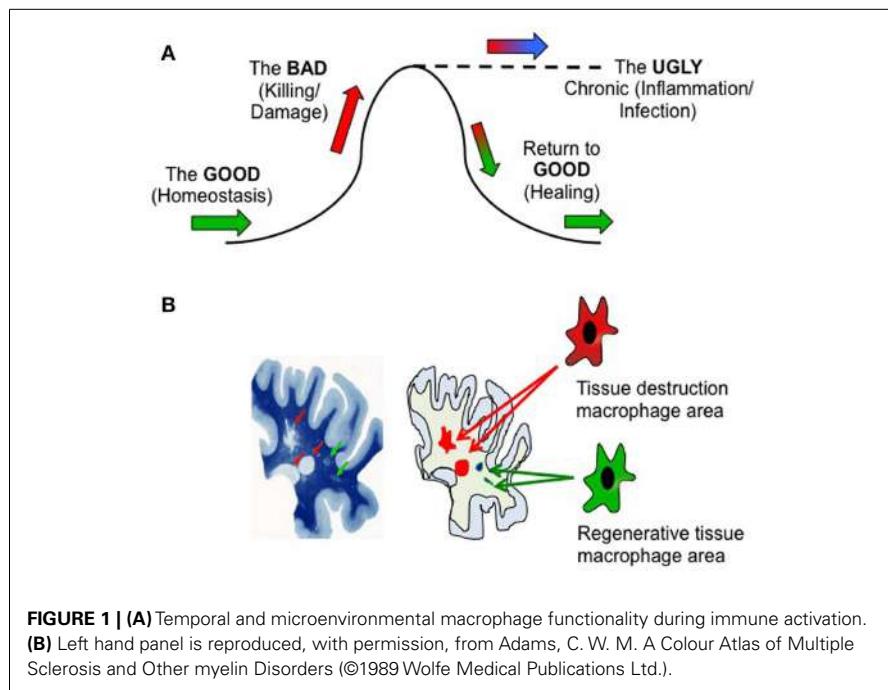
phase in which potentially tissue-damaging macrophage functionality is initiated due to damage, infection, or autoimmunity (pro-inflammatory) or tumor development (anti-inflammatory) (red); and the “Ugly” phase representing a failure to down-regulate the initial response that results in chronic pathogenesis and tissue damage (blue), i.e., an inability to return to the “Good” phase through healing. The definition of “good” and “bad” in this sense will depend on the setting – a pro-inflammatory response to an infection may be desirable, but if uncontrolled may lead to tissue damage. Likewise, while anti-inflammatory responses beneficially modulate autoimmune reactions, they contribute to tumor development. The salient point is that macrophage function will vary during these different phases.

MICROENVIRONMENTAL VARIATION IN INFLAMMATION

The aforementioned basic view reflects the *overall* functionality of an inflamed tissue, but does not take into account the potential compartmentalization of a tissue, and that these processes may be occurring *simultaneously* in different areas of the affected tissue. Consider the image of a slice of multiple sclerosis subcortical white matter brain tissue (Figure 1B). Luxol fast blue staining (left) reveals healthy regions of myelin (dark blue), focal areas of demyelination (red arrows), and focal “shadow plaques” that represent remyelination (green arrows) (5). Two disparate processes – *pathogenic demyelination* and *healing remyelination*, occurring side-by-side, implicating that within

these physically separate microenvironments immune cells such as macrophages might be conducting different processes – damage or repair (right image). In support of this, recent histopathological evidence reveals mixed macrophage phenotypes in human MS lesions (6).

Understanding of the concept of specific microenvironments within tissues is increasing, not least within tumor immunology (7). The ambiguous role of tumor associated macrophages (TAMs) in tumor progression is reflected by TAMs both actively augmenting cancer cell proliferation, invasion, metastasis, and angiogenesis by releasing cytokines, growth factors, enzymes, and angiogenic factors, but they also kill cancer cells. These varied activities encompass both M1 and M2 macrophage properties. It is counterintuitive that such diverse tumor-promoting, or conversely anti-tumoral, activities are performed by a single TAM cell type, so the existence of distinct TAM subpopulations associated with different intra-tumoral microenvironments is predicted (8). The source of the TAM may also influence their functions. For example, in gliomas TAM may be either resident brain microglia or blood infiltrating macrophages. Molecularly and functionally distinct TAM subpopulations may thus coexist in tumors, the heterogeneity depending on cancer type, stage of tumor progression and specific location within the tumor tissue (9, 10). A dynamic “switch” in TAM phenotype during tumor progression may explain the mixed activation state of TAM subsets present in different established tumors, and in certain models a switch from TAM



is linked to tumor progression (11). It is noteworthy that the “switch” that is often referred to in the literature may rather reflect a *relative predominance* in M1 or M2 cell numbers rather than a full phenotypical/functional change of a single cell. Different macrophage populations induced during tumor progression have also been reported to occupy different microenvironments within the tumor mass (12). In a murine hepatocellular carcinoma model, the MHC Class II^{high} TAM population (M1-like) was associated with tumor growth suppression during early tumor growth while MHC II^{low} TAMs (M2-like) dominated as the tumor progressed (13).

Investigation of the spatial distribution of macrophage phenotypes in human plaques at different stages of atherosclerosis development also reveals microenvironment variations. M1 is the predominant phenotype in rupture-prone shoulder regions, and M2 in the adventitia (14). Likewise, in models of lung inflammation induced by butylated hydroxytoluene or *Mycobacterium tuberculosis* there is an initial M1 activation that progresses to M2 (15). However, granuloma-associated macrophages during active infection may retain an M1 phenotype while nearby uninfected alveolar macrophages are M2.

Clearly, at various sites of inflammation then individual subpopulations of cells contribute to specific microenvironments in different ways.

INFLAMMATION IS A TEMPORALLY EVOLVING CIRCUMSTANCE

Another aspect to consider when homogenizing tissues for cell purification and subsequent FACS or RT-PCR analyses is not only the geographical gradient of macrophage activation states that will be lost but also a temporal element. For example, given the realization that resident macrophages and circulating monocytes are fundamentally different cell types (16, 17), consider the macrophage disappearance reaction (18) in which initial immune activation within a tissue results in efflux of resident cells and infiltration of circulating cells, this cellular flux being reversed on eradication of the offending stimulant. If one would take a snapshot in the tissue and sample the phenotype of the macrophages present at a given time, it is difficult to be sure whether they are coming or going from the site of interest. Obviously, this can impact on their functional relevance to the ongoing immunological process and especially in our scientific interpretation. Now that small rodent PET/CT/MRI imaging is becoming more standardized, one can

expect this issue to be further addressed in forthcoming years.

FUNCTION VERSUS FORM DURING INFLAMMATION

A host requires basic macrophage functions in order to survive. These functions are sometimes less easily measured than other surrogate markers such as cell surface proteins. Consequently, there is a wide range of surface markers, cytokines and chemokines reported to distinguish M1 and M2 activation states (mostly *in vitro*). However, the use of such “markers” without parallel assessment of functions can result in conflicting results.

Variation in published results might be explained by variation in employed activation protocol, difference in rodent strain (19) or human donor to which the same protocol is applied. Take IL-10, for example, a prototypic anti-inflammatory cytokine that can be produced by M2 and M1 cells. Both the production and lack of production of IL-10 in M-CSF-stimulated human M2 monocytes have been described (21). Reliance on surface marker expression can also be particularly misleading – if anti-inflammatory M2 states receive an additional LPS stimulation *in vitro*, then while expression of CD86 and MHC II might become upregulated (“M1”) the IL-10 production is actually enhanced (22). Given the dominant functional role of IL-10 as an immunosuppressive cytokine, then functionally such cells are more potent M2, despite starting to develop an M1 surface. Similarly, in alcoholic hepatitis, liver M2 macrophages were determined to express M1-associated receptors (23). It would thus seem that there is a necessity to distinguish between surface and functional phenotypes.

Clearly, the biological functions in microenvironments should be more important than any other phenotype, and one expects as much functional variation as there is in M1–M2 phenotypic definition. Even the basic morphology of activated cells *in vitro* is reported to be exact for different phenotypes, yet closer examination reveals this is not necessarily the case (20, 24, 25). In our own study in which we applied IL-4, IL-10, and TGF β simultaneously, cells of three different morphologies were apparent in the same culture well, representing the

three distinct morphologies observed if single cytokines were applied in separate cultures (26). Does this imply microheterogeneity even in the cell culture medium containing a mixture of cytokines, such that the first cytokine receptor ligated on a cell surface dictates the morphology of that specific cell? Whether the sequence of activation is of any consequence will depend on whether there are actually any kinetic effects that impinge on the final morphology or function. This may seem a trivial issue but a recent publication elegantly demonstrated precisely why this is important by studying human macrophage transcriptomes using different combinations of activation stimulants (4).

The challenge is thus how to quantify functional phenotypes in microenvironments. We have a limited range of markers that can be applied immunohistologically. Detailed knowledge about expression/regulation of expression for *each macrophage population in each tissue*, within *each microenvironment* within a tissue, during *both* resting and inflammatory states, is currently lacking. Development of conditional knockout mouse strains lacking resident/peripheral macrophage populations is one approach that is warranted, for example, to distinguish the relative roles of infiltrating macrophages and resident microglia during brain tumor development. Alternatively, the use of single cell laser capture and proteomic or genetic analyses might be one modern approach to explore what individual cells within a particular microenvironment do within their niche, although function will only be inferred from these analyses (27, 28). The basic assumption is that all cells are equal and that the relative numbers of different subpopulations will ultimately define the functional state of the tissue. Increasing evidence challenges this assumption, and in our hands co-culture of pre-activated M1- and M2-type populations demonstrated a clear dominant phenotype of M2 cells (pre-activated with a combination of IL-4/IL-10/TGF β) (29). Even if single cells are phenotyped and their relative numbers are quantified within a tissue or microenvironment, it remains difficult to predict the net functional activity or interplay *in vivo* if they are not functionally equivalent.

THERAPEUTIC MANIPULATION OF CHRONIC INFLAMMATORY MICROENVIRONMENTS

A final question is whether chronic inflammatory states such as autoimmune diseases represent a failure to down-regulate pro-inflammatory M1-mediated tissue destruction (i.e., a deficiency in anti-inflammatory M2 function), or whether this reflects a lack of healing M2 functionality. It follows that a stochastic alteration of the relative M1/M2 functions within microenvironments represents a feasible therapeutic approach. Earlier work indicated that M2 cells accumulate at the edge of the tissue damage in the setting of spinal cord injury (30). If large numbers of “therapeutic cells” could be applied to inflammatory microenvironments (e.g., through local stimulation or cell transfer) then they should be able to exert tailored “local” immunomodulatory effects. In our experience adoptively transferred pre-activated anti-inflammatory M2 cells resulted in clinical abrogation of both T1D (22) and MOG-EAE (26) disease courses. In addition to a cell therapy approach, there are new generations of agents aimed at specific conversion of macrophage phenotypes within microenvironments, such as TAM conversion to M1 within tumors using docetaxel and phosphatidylserine-targeting antibody (31). The effectiveness of this approach was first reported many years ago (32). The major challenge will be to access the microenvironments specifically rather than systemically administering an agent and hoping for its specific access to the target area.

LOOKING BACK AND LOOKING AHEAD

The last decade has heralded a revolution in our understanding of immune mechanisms and particularly the critical role of macrophages in both innate and adaptive responses. During the coming decade, we can expect a refinement of this knowledge when the functions of individual cells and their specific contributions to a specific microenvironment become better understood. This may lead to yet further refinement of macrophage nomenclature as function supersedes form in importance. The next era may well also herald the successful therapeutic manipulation of inflammatory microenvironments in order to slow or abrogate inflammatory

disease courses in human beings. One can be certain that the good macrophages vanquishing the bad macrophages will be a component aspect, and that restoration of the damaged, ugly tissue will also be macrophage-dependent.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 09 October 2014; accepted: 13 November 2014; published online: 03 December 2014.

Citation: Harris RA (2014) Spatial, temporal, and functional aspects of macrophages during “The Good, the Bad, and the Ugly” phases of inflammation. *Front. Immunol.* **5**:612. doi: 10.3389/fimmu.2014.00612

This article was submitted to Molecular Innate Immunity, a section of the journal *Frontiers in Immunology*.

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"Of mice and men": arginine metabolism in macrophages

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Edited by:

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Reviewed by:

Charles Dudley Mills, BioMedical Consultants, USA

Sidney Morris, University of Pittsburgh, USA

Keywords: arginase, human macrophage, macrophage polarization, nitric oxide synthase, macrophage activation

INTRODUCTION

Macrophages are involved in inflammation from induction to resolution. Polarization of macrophages along the M1 (classical) or M2 (alternative) axis occurs during inflammation and can be at least partly categorized by the route of arginine metabolism within the macrophage, balancing the activities of the arginase and nitric oxide synthase (NOS) enzyme families (1, 2). Arginase activity is associated with tissue repair responses (via ornithine production and pro-proliferative effects). In contrast, NOS2 generates nitric oxide (NO) species with anti-proliferative effects that is necessary for protection against pathogens and aberrant cells (2, 3). Other NOS enzymes produce NO that acts in the regulation of smooth muscle tone and other cellular processes (4). Macrophages preferentially expressing the arginase or NOS2 pathways enzymes also influence T-cell activation, proliferation, signaling, and apoptosis in different ways (1).

While arginase and NOS enzymes can be used to ascertain the pathway of macrophage activation in rodents, there has been debate as to whether they are present in macrophages from humans and other mammals. The arginase and NOS enzymes are extensively conserved, and the NOS forms found in mammals are similar to those in cnidarians, mollusks, and other chordates (5, 6). These arginine-metabolizing enzymes are present in some human leukocytes, and there is evidence that they are also present in macrophages from other vertebrates, including chickens, rabbits, cows, and primates (7–12). However, comparisons of tissue macrophages of different species are lacking, which limits our understanding (13). Many studies in

humans have principally focused on blood monocytes, leading some researchers to question the suitability of rodents as model of macrophage activation, as there is not always a direct correlation with human cells. Was Robert Koch correct when he said "Gentlemen, never forget that mice are not humans," or can the differing results between species be explained, in part, by differences in the types of monocyte or macrophage studied? Our purpose here is to examine this question.

ARGININE METABOLISM IN MAMMALIAN CELLS

Many mammalian cells, including neutrophils, granulocytes, erythrocytes, hepatocytes, cardiac myocytes, dendritic cells, myeloid-derived suppressor cells, foam cells, natural killer cells, endothelial cells, and smooth muscle cells, have arginase (12, 14–16) or NOS activity (8, 17–19), albeit to different degrees. Macrophages are the primary circulating cells that can express either of these enzymes, depending on the inflammatory circumstance. Experiments that detect NO, ornithine, or urea production (via NOS2 or arginase) have most often been performed on rodent macrophages. Macrophages from some mouse strains (e.g., the M1-biased C57BL/6 strain) can be stimulated by lipopolysaccharide (LPS) to produce considerable quantities of NO. Macrophages from others strains (e.g., M2-skewed BALB/c mice) produce much less NO (20) and produce more ornithine instead. Some researchers did not detect any NO production in macrophages from humans, pigs, and rabbits (8, 11, 14, 21–23), but others (including ourselves) have observed NOS or arginase activity in macrophages from

rabbits, humans, and other primates (4, 7, 10, 12, 17, 24–26).

WHY IS THERE CONTROVERSY?

One main difference between the studies from laboratories is that some use monocyte-derived macrophages (MDM), while others study tissue macrophages directly. A number of groups have detected NOS or arginase activity in human monocytes or macrophages (3, 27–29); but others have not. Why is this so? Part of the explanation lies in the fact that *in vitro*-derived macrophages can generate different responses from macrophages obtained *in vivo* as discussed below (and shown in Table 1). Another explanation is that many groups use the identification of enzyme protein rather than detection of enzyme activity as evidence of enzyme expression. Failure to detect the presence of a protein is not definitive evidence for absence of expression (especially when considering potentially different detection thresholds of antibodies or the high V_{max} of arginase, i.e., very little enzyme is required for ornithine production).

MACROPHAGES PRODUCED *IN VITRO*

Macrophages have been produced *in vitro* in a number of ways. Cells from bone marrow have been isolated and "differentiated" in culture medium containing high levels of cytokines (such as colony stimulating factors, CSFs) to produce bone marrow-derived macrophages (BMDM) (13, 23, 44–46). Macrophages have also been produced by isolating and culturing monocytes from blood, to produce MDM (10, 13, 22, 30, 37, 47, 48). Production of these *in vitro*-derived macrophages is cheap, simple, and reproducible, but they may not be a

Table 1 | The presence of arginine-metabolizing enzymes in human monocytes and macrophages varies with cell source, treatment and health status/stress level of the individual.

Cell origin	Cell	Treatment	NOS test	ARG test	Result	Reference
Blood monocytes	Monocyte, mono-mac	0, 2, 3, or 5d culture	RNA, citrulline, FC	RNA, urea	NOS, ARG1 and ARG2 levels vary between monocyte subpopulations	(27)
Blood monocytes	Monocyte, mono-mac	0, 3, or 7d culture or 7d M-CSF, 0.75d IFN γ /LPS, or IL4		Gene array	No difference (\leq 2-fold cut-off, therefore genes with smaller differences discounted)	(30)
Blood monocytes (filaria-infected)	Monocyte	1d culture	RNA	RNA	\uparrow ARG1, \downarrow NOS2	(28)
Blood monocytes (burns victims)	Monocyte	2d culture		Urea	\uparrow ARG1	(29)
Blood monocytes	Monocyte	2d microfilaria, M-CSF, IL4, or IFN γ /LPS	RNA	RNA	Most donors had low but detectable NOS2 and ARG1 RNA expression which did not change with any treatment.	(31)
Blood monocytes	Mono-mac	3d IFN γ and/or IL4 (No M-CSF)		RNA	\downarrow ARG1, but detectable in all conditions	(32)
Monocyte/macrophage cell line (U937)	Mono-mac	?d LPS and/or IFN γ	Transcription run-on assay		No induction of NOS2 gene transcription (for that particular region of the promoter region)	(33)
Monocyte/macrophage cell line (U937)	Mono-mac	1d selenomethionine and 1d LPS and/or IFN γ	Griess, RNA Western		Selenomethionine \downarrow LPS-induced NOS2 expression (RNA and protein) and nitrite production	(34)
Blood monocytes, peritoneal macrophages	Mono-mac, macrophage	?d culture, 2d LPS, IFN γ , or TNF α /GM-CSF	Griess, amino acid HPLC		No nitrite, ornithine, citrulline production, no arginine consumption	(22)
Blood monocytes, peritoneal macrophages	Monocyte, mono-mac	0d or 3d LPS or cytokine	RNA, IB, ICC, biopterin, citrulline, Griess		NOS2 mRNA and protein present in monocytes, \uparrow peritoneal macrophages (\uparrow with LPS). Both cell types produce neopterin, nitrite/nitrate and citrulline (low levels)	(35)
Blood monocytes (MS sufferers)	Macrophage	6d GM-CSF 0.75d IL4, IFN γ , LPS, or TNF α	RNA, Griess	RNA, WB, urea	ARG1 and NOS2 mRNA and nitrite production in MS and controls, \uparrow with M1 or M2 cytokine challenge. ARG1 protein and urea production present in controls, \uparrow in MS	(36)
Blood monocytes	Macrophage	8d M-CSF, 5d oxLDL		RNA	No change in ARG1 levels	(10)
Blood monocytes	Macrophage	10d M-CSF, 1d IL4, or IL10		Urea, WB arginine	No ARG1 after induction by IL4 or IL10	(14)
Blood monocytes	Macrophage	14d IFN γ /LPS	Griess		No nitrite production	(37)
Alveolar macrophages (volunteers)	Macrophage	IFN γ	Griess, citrulline		No NO production, no effect of NOS inhibitor	(21)
Alveolar macrophages	Macrophage	?d (short), 0.8d IL4, or forskolin (i.e., \uparrow cAMP)		Urea	Untreated macrophages have ARG activity similar to unstimulated RAW cells. \uparrow ARG with IL4/forskolin but not IL4 alone	(38)
Alveolar macrophages (cancer sufferers, volunteers)	Macrophage	0.75d IFN γ /LPS or IL-10	RNA, WB	RNA	\uparrow ARG with IL10 stimulated cells, \uparrow NOS2 with IFN γ /LPS stimulated cells	(39)

(Continued)

Table 1 | Continued

Cell origin	Cell	Treatment	NOS test	ARG test	Result	Reference
Alveolar macrophages (TB patients, volunteers)	Macrophage	None	IHC, WB, RNA, diaphorase		45–49% of cells from TB patients have NOS2. Smoking controls had some NOS2-positive macrophages, non-smoking controls have few NOS2-positive cells	(24)
Alveolar macrophages (TB patients)	Macrophage	None	IHC		Macrophages in TB granulomas stain for NOS1, NOS2 and nitrotyrosine (i.e., active)	(26)
Alveolar macrophages (TB patients)	Macrophage	None	IHC	IHC	ARG1 in macrophages in TB granulomas, few have Arg2. Some macrophages on outer margins have both NOS2 and ARG1, some near center have NOS2, NOS3 and ARG1	(12)
Atherosclerotic plaque macrophages	Macrophage	None	ISH, IHC		NOS2 in macrophages and smooth muscle cells, co-localized with oxidized lipoproteins and peroxynitrite (i.e., NOS is active)	(7)
Atherosclerotic plaque macrophages	Macrophage	None	IHC, WB		Fatty streaks: no NOS2. Advanced plaques: NOS2 present in macrophages near necrotic core, associated with ceroid accumulation and nitrotyrosine (i.e., active)	(25)
Atherosclerotic plaque foamy macrophages	Macrophage	None		IHC	↑ARG1 in macrophages in superficial layers, ↓ARG1 in macrophages surrounding lipid core	(10)
Atherosclerotic plaque	Macrophage	None	IHC, ISH		NOS2 and nitrotyrosine localized to smooth muscle cells, macrophages and foam cells (i.e., active)	(17)
Oral macrophages	Macrophage	None	IHC, nitrate		NOS2 present in macrophages from gingivitis samples	(40)
Placental macrophages	Macrophage	None		FC	Some M2 macrophages have ARG1	(16)
Skin macrophages (wound)	Macrophage	None	IHC, HPLC	IHC, WB, ELISA, HPLC	NOS2 present in macrophages, some have ARG2, but none have ARG1. Controls: no ARG2	(41)
Tumor-associated macrophages	Macrophage	None	IHC		NOS2 present in some macrophages (bladder)	(42)

While changes in RNA expression of arginine-metabolizing enzymes have been used to identify macrophage activation states, protein changes [such as western blotting (WB) or immunohistochemistry (IHC)] are also useful. Nitric oxide synthase (NOS) activity can be assessed directly [e.g., production of citrulline or NO (e.g., Griess assay)] or by the presence of markers of NO production (such as peroxynitrite, nitrotyrosine or ceroid, a complex of oxidized lipids and proteins). Arginase (ARG) activity can be measured as urea or ornithine production (e.g., urea assays, amino acid HPLC).

d, number of days; ?d, unspecified number of days; FC, flow cytometry; M-CSF, macrophage colony-stimulating factor; IFNy, interferon- γ ; LPS, lipopolysaccharide; IL, interleukin; TNFa, tumor necrosis factor- α ; GM-CSF, granulocyte-macrophage colony-stimulating factor; IB, immunoblot; ICC, immunocytochemistry; MS, multiple sclerosis; oxLDL, oxidized low density lipoprotein; TB, tuberculosis; ISH, in situ hybridization; Griess, Griess assay for nitrite/nitrate production.

It should be noted that NO production below the detection levels of this relatively insensitive assay may still have functional effects (43).

full representative of tissue macrophages, as the preparation and culture procedures may not be sufficient to induce cell activation (4). The differences between tissue macrophages and *in vitro*-derived

macrophages are at least partly dependent on cell source, time in culture, and the degree of manipulation in culture. Each research group will use different types and sources of culture media and sera, which

vary greatly in the concentrations of factors that influence NOS2 or arginase expression, such as transforming growth factor β (TGF β) (4, 20, 49). Another confounding issue is that circulating monocytes and

tissue macrophages arise from different stem cell populations (50), although some macrophages found at sites of infection or inflammation may derive from infiltrating monocytes (51). Together, these factors may account for many of the differences observed in NO and urea production in these macrophages (8, 20).

Monocyte-derived macrophages or BMDM from different strains of mice can differ in their response to interferon- γ (IFN γ), LPS, and tumor necrosis factor- α (TNF α) (4, 8), and differences in the rodent background can result in differences in macrophage gene expression (13, 20, 49). Human *in vitro*-derived macrophages also show variability in their responses to LPS (4, 22, 46). It may be that the same stimulus is able to generate quite different responses in genetically diverse individuals, as it does between mouse strains (38, 49, 52). In general, human macrophages are not as responsive to LPS as mouse macrophages, possibly because of the lower environmental exposure of humans to LPS. It is also possible that human monocytes may be more effectively stimulated to become M1-activated macrophages by cytokines other than IFN γ and LPS/TNF α (e.g., IFN α) (4, 18, 43). Human macrophages take longer time to respond to the stimulatory factors *in vitro* than mouse macrophages, and some experiments using human MDM may have ended before a response was detected (48). There are other indications that the timing and length of the exposure of the cells to varying cytokines *in vitro* are important. For example, when M1-polarizing cytokines were removed from the culture medium, NOS2 levels in mouse BMDM were reduced and NO production (measured as nitrite) ceased (45). In addition, whichever arginase or NOS enzyme was induced earliest, the alternative enzyme decreased in expression and activity, unless arginine was present in excess (15, 45, 53). Macrophages require the local environment to continuously give appropriate activation cues. Changes in environmental cues can stimulate macrophage populations *in vitro* to express varying percentages of M1 or M2 dominant activity (54). When activation cues are reduced or removed, macrophages may become deactivated (e.g., M2c) or indeterminate (e.g., have features of M1 and M2).

MACROPHAGES OBTAINED *IN VIVO*

Macrophages can be identified in whole tissues and organs or isolated in large numbers from *in vivo* sources such as the peritoneum or granulomas, and either examined immediately or used *ex vivo*. Macrophages obtained *in vivo* or made from monocytes can respond differently to the same stimulus (35, 47). In one study, monocytes and tissue macrophages were obtained from patients with an inflammatory disease (either rheumatoid or psoriatic arthritis). Compared with tissue macrophages, the MDM had a blunted response to the M2 cytokines interleukin-4 (IL-4) and IL-13, at least partly due to a reduction in some of the receptor elements for these cytokines (47). These results suggest that the response of the macrophages to M2 cytokines may be source specific, but it is possible that these cytokines alone were not sufficient to fully stimulate the MDM (38). Several lines of evidence suggest that macrophages *in vivo* express functional NOS2. Blood monocytes and peritoneal macrophages obtained from women during laparoscopic procedures contained NOS2 mRNA and protein. The macrophages had higher NOS levels than the monocytes, and this could be increased by treatment with LPS. The monocytes and macrophages also produced neopterin, nitrite/nitrate, and citrulline (suggesting that the enzyme was active). Although the production of NO from these macrophages was low, it would probably have been sufficient to cause functional changes (35).

Macrophages can also be obtained from alveolar aspirates, skin, and the placenta (10, 16, 21, 38, 39, 55, 56). For example, sponges placed subcutaneously into mice, rats, or rabbits attract large numbers of macrophages. The sponges can be removed from the animal and the macrophages were isolated and purified (10, 55, 56). It is a little more difficult to obtain and purify macrophages from other tissues, such as atherosclerotic vessels (44), but intact biopsy, surgical, or cadaveric specimens can also be investigated. It should be noted that resident macrophages from different tissues observed at different times (and different health states) may not necessarily have identical properties (51, 57).

In order to perform their full range of functions, macrophage populations exhibit “plasticity” of phenotype (52, 58), regardless of whether they are found *in vivo* or derived *in vitro*. As macrophages adapt or change their functions, they can simultaneously express markers of M1 and M2 activation, including NOS2 and arginase-1 (12, 59, 60). For example, tissue macrophages (and MDM) from *Mycobacterium tuberculosis*-infected cynomolgus macaques have been observed to co-express functional NOS and arginase enzymes (12). We suggest that *macrophages display a spectrum of activation phenotypes, and it is the relative (and not absolute) proportion of M1 or M2 markers that we can use as a ‘handle’ to determine the type of activation state*.

EFFECT OF DISEASE AND TRAUMA ON MACROPHAGE ACTIVATION

Blood monocytes from healthy volunteers do not usually need to produce NOS or arginase, so it is not surprising that many studies have not detected NOS or arginase in these cells (10, 14, 21, 22, 29, 30, 37). However, studies performed on tissue or cells from people undergoing stress, trauma [e.g., burns (29)], pregnancy (16), or disease {such as infection [e.g., tuberculosis (12, 24, 26) or filarial infection (28)], atherosclerosis (7, 10, 17, 25), autoimmune diseases (27, 36) and cancer (42, 61)} demonstrate that human macrophages (and sometimes monocytes) can produce active forms of the arginine-metabolizing enzymes (**Table 1**).

Trauma results in a pattern of gene expression in macrophages that is consistent with a wound-healing response, with an initial increase in NOS followed by decreased NOS production and activity, elevated IL-4, IL-10, and TGF β levels, and increased arginase expression and activity, resulting in decreased plasma arginine levels (28, 29, 62).

Disease, however, causes different patterns of gene expression. For example, monocytes from multiple sclerosis sufferers not only have higher levels of arginase-1 and increased urea production, but also have increased NOS2 mRNA and nitrite production (particularly when stimulated by M1 cytokines or LPS) (36). Macrophages from patients with inflammatory diseases, such as tuberculosis,

malaria, or rheumatoid arthritis, have increased levels of NOS2 mRNA and active protein (4, 8, 24, 26, 63), which may contribute to elevated plasma NO levels (64). Atherosclerosis is another inflammatory disease with a considerable macrophage contribution, with oxidized low-density lipoproteins taken up by macrophages during their transformation into foam cells. Plaque macrophages express NOS2 RNA and protein, as well as markers of NOS activity (including the presence of nitrotyrosine or ceroid) (4, 7, 17, 25). Plaque macrophages and foam cells express arginase-1 (10), and macrophages laser-dissected from plaque have upregulated levels of arginase-2 and NOS2 (65). Macrophages present in some neoplastic diseases also produce active NOS2 (4, 42, 66). Reducing the local levels of arginine has been proposed as a treatment for these diseases, by reducing inflammation-triggered immune dysfunction, tumor escape, fibrosis, and immunosuppression (61). Possible pharmacological interventions include treatment with arginine degrading enzymes, NOS competitors and inhibitors, asymmetric dimethylarginine, NO-releasing aspirins, cyclooxygenase, and phosphodiesterase or arginase inhibitors (8, 61). *These studies suggest that an inflammatory environment is necessary in order to observe NOS or arginase in human monocytes and macrophages. The in vitro experiments that do not demonstrate arginase or NOS expression may simply be lacking the additional cues needed for expression rather than demonstrating an inability to actually express these factors.*

CONCLUSION

The modulation of macrophages to express NOS or arginase has clear benefits for treating disease in humans (and other species). To do this, one needs to either determine suitable signals to stimulate these pathways or obtain a sufficient number of human macrophages (e.g., by tissue culture) that function like tissue macrophages.

Because macrophages from different inbred strains of mice vary greatly in their macrophage NOS and arginase balance, one would predict similar variability to be found in humans as well. In addition, the source of the macrophages being studied has been found to be important. Several groups have reported that

human monocytes from healthy volunteers that have been differentiated or manipulated *in vitro* using current protocols tend not to have detectable levels of arginase and NOS enzymes, whereas MDM from diseased or stressed individuals or tissue macrophages obtained from normal, diseased, or stressed individuals do express NOS and/or arginase. Together these observations suggest that the current system of differentiating macrophages from human peripheral monocytes *in vitro* needs further refinement before it can be considered to be an accurate model of human macrophage behavior *in vivo* (63). In turn, we need to understand the differences and similarities between the different species and the cells being studied to develop experimental models that will answer some of the outstanding questions regarding macrophage M1/M2 or other activation states: What regulates macrophage activation in tissues? What mechanisms regulate macrophage plasticity and stability? How does plasticity of phenotype affect tissue macrophages? What are the full *in vivo* ramifications of the M1/M2 paradigm?

Further work is important to be sure that our observations of the human system *in vitro* are real, and not due to our cell source, measurements, or manipulations. We suggest that macrophages obtained from mice remain useful for investigating aspects of these questions in humans/human macrophages. So, although mice are not men (as Robert Koch observed), we agree with Rudolf Virchow that “Between animal and human medicine there is no dividing line – nor should there be. The object is different but the experience obtained constitutes the basis of all medicine” [Rudolph Virchow, 1821–1902].

ACKNOWLEDGMENTS

Anita C. Thomas is supported by funding from the British Heart Foundation. Joshua T. Mattila is supported in part by NIH RO1A103785 and Bill and Melinda Gates Foundation grants to JoAnne Flynn (University of Pittsburgh).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 01 August 2014; accepted: 19 September 2014; published online: 07 October 2014.

*Citation: Thomas AC and Mattila JT (2014) “Of mice and men”: arginine metabolism in macrophages. *Front. Immunol.* **5**:479. doi: 10.3389/fimmu.2014.00479*

This article was submitted to Inflammation, a section of the journal Frontiers in Immunology.

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Rethinking regenerative medicine: a macrophage-centered approach

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Regenerative medicine, a multi-disciplinary approach that seeks to restore form and function to damaged or diseased tissues and organs, has evolved significantly during the past decade. By adapting and integrating fundamental knowledge from cell biology, polymer science, and engineering, coupled with an increasing understanding of the mechanisms which underlie the pathogenesis of specific diseases, regenerative medicine has the potential for innovative and transformative therapies for heretofore unmet medical needs. However, the translation of novel technologies from the benchtop to animal models and clinical settings is non-trivial and requires an understanding of the mechanisms by which the host will respond to these novel therapeutic approaches. The role of the innate immune system, especially the role of macrophages, in the host response to regenerative medicine based strategies has recently received considerable attention. Macrophage phenotype and function have been suggested as critical and determinant factors in downstream outcomes. The constructive and regulatory, and in fact essential, role of macrophages in positive outcomes represents a significant departure from the classical paradigms of host–biomaterial interactions, which typically consider activation of the host immune system as a detrimental event. It appears desirable that emerging regenerative medicine approaches should not only accommodate but also promote the involvement of the immune system to facilitate positive outcomes. Herein, we describe the current understanding of macrophage phenotype as it pertains to regenerative medicine and suggest that improvement of our understanding of context-dependent macrophage polarization will lead to concurrent improvement in outcomes.

Keywords: regenerative medicine, biomaterials, host response, foreign-body reaction, stem cells, macrophages

INTRODUCTION

The macrophage has long been known to play an important role in the tissue remodeling response which occurs following injury. In brief, macrophages arrive at the site of tissue injury 24–48 h post-injury, serve as phagocytes clearing the wound bed and initiating the processes that lead to the default outcome of scar tissue formation (1, 2). However, only recently it has been recognized that macrophages can have positive impacts upon tissue remodeling following injury (3–9). While the specific mechanisms by which macrophages direct tissue remodeling responses remain a subject of ongoing research, it has been suggested that a transition from a pro-inflammatory (M1) phenotype to a more regulatory or anti-inflammatory M2 phenotype is a key aspect of tissue remodeling which promotes functional outcomes as opposed to scar tissue formation.

A correlation of macrophage phenotype with functional recovery in wound healing has been suggested for more than two decades (9). With the introduction and general acceptance of the M1/M2 phenotypic dichotomy (10), correlation of macrophage polarization states and functional recovery has now been reported in several other tissues and organ systems and

represents an area of increasing interest for those in the area of wound healing and regeneration. The central dogma of this macrophage-centered approach is that treatments which facilitate an efficient and timely switch from a pro-inflammatory to an anti-inflammatory and regulatory phenotype, will logically promote functional tissue remodeling over scar tissue formation.

The M1/M2 paradigm has been widely studied in the context of disease pathogenesis, particularly cancer, for more than two decades (11–13). The participation of M1 and M2 macrophages in a diverse set of diseases including atherosclerosis, endometriosis, and pulmonary fibrosis is also now recognized (2, 12, 14–18). In addition, there is evidence for the importance of macrophages in tissue and organ development and in processes such as limb regeneration in the axolotl (19, 20). Loss of macrophages during these processes leads to defects in development or retardation of the regenerative process in the axolotl. Similar findings have been reported in other regenerative species including zebrafish, where ablation of macrophages results in defects in fin regeneration following injury (21). This ability to promote a regenerative response is lost in higher order species with increasing complexity of the

immune system, having been replaced with a default mechanism of “rapid resolution” (i.e., scarring). While the mechanisms which underlie the loss of regenerative potential remain largely unknown, a better understanding of the role of the innate immune system in the regenerative process of lower organisms may provide targets for regeneration strategies in humans (22).

Regenerative medicine approaches to tissue reconstruction or organ replacement seek to restore the form and function of lost, damaged, or diseased tissues. These approaches logically rely upon our understanding of wound healing, development, and regeneration as guideposts for design. These approaches may incorporate one or more biomaterials, biologically active molecules, and/or cell sources. Recent advances in these areas have enabled highly innovative and promising therapies, but translation of such strategies, without exception, requires in depth investigation and understanding of the host response following delivery.

The purpose of the present review is (1) to provide rationale for a macrophage centric approach to tissue reconstruction; and (2) to give an overview of the current state-of-the-understanding of the implications associated with host macrophage responses in regenerative medicine.

It should be noted that the description of macrophages as having either an M1 or M2 phenotype is a simplification of the *in vivo* reality. Though it is now clear that M1 and M2 macrophages each play distinct roles in tissue remodeling following injury, the inflammatory process which occurs following injury is dynamic both spatially and temporally and macrophages may express transitional phenotypes. Logically, these cells will also express functions such as phagocytosis, antigen presentation, and effector molecule production to differing degrees during the inflammation and remodeling process. For the purposes of simplicity and general discussion, and as the M1/M2 terminology are used ubiquitously throughout the literature, we describe macrophage phenotype as M1 and M2 in the below examples with further discussion of the spectrum of possible phenotypes and their potential roles in regenerative medicine thereafter.

A MACROPHAGE CENTRIC APPROACH

There is evidence for both pathogenic and protective roles of macrophages in many biologic processes (12, 23). It is well understood that uncontrolled inflammation can be a detrimental process (e.g., inflammatory bowel disease, rheumatoid arthritis). However, an organized and well regulated macrophage response has been shown to be a determinant of tissue remodeling following injury, with the potential for positive outcomes and functional recovery. The key role of macrophages in functional recovery following injury suggests that methods which are capable of modulating the macrophage response in a controlled, reproducible, and well-defined manner, may also meet with improved outcomes in regenerative medicine applications. Below, we review the role of macrophages in the response to tissue injury and the subsequent remodeling process in three different tissue environments as a baseline from which to understand the potential role of macrophages in regenerative medicine approaches to tissue reconstruction and to provide the rationale for a macrophage centric approach.

THE M1/M2 PARADIGM DURING THE SKELETAL MUSCLE INJURY RESPONSE

The role of the M1/M2 paradigm during the skeletal muscle injury response is relatively well characterized. Skeletal muscle tissue possesses inherent regenerative capacity following acute injury such as exercise-induced trauma. The capacity of muscle tissue to regenerate relies heavily upon a population of normally quiescent muscle specific progenitor cells, referred to as “satellite cells,” and their interactions with inflammatory cells that infiltrate the injured muscle microenvironment (24, 25). An efficient skeletal muscle injury response which successfully restores the injured muscle tissue requires satellite cell progression through a structured process of activation including proliferation and subsequent maturation into committed myoblasts, myoblast alignment, and finally, fusion and differentiation into new contractile skeletal muscle myotubes (26–29). This carefully regulated process of satellite cell differentiation is controlled, in large part, by the activity of an orchestrated heterogeneous inflammatory response consisting predominantly of M1 and M2 polarized macrophages (30, 31).

Following acute skeletal muscle injury, one of the earliest events is the infiltration of the damaged tissue by inflammatory cells. Neutrophils comprise the initial wave of cells to enter the damaged muscle tissue and reach elevated levels as soon as 2 h post-injury and maximum numbers after 6–24 h (30, 32, 33). Neutrophils phagocytose host necrotic cellular or bacterial debris and propagate a pro-inflammatory response through the release of reactive oxygen species and T-helper (Th)1 associated pro-inflammatory cytokines, which recruit monocytes and macrophages. However, neutrophil numbers decline through apoptosis and the neutrophil response is generally resolved by 3–4 days post-injury (30). Monocyte-derived macrophages recruited to the damaged tissue shortly after neutrophil infiltration represent the predominant immunologic participant in the skeletal muscle injury response thereafter.

Monocytes originate in the bone marrow and express chemokine receptors and adhesion molecules which facilitate their extravasation and migration from the blood to the injured skeletal muscle tissue site where they differentiate into macrophages (34, 35). Tissue resident macrophages normally found within the skeletal muscle microenvironment are also likely to play a role. However, the relative contributions of tissue resident macrophages as compared to circulating macrophages to the tissue remodeling process remain unknown. Immediately after injury, infiltrating macrophages become polarized toward a pro-inflammatory or M1 phenotype. The mechanisms behind this M1 activation remain only partially understood and include macrophage exposure to pro-inflammatory cytokines (i.e., IFN- γ , TNF- α) and/or necrotic cellular or bacterial debris (30, 35–37). M1 macrophages within the injured muscle microenvironment phagocytose necrotic muscle debris and participate in a transient pro-inflammatory response, reaching elevated levels at 24 h post-injury and maximum numbers after 2 days (30, 35). In addition to producing large amounts of pro-inflammatory cytokines (i.e., TNF- α , IL-1 β , IL-12), M1 macrophages process and present antigen and express high levels of iNOS which facilitates NO production (38, 39).

After 2 days, macrophages participating in the remodeling of injured skeletal muscle show a transition from the pro-inflammatory M1 to the immunoregulatory and anti-inflammatory M2 phenotype. The mechanisms behind this M1 to M2 phenotypic switch remain only partially understood but include exposure of M1 macrophages to increased IL-10 concentrations from skeletal muscle at 48 h post-injury (40, 41); M1 macrophage mediated phagocytosis of apoptotic, as opposed to necrotic, cells (40, 42); and exposure of M1 macrophages to degradation products from extracellular matrix (ECM) (43). M2 macrophages reach peak numbers within areas of injured muscle at 4 days post-injury and remain a predominant cell-type present in the remodeling muscle microenvironment for several days (44, 45). M2 macrophages facilitate resolution of inflammation through the release of anti-inflammatory cytokines (i.e., IL-10, IL-13), which deactivate pro-inflammatory cell phenotypes and promote tissue remodeling and repair (35, 44, 45).

This transition of the initial response dominated by M1 macrophages to a more M2 dominated population following acute muscle injury facilitates skeletal muscle remodeling and is required for efficient and complete functional restoration. Specifically, the pro-inflammatory products of M1 macrophages promote the activation and expansion of quiescent muscle satellite cells within the tissue injury site (35, 38, 46, 47). For example, TNF- α produced in large quantities by M1 macrophages represents a well-accepted mitogen for satellite cell-derived skeletal muscle myoblasts (31, 48). Following satellite cell and myoblast expansion, paracrine signals from M2 macrophages facilitate the alignment, fusion, and differentiation, of these skeletal muscle progenitor cells. For example, IL-10, an immunomodulatory cytokine produced by M2 macrophages, is myogenic for skeletal muscle progenitor cells (4, 31).

The participation of a heterogeneous population macrophages following muscle injury is highly regulated. For example, the perturbation or prolongation of either the M1 or M2 macrophage population during the skeletal muscle injury response results in impaired skeletal muscle regeneration. Depletion of phagocytic leukocytes, including macrophages, prior to toxin induced skeletal muscle injury blocks the removal of cellular debris and impairs regeneration (31). Depletion of macrophages at the time of injury prevents the participation of M1 macrophages in the early response and therefore shows their importance to muscle regeneration (31, 49). Furthermore, immediately following injury, skeletal muscle shows decreased activity of muscle specific transcription factors in TNF- α knockout animals when compared to their wild-type counterparts (50, 51), suggesting that TNF- α from M1 macrophages promotes the early or proliferative stage of myogenesis. However, prolonging TNF- α activity beyond the early proliferative stage of myogenesis has deleterious effects. While TNF- α promotes skeletal muscle precursor cell mitogenesis, it also inhibits myogenesis (52–54).

The participation of M2 macrophages is also required for the skeletal muscle injury response. As stated above, these immunomodulatory cells drive the late or differentiation stage of myogenesis. For example, when macrophages were deleted after 2 days post-injury, a time point consistent with the transition to an M2 macrophage response, myoblast differentiation, and

subsequent regeneration was impaired (55). Furthermore, transgenic animals unable to mount a M2 macrophage response show an accumulation of proliferative myoblasts and a lack of myogenic differentiation following injury (7). These studies show the importance of a present, yet regulated, M1 to M2 phenotypic transition of macrophages for efficient skeletal muscle regeneration.

THE M1/M2 PARADIGM DURING CUTANEOUS WOUND HEALING

Adult mammalian cutaneous wound healing is another highly regulated process that follows a sequence of events comprising the following three interdependent and overlapping phases: (1) the inflammatory phase; (2) the granulation tissue formation and wound contraction phase; and (3) the matrix deposition and tissue remodeling phase (56, 57). Multiple studies have now demonstrated distinct macrophage phenotypes associated with each of these phases and with remodeling outcomes following injury.

The onset of wound healing, designated the inflammatory phase, can be further sub-divided into an early and late inflammatory phase. Immediately following injury, hemostasis provides a provisional matrix for cell migration. During the early inflammatory phase, which occurs at 1–4 days post-injury, neutrophils and monocyte-derived macrophages respond to pro-inflammatory signals released from the wound microenvironment including growth factors, cytokines, damage associated molecular patterns (DAMPS), and pathogen associated molecular patterns (PAMPs) (58). These pro-inflammatory effector molecules along with the presence of necrotic cellular and bacterial debris facilitate the polarization of infiltrating macrophages toward the M1 phenotype (59). M1 macrophages associated with the early inflammatory phase are highly phagocytic and participate in the inflammatory phase by producing large quantities of pro-inflammatory cytokines (i.e., TNF- α), proteases, and ROS with the ultimate goal of pathogen control and removal of necrotic cell and tissue debris (57, 59).

The late inflammatory phase, which occurs at 5–7 days post-injury, is marked by an accumulation of apoptotic as opposed to necrotic cells, which upon phagocytosis facilitate the polarization of macrophages toward the immunomodulatory M2 phenotype (59). During the granulation tissue formation and wound contraction phase of cutaneous wound healing at 7–10 days post-injury, paracrine effector molecules, including cytokines such as IL-10 and growth factors such as VEGF, PDGF- β , and TGF- β , produced by M2 macrophages recruit fibroblasts into the wound site and promote myofibroblast differentiation (6, 60). M2 macrophages continue to release anti-inflammatory and pro-angiogenic factors, which facilitate the resolution of inflammation, recruitment of endothelial cells, and deposition of new ECM (61, 62). Activated myofibroblasts bridge the wound gap and develop contractile forces to facilitate wound contraction. Growth factors produced by M2 macrophages and myofibroblasts synergistically promote the proliferation and migration of keratinocytes to facilitate wound re-epithelialization (60, 61).

The matrix deposition and tissue remodeling phase, which occurs after 10 days post-injury, is marked by a decrease in macrophage numbers populating the wound site, along with an overall decrease in total cellularity. Granulation tissue formation

reaches a plateau and tissue present within the wound site is partially remodeled into fibrotic scar tissue at this time (56, 57).

The above stages of cutaneous wound healing are carefully regulated by the activity of responding macrophages. Similar to the M1/M2 macrophage paradigm associated with the skeletal muscle injury response, cutaneous wound healing is dependent upon a heterogeneous macrophage population and an M1 to M2 phenotypic transition. The pro-inflammatory activity of M1 macrophages during the early inflammatory phase is required for efficient pathogen control. Conversely, M2 macrophage activity during the late inflammatory phase is required for the resolution of inflammation and the recruitment of cells, which facilitate granulation tissue formation and wound re-epithelialization. Perturbation of the M1 macrophage phenotype during the early inflammatory phase, either by conditional depletion or due to impaired recruitment, results in delayed granulation tissue formation and wound closure (63, 64). Similarly, prolonging the M1 macrophage phenotype (i.e., preventing the phenotypic transition to M2) through the exogenous addition of TNF- α during the late inflammatory phase also resulted in poor wound remodeling outcomes (65). Depletion of M2 macrophages during the late inflammatory phase results in prolonged inflammation and impaired wound repair (63, 66). These M2 macrophage depleted cutaneous wounds resemble chronic wounds typically associated with the pathogenesis of chronic venous ulcers (CVU) and diabetes. In fact, studies have shown that failure of cutaneous wound macrophages to undergo the M1 to M2 phenotypic transition represents a hallmark of these chronic inflammatory diseases (65, 67, 68). Taken together, these studies show the importance of functional macrophage heterogeneity and the extent to which immunomodulatory effects of M2 macrophages are critical for efficient wound healing and tissue remodeling.

THE M1/M2 PARADIGM DURING THE CNS INJURY RESPONSE

The role of M1 and M2 macrophages following injury in the central nervous system (CNS) is more ambiguous when compared to other tissues, and is made more complex by the presence of the blood–brain barrier. However, similarities to the macrophage heterogeneity associated with the injury response in other tissues are increasingly being reported.

Central nervous system resident macrophages, referred to as microglia, have long been considered the primary responders to injury in the CNS with little to no role having been recognized for circulating cells until recently. Generally, microglia are recruited to, and form a dense barrier around, the lesion site immediately following spinal cord injury (69, 70). These activated microglia produce large quantities of cytotoxic factors and pro-inflammatory cytokines including IL-1 β , IL-6, and TNF- α . This pro-inflammatory response facilitates pathogen control and debris clearance, and also the recruitment of neutrophils and blood-derived monocytes and macrophages (5, 71–73); however, this response is also commonly cited as a driver of poor remodeling outcomes following injury in the CNS. As with other tissue injury responses, functional remodeling following CNS injury involves a transition from a pro-inflammatory to an immunoregulatory and homeostatic response. It remains unknown if microglia show M1 to M2 phenotypic plasticity similar to that observed to

monocyte-derived macrophages. However, recent studies suggest that CNS microglia drive an early pro-inflammatory response, but infiltrating macrophages from the circulation may facilitate the M2-like tissue remodeling response (74). Specifically, recruited blood-derived macrophages, showing an anti-inflammatory phenotype consistent with M2 polarization, do not directly enter the lesion center, but are found around the lesion site at 3 days post-injury (74, 75). These immunoregulatory macrophages have been shown to arrive at the site of injury by specifically trafficking through a remote blood–cerebrospinal-fluid (CSF) barrier, the brain ventricular choroid plexus (CP) (8). Once at the injury site, these M2 macrophages produce IL-10 for mitigation of the pro-inflammatory response and contribute to repair mechanisms including remyelination (8, 75, 76).

The participation of M2 polarized macrophages in the CNS injury response is essential to the repair process. For example, the endogenous partial recovery which can be observed following spinal cord injury is abrogated when M2-like macrophages are depleted using antibodies or conditional ablation (74, 75). Consistent with this notion, blockage of CP mediated macrophage trafficking inhibits M2 macrophage recruitment and subsequently impaired recovery following injury (76). These studies show the importance of a heterogeneous macrophage response to CNS injury and, specifically, that M2 macrophages contribute to processes beyond inflammation.

The above studies support an emerging dogma of effective recovery from tissue injury in which initial responses consisting predominantly of M1 macrophages and secondary or later stages consisting predominantly of M2 macrophages drive functional remodeling outcomes. Furthermore, it appears that M2 macrophages contribute to more than immunomodulation during the response which follows tissue injury. Several organ systems, in addition to the above examples of muscle, skin, and CNS tissue have now been shown to undergo similar responses following injury and are also characterized by heterogeneous and temporally shifting macrophage phenotypes.

A DEPARTURE FROM THE “CLASSICAL PARADIGM”

The observation of dichotomies in macrophage phenotype in disease pathogenesis as well as tissue remodeling following injury represents a departure from the classical understanding of the macrophage as a primarily phagocytic and pro-inflammatory cell. The foreign-body reaction (FBR) has been well studied over the last three decades (77). Logically, this response is an extension of normal wound healing as the implantation of a biomaterial necessarily requires the creation of a surgical injury. The seminal works in this area by Anderson (77, 78) and others describe the host response to implanted materials as occurring in stages including injury (implantation), protein adsorption, acute inflammation, chronic inflammation, FBR, granulation tissue formation, and encapsulation. These processes are well recognized to be dominated by mononuclear cells, and macrophages in particular.

During a FBR persistent inflammatory stimuli, such as the presence of a non-degradable biomaterial, lead to chronic inflammation and the formation of multinucleated foreign-body giant cells (79). Multinucleate giant cells are formed by the fusion of persistent pro-inflammatory macrophages, consistent with the M1

phenotype, located at the surface of the biomaterial and further exacerbate the deleterious inflammatory response through a process known as “frustrated phagocytosis” (80, 81). Failure to resolve the inflammatory response results in a FBR, leading to the deposition of disorganized fibrous tissue consistent with scarring and encapsulation of the implant (82, 83). This dense fibrous scar isolates the implant and prevents its integration with the surrounding host tissue.

The interpretation of the FBR as a negative occurrence in this context led to the development of materials with a focus on “inertness” and “biocompatibility” (84–86). This focus upon the host response to biomaterials resulted in an associated emphasis upon the material characteristics which determine the host response and downstream outcomes. However, these same characteristics may not be ideal in the setting of regenerative medicine where the focus is upon the restoration of function through the development of new host tissues rather than through the provision of a simple mechanical substitute. These concepts do not imply that medical devices such as hip implants and surgical mesh are not effective for their intended functions, but rather that the intended use, and therefore, the design characteristics and functional requirements, of these materials are incompatible with the goals of regenerative medicine.

The emergence of regenerative medicine and the need and desire for therapies which restore endogenous tissue function has led to a significant increase in our understanding of the role of stem cells in tissue repair as well as innovation and development of new biomaterials as stand alone therapies and/or delivery systems for cells or biologic factors. These materials are most often degradable in nature, and include engineered biologic cues or inherent bioactivity when derived from natural or tissue based sources. As such, the host response to these materials will be significantly different and more complex than the response to mono-component, synthetic or metallic implants. Further complexity is seen when materials are used in combination with cells or other factors.

It is now recognized that certain materials when used alone or in concert with a cellular component can provide an inductive template for constructive and functional tissue remodeling. That is, the provision of a bioactive material and/or cells leads to the formation of new, site-appropriate tissue. One example of such materials is biologic scaffolds composed of ECM (87, 88). These materials are derived through the decellularization of source tissues and organs and are widely utilized in regenerative medicine approaches to tissue reconstruction (89, 90). By the nature of the source (i.e., intact tissue), the materials that result from efficient decellularization can be thought of as degradable reservoirs of tissue specific structural and functional components. These materials have been shown to be effective templates for constructive remodeling in both pre-clinical and clinical applications, and in several body systems (87, 88). However, it should be noted that reports of the effectiveness of ECM based scaffold materials are variable and highly dependent on the methods of scaffold production.

Despite the distinct differences in long-term outcomes which have been observed with various ECM based scaffold materials, all ECM implants have been shown to elicit a histologically similar cellular response in the first week to month post-implantation (3, 91). This response is characterized by an early infiltrate of

neutrophils followed by a dense infiltrate of mononuclear cells. Under the classical paradigm, such a response would commonly be associated with progression to a FBR with negative implications for functional tissue remodeling outcomes. However, the response typically proceeds down one of three distinct pathways: (1) a classic FBR with encapsulation and no signs of constructive remodeling; (2) chronic inflammation and degradation or integration of the material with little to no constructive remodeling; or (3) reduction of the inflammatory infiltrate with subsequent constructive remodeling (3, 91).

Based upon these disparate outcomes, it was hypothesized that, though the early host response to the materials was histologically similar (i.e., characterized by a dense infiltration of mononuclear cells in the site of implantation), differences in the early macrophage phenotype to certain ECM scaffold materials might exist and that these differences may be related to downstream remodeling outcomes. Indeed, this hypothesis was shown to be correct with those ECM scaffolds which elicited constructive remodeling outcomes being associated with a timely transition from an M1 to an M2 macrophage phenotype (3, 92, 93). These studies have provided the impetus for investigation of macrophage phenotype in a number of regenerative medicine applications using biomaterials and cell-based therapies. The results of these investigations now clearly show a correlation between macrophage phenotype and successful outcomes associated with multiple regenerative medicine strategies. A review of selected studies which demonstrate this phenomenon are described below with a focus upon multiple strategies (materials, cells, and bioactive factors) which show associations between macrophage phenotype and remodeling outcomes.

THE M1/M2 PARADIGM IN TISSUE ENGINEERING AND REGENERATIVE MEDICINE

An endogenous host injury response, consisting of immunomodulation, including the participation of M2 type macrophages represents a necessary component of efficient and functional tissue repair. It is therefore logical that regenerative medicine strategies aimed at activating or augmenting endogenous repair mechanisms should utilize a similar strategy. Regenerative medicine strategies aimed at promoting M2 macrophage activation have included cell-therapy and the implementation of synthetic and biologic scaffold materials, among others.

CELLULAR THERAPY

Cellular therapy is generically defined as the transplantation or delivery of exogenous cells to sites of injured or missing tissues. Stem and/or progenitor cells are often used in regenerative medicine applications because of their multi-lineage differentiation potential and well-recognized resistance to oxidative stress (94, 95).

The cell source is most commonly autologous due to immune rejection considerations, although many studies are investigating the use of allogeneic sources. Cellular therapy based strategies aimed at promoting tissue remodeling have been used to treat injured tissues including the myocardium, the spinal cord, and skeletal muscle, among others. Despite moderate pre-clinical and clinical success, cell-therapy is associated with limitations

including failure of the exogenous cells to engraft within host tissue (96–101). It is now increasingly recognized that therapeutic outcomes associated with cellular therapy are largely a result of paracrine effects exerted by the transplanted or delivered cells upon the injured host tissue microenvironment rather than direct differentiation of the transplanted cells into new tissues (102–107). These paracrine effects include modulation of macrophage polarization and beneficial remodeling events facilitated by a transition to the M2 macrophage phenotype (108–110).

Co-culture experiments have shown that the secreted products of different stem/progenitor cells directly promote an M2 macrophage phenotype (111–113). A large number of pre-clinical studies also support these results. For example, following spinal cord injury, transplanted bone marrow-derived mesenchymal stem cells (MSC) modulate the host inflammatory microenvironment by promoting an M1 to M2 transition, which ultimately leads to a permissive environment for axonal extension and functional recovery (114). Furthermore, following traumatic brain injury, intravenous (IV) delivery of multipotent progenitor cells promotes the polarization of microglia to an M2-like phenotype (115). Several studies, using cells of multiple origins (i.e., autologous and allogeneic bone marrow-derived MSCs, adipose derived MSCs, and umbilical derived MSCs, among others), suggest that the therapeutic effects associated with exogenous cell delivery for the treatment of myocardial infarction are a result of enhanced macrophage polarization switching (116–118). Cellular therapy mediated M2 macrophage polarization has been used to promote tissue remodeling and repair in several anatomic locations and disease states including kidney ischemia-reperfusion injury and asthma associated alveolar inflammation, among others (119–121).

SCAFFOLD MATERIALS

Regenerative medicine strategies aimed at promoting tissue reconstruction or replacement often employ the use of surgically implantable synthetic or biologic materials designed to serve as cellular support scaffolds. As described above, implantation of these materials following injury alters the default injury response. For example, following surgical placement, synthetic and/or biologic scaffold materials are able to affect the phenotype of infiltrating inflammatory cells, host progenitor cell activity, as well as fibrosis and fibrous capsule development (78, 122). These effects depend on the scaffold composition, degradability, cellularity, porosity, and implantation site among others (78).

SYNTHETIC SCAFFOLD MATERIALS

As stated above, the surgical placement of non-degradable synthetic scaffold materials is commonly associated with a FBR consisting of persistent M1 macrophage activity and an increased deposition of scar tissue (3, 78, 91, 123–125). Recently, strategies aimed at modulating material properties to reduce the persistent pro-inflammatory M1 macrophage response to synthetic biomaterials have been examined. These strategies have included alterations in scaffold surface chemistry and structural characteristics. However, some of the studies examining these strategies are associated with conflicting results. For example, one study

suggests that synthetic scaffold materials composed of fibers with smaller diameters are associated with more M2-like macrophage activation when compared to their larger diameter counterparts (126). In contrast, another study showed that larger fiber diameter enhanced M2 macrophage polarization (127).

A recent series of studies has demonstrated the effects of material pore size upon integration of the material as well as macrophage phenotype (13, 128–131). In these studies, materials were produced with tight distributions of pore sizes. Results showed that materials possessing pores of roughly 30–40 μm were shown to integrate with reduced encapsulation and higher vascularity when implanted into dermis or cardiac tissues (13, 131). These changes in outcome were also associated with shifts in macrophage phenotype (128, 131). However, interestingly, the shifts in phenotype were observed to be spatially distinct with cells outside of the pore templated implants having an increased M2 phenotype as compared to non-porous implants, and the cells within the implant having a predominantly M1 phenotype (128). These studies suggest that manipulation of the structural and surface characteristics of synthetic scaffold materials can affect macrophage phenotype. Specifically, some of these manipulations appear to alter the macrophage response and are also associated with improved outcomes.

Another manner in which biomaterials can be tailored to promote shifts in macrophage phenotype is through the use of biologically active molecules such as growth factors and cytokines. Examples of these approaches are numerous and are commonly employed in regenerative medicine with resulting improvements in remodeling outcomes. A recent study investigated the effects of incorporation of either M1 (IFN- γ) or an M2 (IL-4) promoting cytokines within a polysulfone tube upon nerve growth across a gap defect when the tubes were used as guidance conduits (132). The results of the study demonstrated that polarization of macrophages toward a more M2 phenotype was associated with increased Schwann cell infiltration and neurite outgrowth. These effects were further examined *in vitro*, with results suggesting that macrophage derived factors were at least in part the cause of the observed chemotaxis of Schwann cells.

BIOLOGIC SCAFFOLD MATERIALS

The biologically derived scaffold materials used in regenerative medicine applications are sourced from a variety of natural sources including mammalian tissues as well as plant, insect, and bacterial sources. These materials offer the inherent advantage of the native ligand landscape and bioactivity resulting from their source material. This inherent bioactivity also leads to added complexity in the host response to these materials. Among these materials, scaffolds derived from mammalian tissues represent the most commonly used materials in pre-clinical and clinical regenerative medicine applications (133–135).

Biologic scaffold materials composed of mammalian ECM have been used to promote constructive tissue remodeling in a variety of clinical applications including hernia repair, rotator cuff reconstruction, esophageal preservation, and skeletal muscle replacement, among others (88, 136, 137). ECM bioscaffolds are derived through the decellularization of mammalian tissue (89, 90, 138). The most common tissue sources are xeno- or allogeneic in nature

and include decellularized dermis, small intestine, bladder, and pericardium among others.

It is now well recognized that the ability of these materials to promote constructive remodeling is tied to their ability to modulate the host macrophage response (3, 92, 93, 135). Multiple studies have shown that ECM based scaffold materials which are properly prepared facilitate a transition from an M1 to an M2 phenotype around 7–14 days post-implantation (3, 92, 93). The exact mechanisms by which these materials facilitate this response remain largely unknown; however, a number of key aspects have been identified. The materials must be adequately decellularized to remove potentially immunogenic cellular constituents and the material must be able to degrade (3, 92, 93, 139). In the presence of excess cellular material or if the material has been chemically crosslinked to prevent degradation, an extended M1 type immune response with no transition to an M2 response is observed and is associated with poor remodeling outcomes or encapsulation.

The necessity of degradation for the transition to an M2 phenotype suggests that breakdown products of the ECM scaffold material may possess immunomodulatory activity. Studies have shown that ECM bioscaffolds can be solubilized and the degradation products formed into a hydrogel under physiologic conditions (140, 141). This hydrogel ECM, when used as a coating for polypropylene surgical mesh, can facilitate a transition from the default M1 and FBR type response to a more M2 type response with a reduction in the FBR and encapsulation (142). These results, as well as other recent *in vitro* studies, further demonstrate the inherent immunomodulatory nature of ECM based biomaterials as well as their ability to improve remodeling outcomes (124, 142).

WORDS OF CAUTION

The above examples clearly illustrate an emerging paradigm in regenerative medicine. That is, strategies which are able to modulate the host response from an M1 to an M2 macrophage response are associated with better outcomes. However, these results should be interpreted with caution. Macrophage phenotypes have been described in many ways (143). “M1” and “M2” (with M2 macrophages including subsets M2a, M2b, and M2c) represents the common terminology used to describe these cells. Macrophage phenotypes have also been described as a spectrum between M1 and M2 with any individual cell being capable of expressing multiple aspects of either phenotype at any given time. Given this phenotypic heterogeneity, and the transient nature of the remodeling process following injury, further study of biomaterials-macrophage and stem cell-macrophage interactions are warranted, as is more thorough definition of the resultant phenotypes and their unique functions. It is unlikely that macrophages which result from interactions with biomaterials, particularly those with inherent naturally occurring ligand landscapes, or stem cells will possess phenotypes which precisely resemble the canonical IFN- γ and LPS (M1) or IL-4, IL-13 (M2a), IC and TLR/IL1-R ligand (M2b), or IL-10 (M2c) activated macrophages.

Adding further complexity to the definition of macrophage phenotypes in regenerative medicine applications is the variability in tissue resident macrophage populations. For example, microglia are the resident macrophages of the brain and derive from the embryonic yolk sac during development and persist in the brain

thereafter, presumably through a process of local replication (144–146). As is described above, these cells have been demonstrated in a number of studies to have phenotypes which are distinct from circulating monocyte-derived macrophages and are known to play distinct roles in a number of CNS disease processes. Other tissue resident macrophage populations also exist, each with a distinct and tissue specific phenotype (147). While a full description of tissue resident macrophages in all body systems and their distinct phenotypic characteristics is beyond the scope of this review, it is important to understand how these differences and the relative contributions of local versus circulating cells will affect outcomes.

At present, studies investigating macrophage phenotype following exposure to biomaterial implants have largely relied upon single surface or gene expression markers as indicators of M1 and M2 polarization. It is now well established that macrophages possess highly complex and plastic phenotypes and that the use of multiple phenotypic markers is essential. Further, a better understanding of the functional implications of these phenotypes is needed to create a mechanistic understanding of the ways in which macrophages may direct tissue remodeling outcomes following biomaterial implantation or stem cell delivery. With this understanding, next generation therapies can be developed to target and modulate specific macrophage phenotypes with desirable characteristics for the given application.

It should further be recognized that baseline macrophage polarization states may be affected by patient characteristics. As is mentioned above, there is now increasing evidence for changes in macrophage phenotype and response to activating stimuli with age and disease both acute and chronic (12, 23, 148, 149). Commonly employed pharmacologic interventions may also affect the response. Also, it is logical that the tissue microenvironment which results following an injury may also be different than that which is experimentally and steriley created in an animal model. Thus, there is a need to investigate macrophage response to regenerative medicine strategies in animal models which, at least in part, can mimic aspects of these complex situations.

CONCLUSION

Macrophage polarization has been clearly shown to be an important determinant of success in regenerative medicine strategies for tissue reconstruction. Macrophages can promote both positive and negative outcomes, which are dependent upon the context in which they are encountered, their phenotype, and function. However, at present, there remains much to be investigated and defined regarding macrophage phenotypes associated with biomaterials, stem cells, and regenerative medicine. Thus, context specific definitions and identification of beneficial phenotypes are needed. Similarly, the unique functions of these cells must also be clearly defined in order to better understand their true role in the remodeling process. Indeed, a focus upon macrophage function during the process of constructive remodeling may prove more useful than further characterization of complex phenotypic markers. Moving studies from correlative to causative and expanding the number of outcome metrics, both phenotypic and functional, will assist in defining both biomaterials and stem cell associated phenotypes and also provides targets for next generation regenerative medicine therapies, which seek to modulate macrophages as a

means of promoting functional tissue recovery – a macrophage centric approach. It is increasingly clear that those strategies that adopt such an approach to regenerative medicine will meet with improved success.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 28 August 2014; accepted: 01 October 2014; published online: 04 November 2014.

*Citation: Brown BN, Sicari BM and Badylak SF (2014) Rethinking regenerative medicine: a macrophage-centered approach. *Front. Immunol.* **5**:510. doi:10.3389/fimmu.2014.00510*

*This article was submitted to Inflammation, a section of the journal *Frontiers in Immunology*.*

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Hepatic localization of macrophage phenotypes during fibrogenesis and resolution of fibrosis in mice and humans

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Macrophages have been found to both promote liver fibrosis and contribute to its resolution by acquiring different phenotypes based on signals from the micro-environment. The best-characterized phenotypes in the macrophage spectrum are labeled M1 (classically activated) and M2 (alternatively activated). Until now the *in situ* localization of these phenotypes in diseased livers is poorly described. In this study, we therefore aimed to localize and quantify M1- and M2-dominant macrophages in diseased mouse and human livers. The scarred collagen-rich areas in cirrhotic human livers and in CCl4-damaged mouse livers contained many macrophages. Though total numbers of macrophages were higher in fibrotic livers, the number of *parenchymal* CD68-positive macrophages was significantly lower as compared to normal. Scar-associated macrophages were further characterized as either M1-dominant (IRF-5 and interleukin-12) or M2-dominant (CD206, transglutaminase-2, and YM-1) and significantly higher numbers of both of these were detected in diseased livers as compared to healthy human and mouse livers. Interestingly, in mouse, livers undergoing resolution of fibrosis, the total number of CD68⁺ macrophages was significantly lower compared to their fibrotic counterparts. M2-dominant (YM-1) macrophages were almost completely gone in livers undergoing resolution, while numbers of M1-dominant (IRF-5) macrophages were almost unchanged and the proteolytic activity (MMP9) increased. In conclusion, this study shows the distribution of macrophage subsets in livers of both human and murine origin. The presence of M1- and M2-dominant macrophages side by side in fibrotic lesions suggests that both are involved in fibrotic responses, while the persistence of M1-dominant macrophages during resolution may indicate their importance in regression of fibrosis. This study emphasizes that immunohistochemical detection of M1/M2-dominant macrophages provides valuable information in addition to widely used flow cytometry and gene analysis.

Keywords: cirrhosis, fibrosis, resolution, M1, M2, IRF-5, IL-12, TGM-2

INTRODUCTION

Chronic injury of the liver leads to induction of fibrogenic processes that ultimately can progress to cirrhosis, a state in which excessive extracellular matrix deposition hampers normal liver functions. Hepatic stellate cells (HSC) are regarded as the principal cells that are involved in scar tissue deposition (1, 2). Recent studies indicate that the role of Kupffer cells has been underestimated in fibrogenesis and consequently hepatic macrophages have gained more attention recently (3–7). Kupffer cells are well-known producers of reactive oxygen species, cytokines and chemokines, that perpetuate hepatic inflammatory responses, and of matrix-degrading enzymes. In addition, these macrophages can phagocytose micro-organisms, apoptotic cells, and cellular debris generated during tissue injury and remodeling. Duffield et al. (8) clearly showed that Kupffer cells exert different, even opposing roles during various stages of liver fibrosis. They showed that macrophage activities during the injury phase

were predominantly associated with promotion of matrix deposition and HSC activities, while during recovery macrophages were associated with enhanced resolution and higher production of matrix metalloproteinases (MMPs) (8, 9). These diverse roles may indicate that activated macrophages differentiate into diverse phenotypes during various stages of liver disease.

Activated macrophages are described to polarize into different phenotypes depending on signals they receive from their environment. Many types can be distinguished, and the most-used, but rather simplified, classification system discerns classically activated macrophages (also called M1) and alternatively activated macrophages (also called M2) (10–12). In fact, these phenotypes represent their dominant appearance in a wide spectrum of overlapping activation types. Other M2-like transitional phenotypes have been described as well, but to date these have been difficult to distinguish from M2 macrophages *in situ* in tissues due to lack of phenotype-specific markers (6, 13–15).

In general, M1-dominant macrophages have enhanced microbicidal and tumoricidal capacity and secrete high levels of pro-inflammatory cytokines like interleukin-12 (IL-12). M1-dominant macrophages can also inhibit fibrotic activities of fibroblasts by releasing antifibrogenic or fibrolytic factors such as MMPs (16, 17). M2-dominant macrophages, activated by interleukin-4 and interleukin-13, are associated with increased fibrogenesis, tissue remodeling, and angiogenesis (17–19). *In vitro*, Song et al. (17) showed that the M2-dominant macrophages produce profibrogenic factors like platelet-derived growth factor-BB (PDGFBB) and transforming growth factor- β (TGF β) and that these M2-dominant cells increase collagen production and proliferation of fibroblasts. Although M2 macrophages are predominantly considered to be pro-fibrotic, they are also associated with anti-fibrotic properties, which may be explained by the different and overlapping M2 phenotypes that exist (5, 11). For instance, M2 macrophages can also aid resolution of fibrosis by phagocytizing apoptotic cells and matrix components via mannose and scavenger receptors (20–22). In addition, Pesce et al. (23) showed that arginase-1 expressing M2 cells were related to suppression rather than induction of fibrosis.

Thus far, most of the knowledge generated about the different macrophage subsets is derived from *in vitro* studies, from flow cytometry analyses of isolated liver macrophages (6), and from gene analysis of liver homogenates (24). Although these techniques generate useful quantitative information, histological detection of macrophages gives unique and additional information with regard to their tissue localization without selection due to isolation limitations or with minor risk of missing changes because other cells express the same markers, such as observed in tissue homogenates (25).

How the different phenotypes are distributed in diseased liver tissue is still largely unexplored. Therefore, we aimed to illustrate, using immunohistochemical techniques, how different macrophage phenotypes are distributed *in situ* during fibrogenic responses and resolution of fibrosis using the general M1 and M2 classification as a starting point. Of the markers commonly used, we chose IL-12 and IRF-5 as markers for the M1-dominant subtype (26). Inducible nitric oxide synthase (iNOS), another commonly used M1 marker, was not chosen because its dominant expression in hepatocytes would make distinguishing neighboring iNOS expressing macrophages difficult (27, 28). To detect M2 polarization, we used upregulation of the mannose receptor (MRC1; also known as CD206), transglutaminase-2 (TGM-2), and chitinase-like secretory protein YM-1 (mouse only) (29–32). TGM-2 was recently identified as a new human and murine M2 marker (33). The commonly used M2 marker arginase could not be used for reasons similar to iNOS (27).

MATERIALS AND METHODS

ANIMALS

Male mice (BALB/c, ± 25 g) were obtained from Harlan (Zeist, The Netherlands) and housed in a temperature-controlled room with 12 h light/dark regimen. The animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Groningen (The Netherlands) and were

performed according to strict governmental and international guidelines on animal experimentation.

ANIMAL MODELS

Chronic liver injury (fibrosis) model

Mice received twice-weekly intraperitoneal injections of CCl4 for 4 or 8 weeks. The dose of CCl4 was gradually increased (diluted in olive oil; week 1: 0.5 ml/kg, week 2: 0.8 ml/kg, week 3–8: 1 ml/kg). Mice were sacrificed after 4 or 8 weeks reflecting early and advanced fibrosis, respectively.

Resolution model

Mice received CCl4 for 4 weeks (with increasing CCl4 doses as described in the previous section). After 4 weeks, CCl4 administration was stopped and the mice were allowed to recover for a week after which they were sacrificed ($n = 6$ per group).

HUMAN LIVERS

Residual human liver tissue samples were obtained from the Department of Hepato-Pancreato-Biliary Surgery and Liver Transplantation [University Medical Center Groningen (UMCG), the Netherlands]. At the UMCG, all patients eligible for organ transplantation are asked to sign a general consent form for the use of left-over body material (after diagnostic procedures) for research purposes. The experimental protocols were approved by the Medical Ethical Committee of the UMCG (Groningen) and the anonymized tissue samples were used according to Dutch guidelines. Normal human liver tissue ($n = 7$) was obtained from residual liver tissue from donor livers discarded for transplantation because of technical reasons. Cirrhotic human liver tissue ($n = 6$) was obtained from patients undergoing liver transplantation. Indications for transplantation were a.o. primary sclerosing cholangitis (PSC), primary biliary cirrhosis (PBC), congenital cirrhosis, and Wilson's cirrhosis. Although all human liver material is anonymized, some available patient characteristics are listed below (see Table 1).

TISSUE PROCESSING

Tissue specimens from at least three different mouse liver lobes were snap frozen in isopentane (-80°C) for immunohistochemical analysis, or in liquid nitrogen for western blot analysis.

A wedge (10–60 g) of freshly obtained human liver was cut, perfused with cold University of Wisconsin organ storage solution (DuPont Critical Care, Waukegan, IL, USA) immediately after resection, and pieces were snap frozen in isopentane (-80°C).

Table 1 | Available patient characteristics of the used human livers.

Patient characteristics	Normal livers	Cirrhotic livers
	<i>N</i> = 7	<i>N</i> = 6
Age (years)	41 (10–57)	49 (35–66)
Gender	<i>N</i> = 4: F <i>N</i> = 2: M <i>N</i> = 1: not known	<i>N</i> = 3: F <i>N</i> = 1: M <i>N</i> = 2: not known

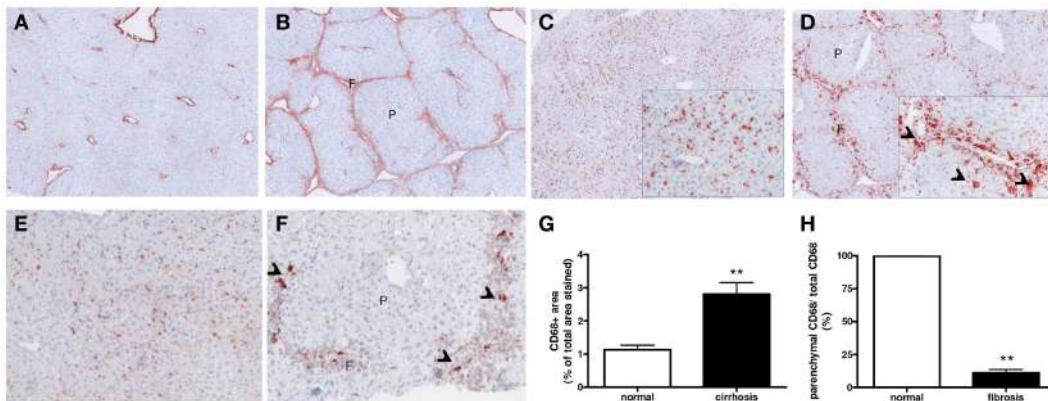


FIGURE 1 | Expression and localization of macrophages in normal and fibrotic mouse livers (8 weeks CCL4). Immunohistochemical analysis shows increased extracellular matrix deposition [collagen type I (**A,B**)] and presence of macrophages [CD68 (**C–F**)] in fibrotic (**B,D,F**) as compared to healthy livers (**A,C,E**). Note the increased size of certain CD68-positive macrophages in the fibrotic areas (**F**) in the CCL4 livers

[arrow heads in insert (**C,F**)]. (**G,H**) Image analysis of CD68 staining. While the total area of CD68+ cells was increased in fibrotic livers, a significantly lower CD68-stained area was found in the parenchyma (*p*) of fibrotic livers as compared to normal. Magnifications: 40× (**A–D**), 100× (**E,F**), and 200× (inserts). f, Fibrotic matrix; p, liver parenchyma. N = 6/group. **p < 0.01.

IMMUNOHISTOCHEMICAL ANALYSIS

Acetone-fixed cryostat sections (4 μm) were stained according to standard immunohistochemical procedures with 3-amino-9-ethyl-carbazole to detect expression of relevant markers (32). Sections were incubated with the primary antibody for 1 h. Primary antibodies to detect fibrotic extracellular matrix [polyclonal goat anti-collagen type I from Southern Biotech], macrophages [mouse anti-human CD68 (DAKO), monoclonal rat anti-mouse CD68 (AbD Serotec, Düsseldorf, Germany), and polyclonal rabbit anti-human CD68 (Santa Cruz Biotechnology)], M1 macrophages [polyclonal rabbit anti-human and mouse IRF-5 (Protein Tech, Manchester, UK), goat polyclonal anti-human IL-12 p40 antibody (ThermoScientific), and goat polyclonal anti-human and mouse MMP9 (Santa Cruz)], and M2 macrophages [polyclonal goat anti-mouse chitinase 3-like/ECF-L (YM-1; R&D), rabbit anti-human TGM-2 (AbD Serotec) and CD206 (rat anti-mouse CD206 and mouse anti-human CD206) both from BioLegends (ITK Diagnostics, Uithoorn, The Netherlands)] were used. Staining of CD68 was quantified by image analysis with Cell^D analysis program (Olympus, Zoetermeer, The Netherlands).

To detect co-localization, we used double-staining techniques with peroxidase and AEC (red) and alkaline phosphatase and Naphtol AS-MX phosphate/Fast Blue BB (blue) (34). Double stainings for IRF-5, IL-12, and CD206 were visualized with NovaRed (red) and BCIP/NBT (blue) from Vector Laboratories.

WESTERN BLOTTING ANALYSIS

Tissue samples were homogenized on ice in cold RIPA buffer [50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.1% Igepal in 0.5% sodium deoxycholate with one tablet of protease inhibitor cocktail and one tablet of phosphatase inhibitor (Roche Diagnostics, Mannheim, Germany)] and lysates were centrifuged for 1 h (13,000 rpm, 4°C). The supernatants were stored at -80°C. Total protein (100 μg) from each sample was applied on SDS-PAGE (10%), transferred to polyvinylidene difluoride membranes, and

incubated overnight at 4°C with the indicated primary antibodies. After washing and incubation with secondary horseradish peroxidase-coupled antibodies, the protein bands were visualized with ECL (Perkin-Elmer, Groningen, The Netherlands) and quantified by G-Box (Syngene, Cambridge, UK).

In order to quantify the marker of interest, we corrected the expression with the expression of the housekeeping protein GADPH (for human samples) or β-actin (for mouse samples).

STATISTICAL ANALYSIS

Results are expressed as means ± SEM. All data were analyzed with the Mann-Whitney U test (Graph Pad software). Differences were considered significant at *p* < 0.05.

RESULTS

LOCALIZATION OF CD68-POSITIVE MACROPHAGES IN HUMAN AND MOUSE FIBROTIC LIVERS

After chronic CCl4 damage (8 weeks CCl4), pericentral necrosis led to wound-healing responses with influx of myofibroblasts and increased collagen deposition (**Figures 1A,B**) in mouse livers. As compared to normal livers, a higher number of CD68-positive cells was found in fibrotic livers, and these macrophages predominantly concentrated in scars during advanced fibrosis (**Figures 1C,D,G**). Furthermore, several scar-associated macrophages differed in appearance from macrophages in normal livers, even some resembled giant cells (**Figures 1D,F**). Remarkably, significantly less staining for CD68+ cells was found in the parenchymal areas of fibrotic livers as compared to normal (**Figures 1E–H**).

Collagen deposition was also greatly increased in end-stage human cirrhotic livers (**Figures 2A,B**), and a similar hepatic distribution of macrophages as in mice was found (**Figure 2**). Macrophages (CD68+) were prominently present in cirrhotic scars irrespective of the origin of cirrhosis (**Figures 2D–F**). The total number of CD68+ cells was somewhat, though not significantly, higher in cirrhotic than normal livers (**Figure 2G**). Again, a trend

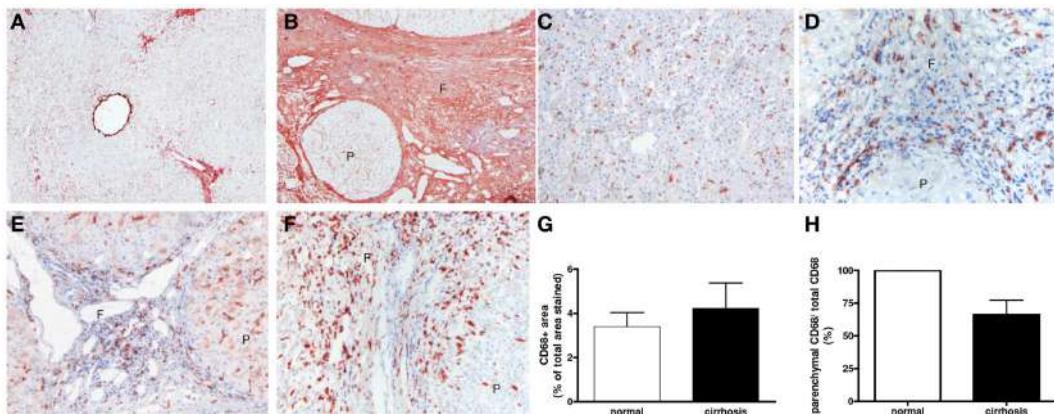


FIGURE 2 | Expression and localization of macrophages in normal and cirrhotic human livers. Immunohistochemical analysis shows enhanced deposition of the extracellular matrix protein collagen type I (**A,B**) and presence of macrophages [CD68 (**C–F**)] in normal (**C**) cirrhotic livers of various origins [**(D)** PBC (**E**) PSC and (**F**) congenital cirrhosis]. Note the abundant

presence of macrophages in the collagenous fibrotic bands (**F**). (**G,H**) Image analysis of CD68 staining in human livers. Reduced CD68 staining was found in the parenchymal area (*p*) of human cirrhotic livers as compared to normal. Magnifications: 40× (**A,B**) and 100× (**C–F**). *f*, fibrotic matrix; *p*, liver parenchyma. *N* = 5 cirrhotic livers, *N* = 6 normal livers.

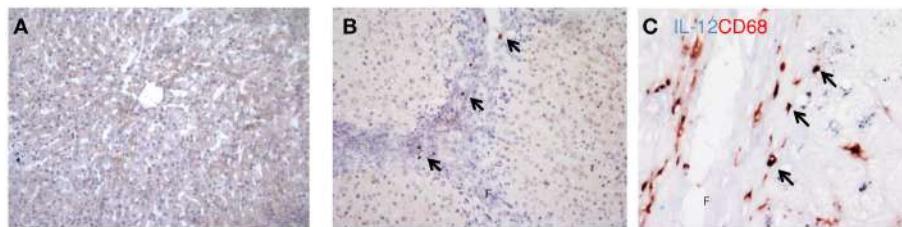


FIGURE 3 | Localization of IL-12 (M1) in cirrhotic human livers. Immunohistochemical staining for IL-12p40 in cirrhotic human livers (**B**), while no staining was observed in normal livers (**A**). (**C**) Co-localization of

IL-12 and CD68. Arrows indicate co-localization, *f*, fibrotic matrix. Magnifications: 100× (**A,B**) and 400× (**C**). *N* = 5 cirrhotic livers, *N* = 6 normal livers.

toward less staining for CD68 was found in the parenchymal areas of cirrhotic livers as compared to healthy livers (Figure 2H).

M1-DOMINANT MACROPHAGES IN MOUSE AND HUMAN LIVERS

Interleukin-12, a major cytokine produced by classically activated macrophages, was used as an immunohistochemical marker to detect the M1-dominant subset in the liver (Figure 3). In human livers, higher numbers of IL-12-positive cells were detected in cirrhotic human livers (Figure 3B) as opposed to barely detectable IL-12 staining in healthy livers (Figure 3A). This increased expression was confirmed by western blot analysis (Figure 4B). IL-12 positive cells were found solely in the cirrhotic collagen bands. The staining for IL-12 co-localized completely with CD68, but only a minor fraction of the CD68 population was positive for IL-12 (Figure 3C).

IRF-5 was also used to identify M1 macrophages in human and mouse livers (Figure 5). IRF-5 staining co-localized completely with CD68 in livers of both species (Figure 5A). Similar to IL-12, only a subset of the total number of macrophages expressed IRF-5. To prove the phenotype-specificity of this M1 marker, we performed double-immunostainings of IRF-5 and the M2 marker CD206 in human livers and found little to no co-localization

(Figure 5B). Microscopic analysis showed that IRF-5 staining was almost absent in normal mouse and human livers (Figures 5C,E). In advanced fibrotic mouse livers, the staining was present in cells residing in the scarred areas (Figure 5D). Similarly, in human livers IRF-5 staining was also predominantly found in cells of the septa (Figure 5F). Western blot analysis of liver homogenates revealed a significantly higher expression of IRF-5 in diseased mouse and human livers as compared to healthy livers (Figures 4A,B).

M2-DOMINANT MACROPHAGES IN MOUSE AND HUMAN LIVERS

Subsequently, we studied the hepatic distribution of alternatively activated macrophages (Figures 6–8). CD206 is a well-known marker for both mouse and human M2-dominant macrophages. CD206/CD68 double-positive cells were present in fibrotic livers and were predominantly found in scars (Figure 6A). In addition to this, CD206 staining was present in liver parenchyma and this staining most likely reflected expression of CD206 on sinusoidal endothelial cells [identified with CD31 (Figure 6F)]. The pronounced endothelial staining of CD206 complicates interpretation of analyses of whole tissue homogenates (like western blot and mRNA expression analyses) used in the macrophage field. Microscopic evaluation of sections stained for both CD206 and CD68

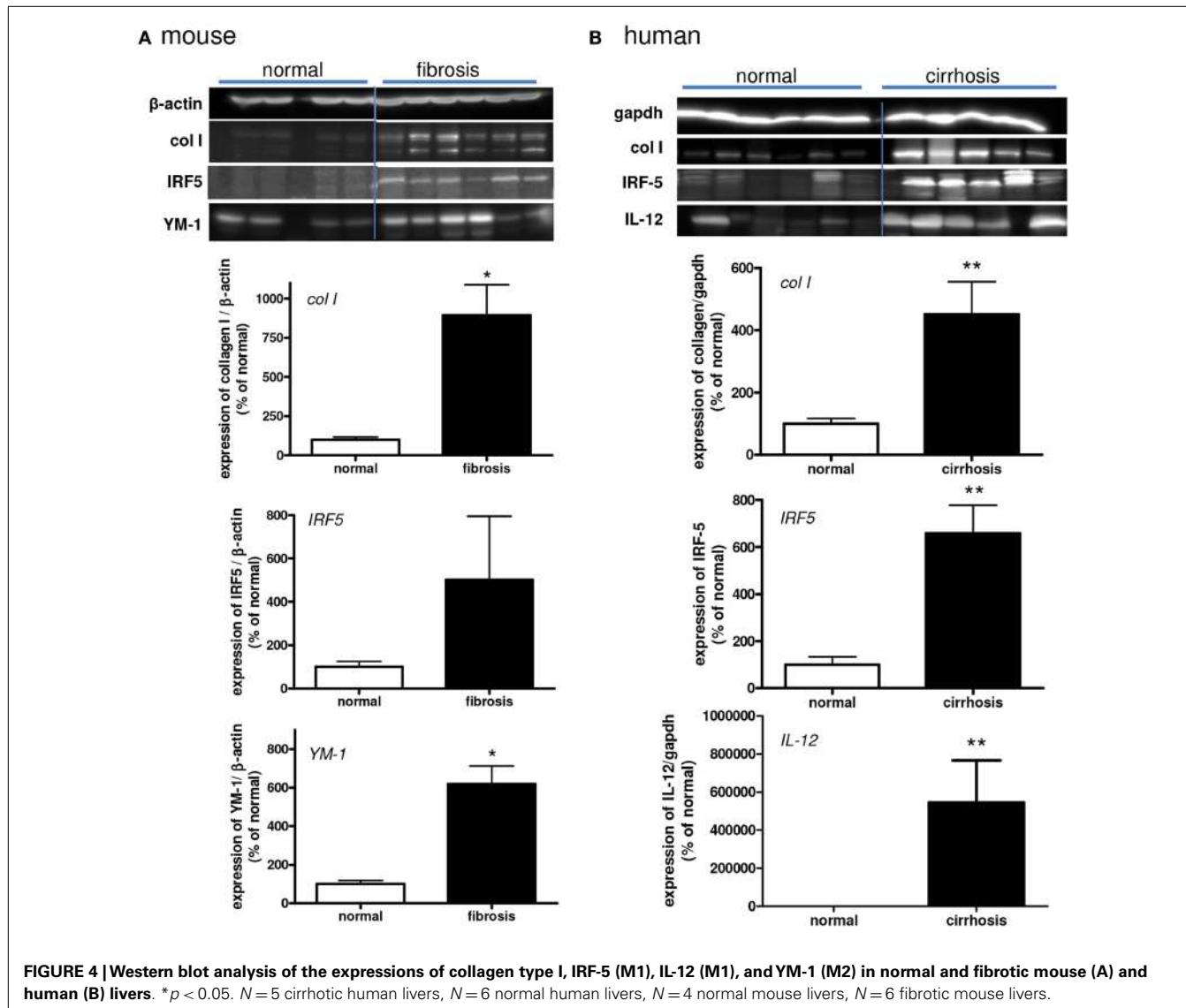


FIGURE 4 | Western blot analysis of the expressions of collagen type I, IRF-5 (M1), IL-12 (M1), and YM-1 (M2) in normal and fibrotic mouse (A) and human (B) livers. * $p < 0.05$. N = 5 cirrhotic human livers, N = 6 normal human livers, N = 4 normal mouse livers, N = 6 fibrotic mouse livers.

indicated that double-positive cells were more frequent in fibrotic liver than in normal livers, whereas western blot analysis revealed reduced expression in both human and mouse fibrotic livers (data not shown).

YM-1 was used as another M2 marker for mouse livers (Figure 7). Expression of YM-1 is restricted to mice and can therefore not be used for human liver tissue (29). YM-1 co-localized with CD68 and with CD206 (Figures 7A,B). All cells that expressed YM-1 were positive for CD68 and CD206, but not all CD68-positive cells stained positive for YM-1. The hepatic expression of YM-1 was clearly higher after chronic CCl4 damage as demonstrated by immunohistochemical staining (Figures 7C–F) and western blot analysis (Figure 4A). YM-1 was present in the fibrotic collagenous bands of the CCl4-damaged livers.

The recently described M2 marker TGM-2 (33) was also used to identify M2-dominant macrophages in human livers (Figure 8). Immunohistochemical staining for TGM-2 resulted in staining of the parenchymal area of normal livers, mostly

staining hepatocytes, but in cirrhotic livers additional strong positive cells were found in septa (Figures 8A,B). TGM-2 staining present in scars co-localized with CD68 (Figure 8C) and with CD206 (Figure 8D) confirming presence of TGM-2 in hepatic M2-dominant macrophages that accumulate in these areas. As with iNOS, arginase-1, and CD206, quantitative evaluation of TGM-2 was confounded by its high expression in hepatocytes. We could not detect differences between normal and cirrhotic livers (data not shown).

M1- AND M2-DOMINANT MACROPHAGES IN A MOUSE MODEL OF RESOLUTION

Cessation of fibrosis-inducing agents induces reversal of the fibrotic process (35). This is also apparent in our mouse model with lower hepatic collagen type I in livers of mice in which CCl4 administration was stopped versus their fibrotic equivalents (Figures 9A–C). Since macrophages are important during resolution (8, 9), we studied the localization and numbers of macrophage

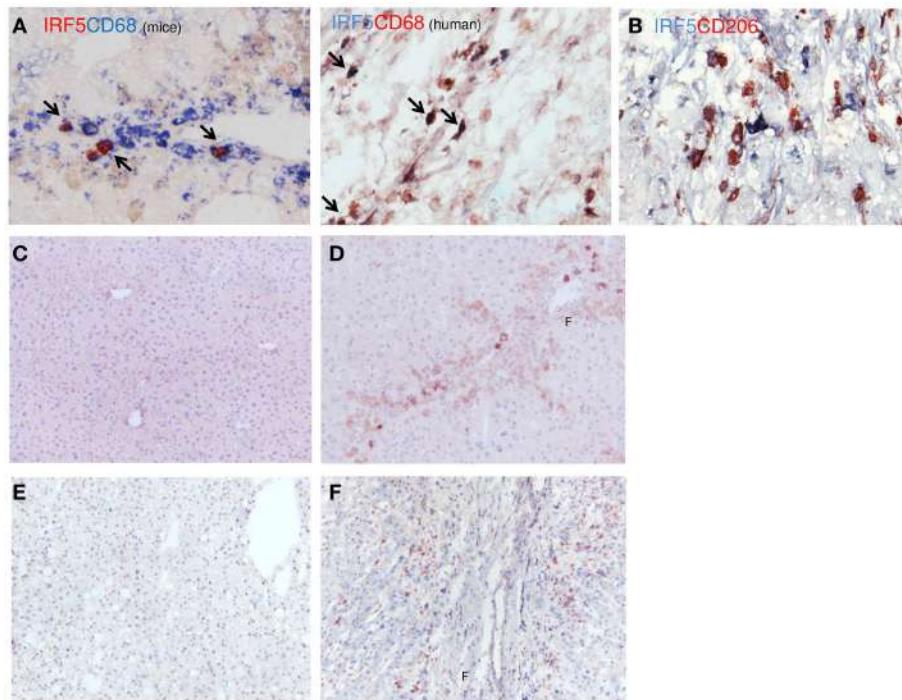


FIGURE 5 | Localization of IRF-5 (M1) in mouse and human livers.

(A) Co-localization of IRF-5 and CD68 in mouse and human livers.
 (B) Double-staining for IRF-5 (blue staining) and CD206 (red staining) showed no co-localization. (C,D) Immunohistochemical staining of IRF-5 in normal

mice livers (C) and in livers after chronic CCl₄ damage (D). (E,F) IRF-5 staining of normal (E) and cirrhotic (F) human livers. f, fibrotic matrix. Magnifications: 100× (C–F) and 400× (A,B). N = 5 cirrhotic human livers, N = 6 normal human livers, N = 4 normal mouse livers, N = 6 fibrotic mouse livers.

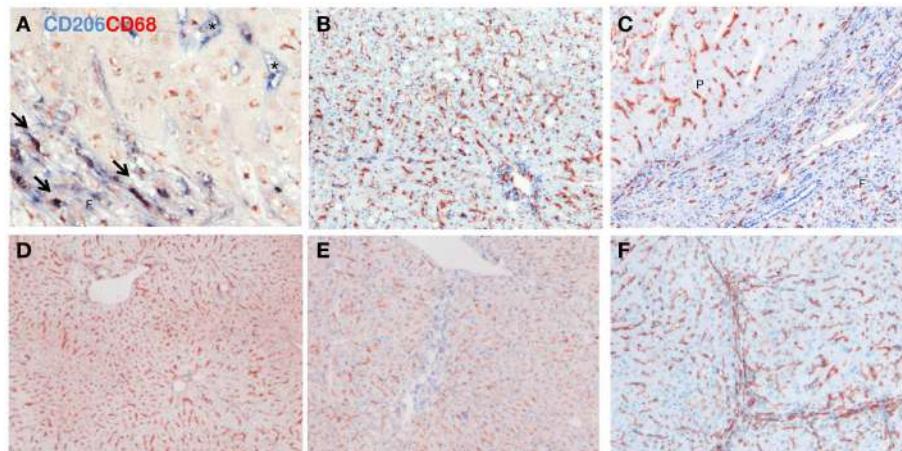


FIGURE 6 | Immunohistochemical staining for CD206 (MCR-1; mannose receptor) in human (A–C) and mouse (D,E) normal (B,D) and cirrhotic (C,E) livers. (A) Co-localization of CD206 (blue staining) and CD68 (red staining). Arrows indicate co-localization, asterisks indicate endothelial

staining of CD206. (F) Immunohistochemical staining for CD31 in fibrotic mouse livers illustrating staining of sinusoidal endothelial cells. Magnifications: 100× (B–F) and 400× (A). N = 5 cirrhotic human livers, N = 6 normal human livers, N = 4 normal mouse livers, N = 6 fibrotic mouse livers.

phenotypes in these two groups of mice. Expression of CD68 was significantly lower in livers undergoing resolution as compared to their fibrotic counterparts (Figures 9D–H).

We detected a slightly reduced expression of IRF-5 with both immunohistochemical and western blot analysis (Figure 10). However, a clear difference in the number of M2-dominant

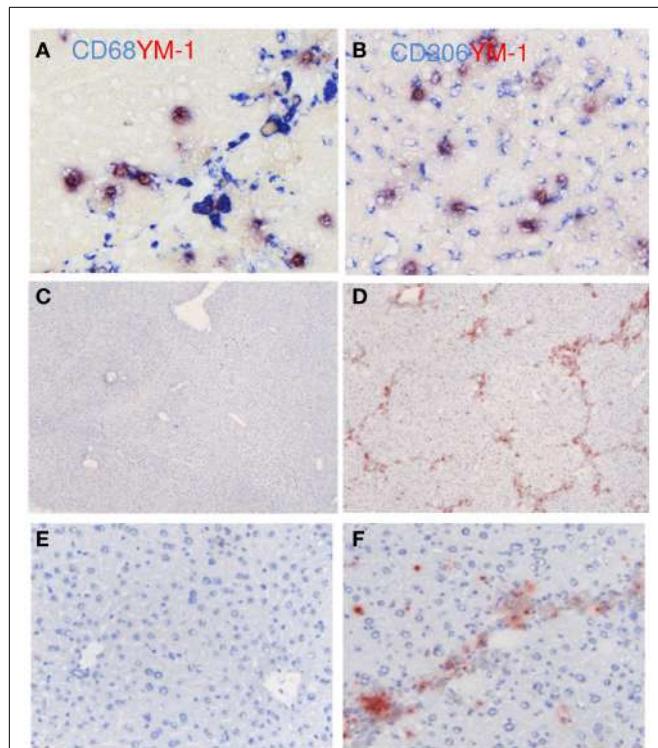


FIGURE 7 | Localization of YM-1 (M2) in mouse livers. **(A)** Co-localization of YM-1 (red staining) and CD68 (blue staining). **(B)** Co-localization of YM-1 (red staining) and CD206 (blue staining). **(C–F)** Immunohistochemical localization of YM-1 in livers of normal mice (**C,E**) and in advanced fibrosis (**D,F**). Magnifications: 40× (**C,D**), 200× (**E,F**), and 400× (**A,B**). $N=4$ normal mouse livers, $N=6$ fibrotic mouse livers.

macrophages was found (Figure 11). YM-1 staining was abundantly present in fibrotic livers, but in livers undergoing resolution this M2 marker was almost completely gone. Western blot analysis revealed a reduction of $81 \pm 8\%$ in YM-1 expression during resolution (Figure 11E).

Since the number of IRF-5⁺ (M1-dominant) macrophages was almost unchanged in fibrotic livers compared to livers undergoing resolution, we measured MMP expression as a functional read out of the presence of these macrophages. M1-dominant cells are known to express MMP9 (17, 21, 36, 37) and western blot analysis of these livers revealed significantly higher expression of MM9 92 kDa, which is known as pro-MMP9, and its processed form (67 kDa MMP9) in the livers undergoing resolution (Figure 10F).

DISCUSSION

Recently, activation of macrophages into different phenotypes has been subject of study in various diseases including in liver diseases. Almost all knowledge obtained thus far is derived from *in vitro* studies or from FACS or PCR analyses of tissues. These *in vitro* studies have been essential to discover markers to distinguish the various macrophages phenotypes and to identify the specific activities of these subsets. How these *in vitro*-generated phenotypes relate to macrophages *in situ* is largely unexplored. In this study, results were obtained from the CCL4 mouse model at several time

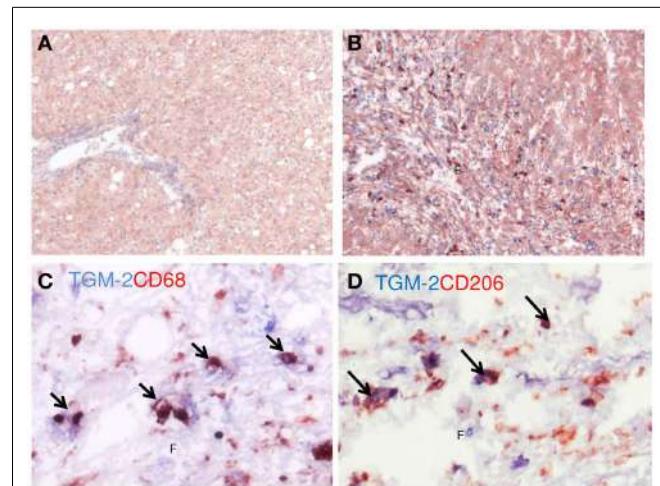


FIGURE 8 | Localization TGM-2 (M2) in human livers.

(A,B) Immunohistochemical localization of TGM-2 in normal (**A**) and cirrhotic (**B**) human livers. Note the presence of the strongly stained cells in the fibrotic matrix (**F**). **(C,D)** Co-localization of TGM-2 (blue staining) and CD68 (red staining). **(D)** Co-localization of TGM-2 (blue staining) and CD206 (red staining). Arrows indicate co-localization. Magnifications: 100× (**A,B**) and 400× (**C,D**). $N=5$ cirrhotic human livers, $N=6$ normal human livers.

points in disease progression (reflecting early and advanced fibrosis) and resolution. Although we are aware that more time points in this mouse model can support broader conclusions, our outcomes with regard to the presence and localizations of the various macrophage phenotypes are first steps toward understanding the dynamics of macrophage phenotypes in relation to localization. A major advantage of our studies is the verification of mouse data in samples of human liver disease. The fact that we find similar distributions of macrophage phenotypes in end-stage disease of a number of different etiologies may point at converging disease mechanisms irrespective of cause.

We used many commonly used markers M1- and M2-dominant phenotypes and found that not all of them can be used reliably for liver tissue. With the ones that can be used, we demonstrated that M1- and M2-dominant subsets are localized side by side in scars of human and mouse cirrhotic livers. Although M1 and M2 markers can be expected to be present on the same cell, based on the theory of overlapping spectra of macrophage subsets (13–15), with our markers (IRF-5 and CD206), we found little to no co-localization. We showed that IL-12 and IRF-5 are useful immunohistochemical markers for M1-dominant macrophages in liver tissue (both mouse and human) and YM-1 for M2-dominant macrophages in murine liver tissue. CD206 and TGM-2 can be useful for immunohistochemistry of M2-dominant macrophages in human liver tissue, but are much less specific and therefore are hard to quantify. Furthermore, in fibrotic livers undergoing resolution we found that M2-dominant macrophages (YM-1 positive cells) disappeared, while M1-dominant macrophages (IRF-5 positive cells) persisted in the scarred areas producing MMPs.

Interleukin-12 and IRF-5 were used to identify the classically activated macrophages in fibrotic livers. Krausgruber et al. (26)

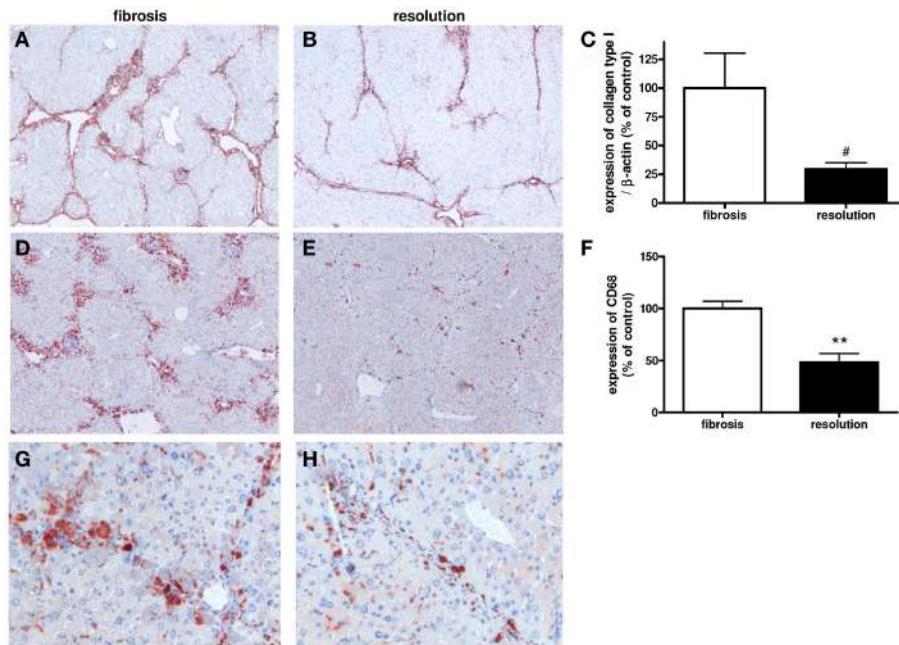


FIGURE 9 | Immunohistochemical and western blot analysis of the hepatic expressions of collagen type I (A–C) and macrophages [CD68 (D–H)] in fibrosis [4 weeks of CCl4 in mice (A,D,G)] and in livers

undergoing resolution [after cessation of CCl4 administration, resolution (B,E,H)]. ** $p < 0.01$, * $p < 0.1$. Magnifications: 40× (A–E) and 200× (G,H). N = 6/group.

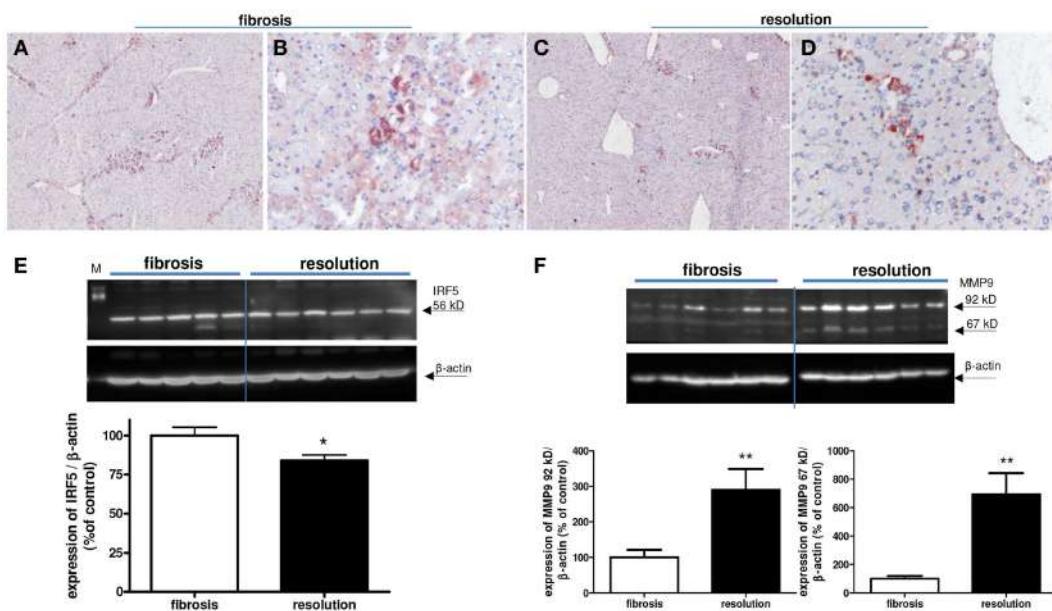


FIGURE 10 | Expressions of IRF-5 and MMP9 (M1) in fibrotic mouse livers [4 weeks CCl4 (A,B)] and in fibrotic livers undergoing resolution [after cessation of 4 weeks of CCl4 administration (C,D)]. Immunohistochemical pictures demonstrate an overview [(A,C) magnification 40×] and close up [(B,D) magnification 200×]. (E) Western blot quantification of hepatic IRF-5

expression in fibrosis versus resolution group, and (F) western blot quantification of MMP9 expression in fibrosis versus resolution group. A 92 kDa pro-form and a 67 kDa processed form of MMP9 is significantly increasingly expressed in livers undergoing resolution. * $p < 0.05$, ** $p < 0.01$. N = 6/group.

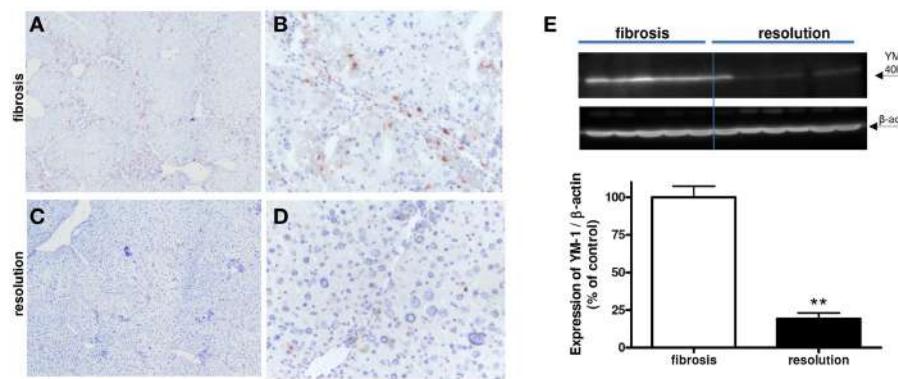


FIGURE 11 | Expressions of YM-1 (M2) in fibrotic mouse livers [4 weeks CCl4 (A,B)] and in fibrotic livers undergoing resolution [after cessation of CCl4 administration (C,D)]. Immunohistochemical pictures demonstrate an

overview [(A,C) magnification 40×] and close up [(B,D) magnification 200×]. (E) Western blot quantification of YM-1 expression in fibrosis versus resolution. ** $p < 0.01$. $N = 6/\text{group}$.

showed high expression of IRF-5 in human M1 macrophages in culture, while M2 and non-activated macrophages did not express IRF-5. We now show that IRF-5 can be used to identify a subset of macrophages *in vivo* in human and mouse livers. Our study clearly demonstrates that M1-dominant macrophages (CD68/IRF-5⁺ cells) are significantly increased in diseased livers as compared to normal. IRF-5⁺ cells are located in fibrous septa in advanced fibrosis. These localizations may correspond with reported *in vitro* M1 activities such as production of pro-inflammatory cytokines and chemokines (5, 11, 17). The observation that M1-dominant macrophages are still present in livers undergoing resolution might be related to their ability to produce MMPs (12, 16, 17, 37). Classical activation of macrophages *in vitro* resulted in higher expression of MMP7 and MMP9 and both may be necessary in the collagenous scars for removal of collagen fibers. Indeed, in our studies we detected higher MMP9 expression in fibrotic livers undergoing resolution. In addition, Fallowfield et al. (9) demonstrated higher hepatic MMP13 expression by scar-associated macrophages in CCL4-damaged livers and it was found that resolution of CCl4-induced fibrosis was retarded in MMP13-deficient mice. However, macrophage phenotypes in these scars were not further characterized. We now show with our localization studies that during fibrogenesis scar-associated macrophages are both of M1- and M2-dominant phenotype, while during resolution the scar-associated macrophages are predominantly M1 cells. It therefore appears that M1 macrophages may also be responsible for the MMP13 production that is necessary for resolution. Co-localization studies with IRF-5 and MMP13 may provide additional insights.

To identify M2-dominant macrophages, we started with the well-known marker CD206 (mannose receptor, MCR-1) (11). While in many organs M2-dominant macrophages specifically express CD206, in livers CD206 expression is found in macrophages as well as in sinusoidal endothelial cells, making quantitative interpretations difficult. In addition, we therefore used the well-known M2-selective marker YM-1, which does not have this disadvantage. However, this marker is only present in rodents (29) and cannot be used for human tissues. TGM-2 is

a novel marker for M2 macrophages recently described by us in lungs (33). The advantage of TGM-2 is that this marker is conserved in mice and humans. TGM-2 is a multifunctional enzyme involved in transamidation and cross-linking of proteins. It is also linked to apoptosis, cellular differentiation, and matrix stabilization (38–40). In liver, Popov et al. (41) showed that TGM-2 is enhanced in mice with CCl4-induced fibrosis, but they found no relationship between TGM-2 and stabilization of fibrotic matrix. However, TGM-2 expression was not related to macrophage activities. Although the hepatic expression is not limited to macrophages, as can be seen in Figure 8, TGM-2 staining in the scar-associated macrophages in cirrhotic livers is much stronger than in other hepatic cells. Therefore, this marker can be used for immunohistochemical stainings but quantification using western blot or PCR will not yield useful results. To summarize, using a combination of the markers CD206, YM-1, and TGM-2, we are able to show that M2-dominant macrophages are present in scar tissue during hepatic fibrogenesis. We now show that TGM-2 is co-expressed in CD68⁺ and in CD206⁺ cells in fibrotic septa in human and mouse livers, confirming its presence in M2-dominant hepatic macrophages.

This study clearly shows the presence of M1- and M2-dominant macrophages side by side in fibrotic lesions in human and mouse livers, indicating that apparently both are necessary in fibrotic responses. At least two questions remain: (1) where do these macrophages come from, meaning are they derived from incoming monocytes and are thus bone marrow-derived or do they develop from tissue-resident Kupffer cells that are embryonic in origin (42). Unfortunately, our study cannot answer this question, as there are no markers discovered yet that can reliably distinguish bone marrow-derived from embryonic macrophages. Previous studies showed that monocytes do infiltrate the liver during fibrogenesis and resolution and also that Kupffer cells do proliferate during injury (43, 44). Understanding the dynamics of all these different macrophages during fibrogenesis/resolution and their interactions is a subject of intense research interest. (2) How these macrophage phenotypes interact with each other and with other resident cells to enhance or dissolve fibrosis. Song

et al. (17) showed that M2-dominant macrophages increased the proliferation index and collagen synthesis of co-cultivated WI-38 fibroblasts, while M1-dominant macrophages markedly reduced collagen production by these cells. Most *in vitro* studies suggest that M2 activation results in enhanced fibrogenesis, while M1 activation inhibits fibrogenesis through antifibrogenic or fibrolytic factors. Just recently, Lopez-Navarrete (18) showed the importance of M2-dominant macrophages in promoting fibrogenesis in a CCl₄-induced model of liver fibrosis in which Kupffer cells were stimulated to polarize to an M2-dominant phenotype after hepatic inoculation of *Taenia crassiceps* larvae. Our results also suggest a more pro-fibrotic character of M2-dominant macrophages, because M2 markers were present in fibrotic lesions in human and mouse livers, but were nearly absent in the livers during resolution of fibrosis.

Recently, Ramachandran et al. (6) suggested a restorative role for macrophages during resolution of fibrosis after cessation of CCL4 intoxications using flow cytometry. The persistence of M1-dominant macrophages during resolution in our studies indicates that this restorative phenotype may have a more M1 bias. M1-dominant macrophages have been reported to be major producers of various MMPs and MMP-producing macrophages were previously reported to be present during liver regeneration in mice (3, 9, 16). However, M2-dominant macrophages can also express MMPs [most notably MMP12 (45)] and were found to be important cells for efferocytosis and phagocytosis of matrix debris (16, 21, 46–48). These characteristics of M2-dominant cells may also be necessary during the resolution phase. The reason we do not see M2-dominant macrophages anymore in our resolution model may be caused by the fact that the resolution is ongoing (based on reduced hepatic collagen deposition) and these functions of M2-dominant macrophages may have less important. Studying macrophage phenotype localizations at more time points during resolution may shed more light on the specific dynamics of the macrophage phenotypes during resolution.

In conclusion, using a set of established as well as recently identified markers we now clearly show local accumulation of both M1- and M2-dominant macrophages in fibrotic septa of mouse and human end-stage cirrhotic livers. This provides a basis for further exploring the different activities of these various macrophage phenotypes during liver fibrosis and resolution of fibrosis. The observation that during liver remodeling M1-dominant macrophages may persist and M2-dominant macrophages may disappear indicates that different combinations of M1 versus M2-dominant macrophages may play a key role in fibrogenesis and resolution. Manipulation of their balance may therefore be of therapeutic value.

ACKNOWLEDGMENTS

We gratefully acknowledge the surgeons of the Department of Hepato-Pancreato-Biliary Surgery and Liver Transplantation (University Medical Center Groningen, Groningen, The Netherlands) for providing us with healthy and cirrhotic human liver tissue. In addition, we thank several students of Pharmacy (J. Loder, K. Feenstra, L. Keyzer, E. Alons, and N. Aughsteen) for their practical contributions to this paper.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 10 July 2014; paper pending published: 02 August 2014; accepted: 22 August 2014; published online: 08 September 2014.

Citation: Beljaars L, Schippers M, Reker-Smit C, Martinez FO, Helming L, Poelstra K and Melgert BN (2014) Hepatic localization of macrophage phenotypes during fibrogenesis and resolution of fibrosis in mice and humans. *Front. Immunol.* **5**:430. doi:10.3389/fimmu.2014.00430

This article was submitted to Molecular Innate Immunity, a section of the journal *Frontiers in Immunology*.

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Functional relationship between tumor-associated macrophages and macrophage colony-stimulating factor as contributors to cancer progression

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The current review article describes the functional relationship between tumor-associated macrophages (TAM) as key cellular contributors to cancer malignancy on the one hand and macrophage-colony-stimulating factor (M-CSF or CSF-1) as an important molecular contributor on the other. We recapitulate the available data on expression of M-CSF and the M-CSF receptor (M-CSFR) in human tumor tissue as constituents of a stromal macrophage signature and on the limits of the predictive and prognostic value of plasma M-CSF levels. After providing an update on current insights into the nature of TAM heterogeneity at the level of M1/M2 phenotype and TAM subsets, we give an overview of experimental evidence, based on genetic, antibody-mediated, and pharmacological disruption of M-CSF/M-CSFR signaling, for the extent to which M-CSFR signaling can not only determine the TAM quantity, but can also contribute to shaping the phenotype and heterogeneity of TAM and other related tumor-infiltrating myeloid cells (TIM). Finally, we review the accumulating information on the – sometimes conflicting – effects blocking M-CSFR signaling may have on various aspects of cancer progression such as tumor growth, invasion, angiogenesis, metastasis, and resistance to therapy and we thereby discuss in how far these different effects actually reflect a contribution of TAM.

Keywords: M-CSF, CSF-1, M-CSFR, CSF-1R, tumor-associated macrophages, M1, M2, cancer progression

INTRODUCTION

CANCER MALIGNANCY

Cancer is a complex multi-step process, in which normal cells acquire a certain growth advantage via a process analogous to Darwinian evolution. These cellular changes can occur under many different circumstances, which contributes to the heterogeneity and variability of the occurrence, development, and outcome of neoplastic disease (1). The traits required for malignant growth include self-sufficiency from external growth signals, insensitivity to negative growth signals, resistance to apoptosis, limitless replicative potential, sustained angiogenesis, acquisition of tissue invasiveness, and metastasis. Recently, genetic instability, altered

energy metabolism, the capacity to evade elimination by the immune system, including active immune suppression, as well as smoldering, non-resolving inflammation, leading to accumulation of random genetic alterations in cancer cells due to inflammatory mediators, have been established as additional hallmarks of cancer (2–6). In this regard, tumors consist not only of neoplastic cells, but should be considered as organ-like structures in which a complex bidirectional interplay exists between transformed and non-transformed cells. The malignant potential of transformed cells requires an apt support structure from the stroma, which can consist of fibroblasts, adipocytes, blood, and lymph vessels, but may also be considerably infiltrated by a wide range of immune cells, such as tumor-associated macrophages (TAM) (7).

PRO- AND ANTI-TUMORAL ROLES OF TAM

Tumor-associated macrophages are the predominant leukocytes infiltrating solid tumors and can represent up to 50% of the tumor mass. The clinical significance of these cells is illustrated by the significant link between TAM number and density and a poor prognosis in 80% of the reported studies. The main exception to this general trend seems to be colorectal cancer, for which a high TAM density is significantly associated with enhanced overall survival (8–10).

Tumor-associated macrophages stimulated by TLR ligands, agonistic anti-CD40, or IFN- γ were shown to have important anti-tumoral activities, provided that cancer cell phagocytosis is

Abbreviations: DAMP, damage-associated molecular pattern; EGF, epidermal growth factor; Fizz1, found in inflammatory zone 1; GAST, gastrointestinal stromal tumor; G-CSF, granulocyte-colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; HUVEC, human umbilical vein endothelial cell; mAb, monoclonal antibody; M-CSF, macrophage-colony-stimulating factor; M-CSFR, M-CSF receptor; Mgl2, macrophage galactose-type C-type lectin-2; MMP9, matrix metalloproteinase 9; MMR, macrophage mannose receptor; MO-MDSC, monocytic myeloid-derived suppressor cells; PAMP, pathogen-associated molecular pattern; PMN-MDSC, polymorphonuclear myeloid-derived suppressor cells; PyMT, polyomavirus middle T oncogene; RNI, reactive nitrogen intermediates; ROS, reactive oxygen species; RT2, RIP1-Tag2; SR-A, scavenger receptor-A; TAM, tumor-associated macrophages; TEM, Tie2-expressing monocytes/macrophages; TGF- β , transforming growth factor- β ; TIL, tumor-infiltrating lymphocytes; TIM, tumor-infiltrating myeloid cells; TMEM, tumor microenvironment of metastasis; VEGF, vascular endothelial growth factor.

not inhibited by CD47 expression on the malignant cells, which is a “don’t-eat me signal” (11, 12). In addition, pro-inflammatory macrophages are able to eliminate cancer cells via the production of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) and secrete chemokines that recruit and prime T cells toward an anti-tumor phenotype in some cancer types, resulting in retarded tumor growth or tumor regression (13–17).

Whereas TAM can exert anti-tumoral activities, the ambiguous role of macrophages in tumor progression is reflected in the finding that TAM can also actively contribute to each stage of cancer development and progression (**Figure 1A**). They can promote cancer cell proliferation, invasion, metastasis, and angiogenesis by releasing cytokines, growth factors, extracellular matrix-degrading enzymes, and angiogenic factors including vascular endothelial growth factor (VEGF), prokineticin (Bv8), and matrix metalloproteinase 9 (MMP9). TAM also inhibit cytotoxic T-cell activity by the secretion of suppressive cytokines such as IL-10 and transforming growth factor- β (TGF- β), high levels of arginase activity, and the production of ROS or RNI (18–22). Finally, TAM contribute to tumor relapse following tumor irradiation and anti-angiogenic therapy (23).

It seems unlikely that the diverse anti-tumoral and tumor-promoting activities of TAM are performed by a single cell type, and the existence of distinct TAM subpopulations, linked to different intratumoral microenvironments, has been predicted (10, 24). Depending on the cancer type, the stage of tumor progression and location within the tumor tissue, molecularly and functionally distinct TAM subpopulations coexist in tumors (25–27). This TAM heterogeneity likely reflects the inherent plasticity of macrophages in response to (micro-)environmental triggers.

MACROPHAGE PLASTICITY

Macrophages have a remarkable plasticity and are found in all tissues, where they display great anatomical and functional diversity. They are implicated in a spectrum of roles required for tissue homeostasis, ranging from host defense against infectious agents, to tissue development, wound healing, and immune regulation. Accordingly, macrophages are able to adopt diverse phenotypes or activation states in response to environmental cues, such as cytokines, pathogen-associated molecular patterns (PAMP), tissue damage-associated molecular patterns (DAMP), and other immune stimuli (7, 28, 29).

Macrophage activation is conventionally categorized on a linear scale, in which the two opposing phenotypes are referred to as the classical (M1) versus alternative (M2) macrophage activation state, originally mirroring the Th1 versus Th2 nomenclature (30–34). M1 macrophage activation is driven by exposure to IFN- γ and TLR ligands. These macrophages secrete pro-inflammatory cytokines (such as IL-12, IL-1, IL-6, TNF, ROS, RNI), promote Th1 responses, exert cytotoxic activities, and are involved in defense against bacterial infections and intracellular pathogens. The M2 activation state refers to macrophages that are not M1 activated and comprise various activation states, induced by a wide array of different stimuli, leading to different macrophage classification systems by different authors. These stimuli include Th2 cytokines (such as IL-4 and IL-13), anti-inflammatory cytokines (such as IL-10 and TGF- β), hormones (such as glucocorticoids), and immune

complexes. Consequently, non-M1 macrophages have very diverse functions, ranging from parasite control to immune suppression, wound repair, tissue remodeling, and angiogenesis. Features of these non-M1 macrophages are the low secretion levels of pro-inflammatory cytokines, high expression of macrophage mannose receptor (MMR) and scavenger receptor-A (SR-A), and an arginine metabolism shifted toward the production of ornithine and polyamines by arginase (35–39). Although the M1/M2 classification has proven useful, any form of classification underscores the complexity of the *in vivo* situation, in which numerous stimuli interact to define the final differentiated state and mixed functional profile of macrophages (40–42). In this context, new nomenclature and experimental guidelines for dealing with macrophage activation and polarization have very recently been proposed (43).

M-CSF AS DRIVER OF BOTH DIFFERENTIATION AND PHENOTYPIC POLARIZATION OF MACROPHAGES

The myelopoietic growth factors macrophage-colony-stimulating factor (M-CSF, also known as CSF-1), granulocyte-macrophage-colony-stimulating factor (GM-CSF) and IL-34 are major cytokines in controlling the proliferation, differentiation, and functional regulation of monocytes, macrophages, and dendritic cells [reviewed in Ref. (44)]. M-CSF and IL-34 are produced by a variety of stromal and epithelial cell types and signal through the M-CSF receptor (M-CSFR, CSF-1R, or CD115), a type III receptor tyrosine kinase (45), encoded by the *Csf1r/c-fms* proto-oncogene (46, 47), that seems to be mainly restricted to cells of the mononuclear phagocyte lineage (48).

Especially, M-CSF instructs the myeloid fate in single hematopoietic stem cells, by inducing the myeloid master regulator transcription factor PU.1 (49). Embryonic yolk sac-derived precursors and fetal liver monocytes have been found to give rise to many tissue-resident macrophages that seed all tissues prenatally and are maintained via self-renewal throughout adult life (50). The importance of M-CSF for establishing and maintaining the tissue-resident macrophage pool is illustrated by the M-CSF-deficient osteopetrotic (*op/op*) mouse, which not only suffers from congenital osteopetrosis due to a severe deficiency of osteoclasts, but also features severe defects in many tissue-resident macrophage populations (51). Besides having effects on macrophage precursor differentiation, M-CSF is known to stimulate macrophage survival (52) and self-renewal during steady-state and inflammation (53). However, macrophage populations in distinct tissues are differentially affected by the M-CSF deficiency. For example, skin Langerhans cells and brain microglia were seemingly normal in *op/op* mice, but were largely absent from M-CSFR-deficient mice, a finding which has been explained by the trophic role of IL-34, whose production is restricted to keratinocytes and neurons under steady-state (54, 55).

In addition to a role in resident tissue macrophage differentiation and maintenance, M-CSFR signaling has also been assigned an important role in polarization of macrophage activation, flowing from the observation of significant differences in the transcriptomes of the macrophage populations primarily generated with the use of M-CSF or GM-CSF. M-CSF-driven macrophage differentiation leads to the expression of a substantial part of the M2 transcriptome, including expression of MMR

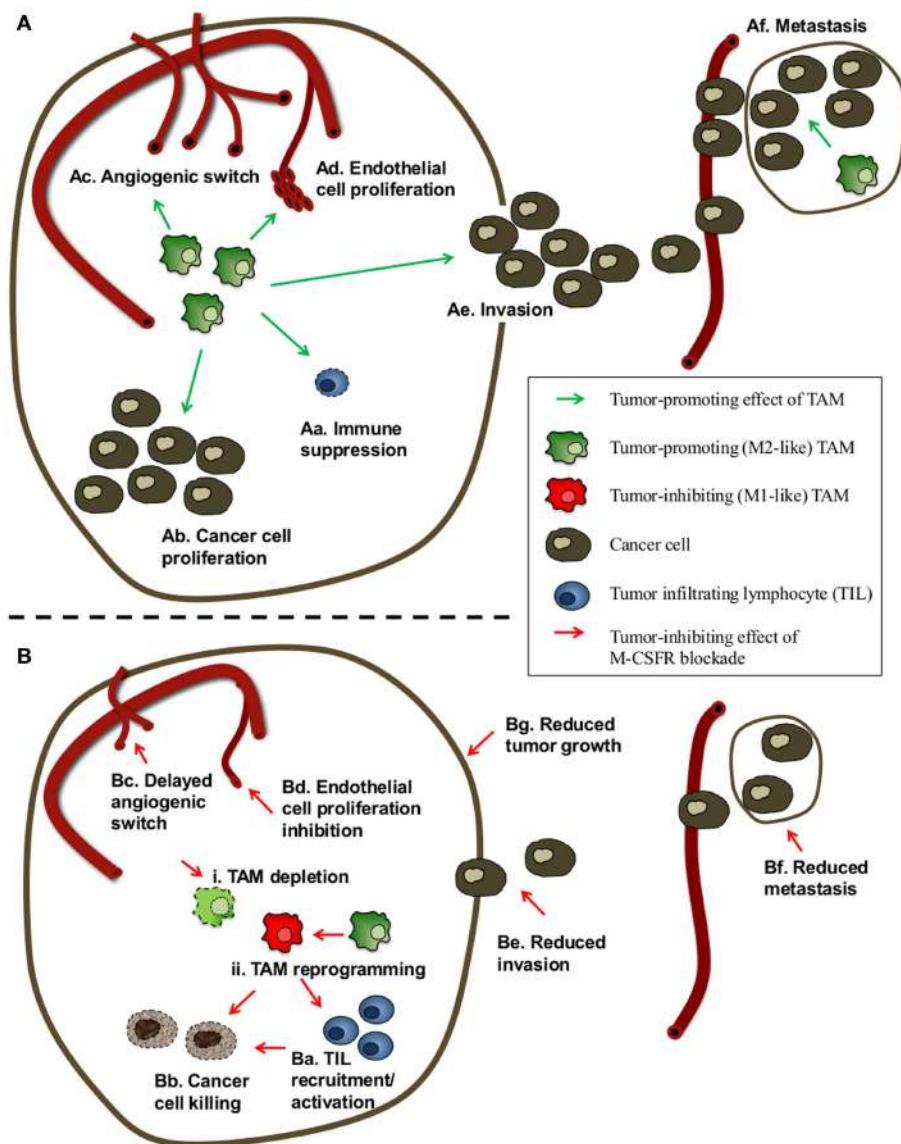


FIGURE 1 | Scheme of the possible effects of TAM and of M-CSFR blockade on cancer progression. (A) Possible tumor-promoting effects of TAM. TAM can promote cancer progression and reduce the efficacy of radiotherapy, chemotherapy, immunotherapy, and anti-angiogenic therapy by combination of different mechanisms. TAM can contribute to enhanced cancer cell numbers by (Aa) inhibiting anti-tumor immune responses and via (Ab) stimulation/maintenance of cancer cell proliferation. TAM can also exert pro-angiogenic activities by enhancing (Ac) angiogenic switching and (Ad) endothelial cell proliferation. Finally, TAM can contribute to cancer malignancy by facilitating (Ae) cancer cell invasion and (Af) seeding, extravasation, survival, and subsequent proliferation of cancer cells at metastatic sites. **(B)** Possible effects of M-CSFR signaling blockade on cancer progression.

and SR-A, while GM-CSF rather induces a pro-inflammatory M1-type of activation (49, 56–58). As such, blocking M-CSFR signaling in myometrial macrophages stimulated the occurrence of an M1-like MHC-II^{high} population at the expense of M2-like MHC-II^{low} macrophages in the pregnant mouse uterus (59).

Depending on the tumor type/model and the blocking agents used to impede M-CSFR signaling, M-CSFR blockade has in most cases been reported to attenuate cancer progression and/or synergistically enhance the effect of chemo-, radio-, and/or immunotherapy via various effects, including (Ba) promotion of tumor-infiltrating lymphocytes (TIL) recruitment and/or activation, (Bb) enhanced phagocytosis/killing of cancer cells, (Bc) a delayed angiogenic switch, (Bd) reduced density of proliferating endothelial cells, (Be) inhibition of both TAM and cancer cell migration and invasion, (Bf) reduced metastasis. In some cases, (Bg) reduction of tumor weight and primary tumor growth has been reported. A number of studies have attributed these effects to (i) ablation of TAM numbers and/or (ii) phenotypic reprogramming of TAM from tumor promoting (often M2-like) TAM to anti-tumor (often M1-like) TAM.

The same study also demonstrated an important role for M-CSF in mediating monocyte extravasation to the tissue, via M-CSF-dependent upregulation of the chemokine CCL2, adding further evidence to the notion that M-CSF affects macrophage dynamics at multiple levels.

Since high M-CSF levels are frequently found in tumor-bearing hosts, the M-CSFR signaling could also play a role in shaping the TAM pool and regulating their activation state.

ASSOCIATION OF M-CSF AND M-CSFR LEVELS WITH HUMAN CANCER PROGRESSION

M-CSF, M-CSFR, AND/OR M-CSF RESPONSE SIGNATURE EXPRESSION IN TUMOR TISSUE

Various studies have documented analyses in which attempts were made to correlate clinical cancer patient parameters such as disease staging and survival with protein and/or mRNA expression levels of M-CSF, M-CSFR, and/or M-CSF response genes. The latter were thereby in turn considered to correlate with the presence of high levels of TAM and thus to represent a macrophage signature.

High M-CSF expression levels, as detected via IHC on tissue sections, have been reported to associate with higher histological tumor grading and in many cases also with more frequent metastases and poor prognosis in various cancer types, including breast cancer (60), serous and mucinous ovarian epithelial tumors (61), endometrioid carcinomas (62), and papillary renal cell carcinoma (63). In gynecological and non-gynecological leiomyosarcoma, expression of individual markers such as M-CSF was found to show at least a trend for correlation with poor outcome, but only the co-expression of M-CSF and three M-CSF-response genes (CTSL1, FCGR3a, and CD163) was independently associated with a worse survival in a multivariate analysis (64).

Studying the expression of M-CSFR via IHC in a large cohort of clinical breast cancer specimens using tissue microarrays revealed that M-CSFR expression was strongly associated with nodal status (65). In fact, in multivariate analysis, M-CSFR was not independent of nodal status as a predictor of survival. The study also revealed that M-CSFR expression was associated with decreased overall survival in non-metastatic breast cancer patients, but not in node-positive patients (65). Of note, in a recent manuscript, low levels of the M-CSFR gene were reported to predict worse overall survival based on online survival analysis tools allowing an evaluation of the prognostic value of genes in breast cancer patients using microarray data (66). In another recent study, a high number of tumor stromal cells – but not the cancer cells themselves – expressing M-CSFR was found to be an independent prognostic marker for lower event free survival and lower overall survival in classical Hodgkin lymphoma (67).

In line with variable results on association of M-CSFR expression with overall survival among different cancer types and patient groups, one report of a study using gene microarray and tissue microarray analyses for evaluating the prognostic value of an M-CSF response signature in breast cancer patients, mentioned a complex relationship of the signature with survival. Indeed, when patients were substratified in subsets, the M-CSF response signature was associated with poor prognosis among low-grade tumors and showed a trend for an association with improved prognosis among estrogen-receptor-negative tumors and among tumors with a TP53 mutation gene-expression signature (68). This variability in the association of M-CSF/macrophage signatures with clinical parameters points to the need to properly identify patient groups in which an M-CSF/macrophage signature correlates with

worse prognosis and which are thus most likely to benefit from M-CSF/macrophage-targeted therapies.

It should also be remarked that the presence of an M-CSF/macrophage signature is not a uniform feature in all cancer patients. In fact, gene microarray and tissue microarray analyses revealed M-CSF and M-CSF response signature genes to be present in 17–25% of breast cancers (68) and in about 27% of myoinvasive endometrioid carcinomas (62). Yet, in the latter case, concordance between the expression of the M-CSF signature in primary endometrioid carcinomas and in their corresponding lymph node metastases was reported. Moreover, in case of breast carcinoma, expression of the M-CSF signature was not only detected in some patients in case of invasive ductal carcinoma, but was already detected at the stage of ductal carcinoma *in situ*. Also in that case, a correlation was found between the presence of the M-CSF signature in ductal carcinoma *in situ* and in invasive ductal carcinoma within the same patient (69). This conservation of the expression of the M-CSF signatures upon disease progression is promising when considering targeting of the M-CSF pathway as a therapeutic option for invasive and/or metastatic disease, and suggests that the presence of a M-CSF/macrophage signature in the primary tumor may be useful for patient stratification to identify those patients who are most likely to respond to M-CSF/macrophage-targeted therapies.

CIRCULATING M-CSF

In some cases, M-CSF is produced to such high levels in cancer patients that it can be detected systemically. Overall trends from studies in patients with newly diagnosed breast tumors indicate that circulating plasma M-CSF levels are not higher in patients with localized tumors than in controls, but are elevated in patients with regionally advanced disease and distant metastases (70, 71). Median M-CSF levels were also reported to be dramatically higher in patients with newly diagnosed tumors of the head and neck, in men with prostate cancer metastatic to bone and women with advanced metastatic breast cancer than those seen in patients with newly diagnosed breast tumors (70).

Prospective studies of the prognostic value of serum M-CSF levels have yielded conflicting results. One study on 471 women with pre-invasive and invasive breast carcinoma reported no significant association between pre-treatment plasma levels of M-CSF and overall/relapse free survival at a median follow up of 5.6 years. In this study, patients were classified into three groups based on the level of initial M-CSF, using median and twice median plasma values as cut-off points (70). In contrast, a recent study of 572 women with early breast cancer, that had not undergone local or systemic anti-cancer treatment prior to serum collection, revealed significantly poorer outcome at a median follow-up of 5.2 years in patients with above-median M-CSF concentrations as compared to those with below-median M-CSF concentrations. In this study population, log M-CSF serum concentrations at study enrollment were predictive of poor survival in both univariate analysis, as well as multivariate analysis adjusted for age, tumor size, nodal status, and tumor grade (71). In a retrospective, nested case-control study of breast cancer risk in 726 breast cancer patients and 734 matched controls with no cancer history, the association of circulating M-CSF levels with the risk of

developing breast cancer was found to vary by menopausal status. High M-CSF levels were associated with a reduced risk of premenopausal breast cancer, whereas they were associated with an increased risk of postmenopausal breast cancer (72). Interestingly, in the aforementioned prospective study, the reported poorer outcome in patients with above-median M-CSF concentrations was confined to postmenopausal women, while no such effect was observed in premenopausal women with early breast cancer (71).

Overall, although the practical use of serum M-CSF levels as prognostic factor for cancer risk and/or outcome may be complicated by a high heterogeneity among patient groups and difficulties in determining optimal cut-off levels for plasma M-CSF, these results do suggest that, at least in some patient groups, M-CSF and M-CSF-dependent macrophages may be directly involved in tumor progression and malignant behavior and thus constitute interesting therapeutic targets.

TAM PHENOTYPIC AND SUBPOPULATION HETEROGENEITY

Originally, TAM were characterized as M2-like cells, proficient in inducing trophic functions like tumor angiogenesis, invasion, proliferation, and expressing the anti-inflammatory cytokine IL-10. These cells were also reported to express M2-specific markers like arginase-1, macrophage galactose-type C-type lectin-2 (Mgl2), found in inflammatory zone 1 (Fizz1), Ym1, TGF- β , SR-A, and MMR (73–76). However, some studies of chronic inflammation-induced cancer indicate the presence of TAM with an inflammatory M1-like phenotype, releasing inflammatory cytokines like IL-12, TNF, IL-6, and IL-1, or with overlapping M1 and M2 characteristics (77–79).

A dynamic switch in the phenotype of TAM during tumor progression might account for the mixed activation state of TAM subsets found in different established tumors. Indeed, in some models, tumor progression is associated with a switch from M1-like to M2-like TAM (80, 81). Hence, M2-like TAM can be linked to tumor promotion and their presence is indicative of poor prognosis (82, 83). Accordingly, a high M1/M2 TAM ratio has been associated with extended survival in many cancer types (84). Moreover, inhibition of monocyte differentiation to M2-like TAM through inhibition of NF- κ B signaling, results in an M1-like phenotype and reduced tumor growth (85). Hence, a picture emerges whereby M2-like TAM are pro-tumoral, and M1-like TAM exert anti-tumoral activities.

Accumulating evidence suggests that different TAM activation states found within the same tumor may reflect responses to divergent local microenvironmental signals (86). As previously mentioned, tumors are complex organoid structures containing peritumoral stroma, perivascular regions, and hypoxic regions, which can all be populated by TAM, albeit with a different molecular profile and exerting specialized functions (86–89). Different studies, using state-of-the-art microscopy, clearly illustrated the existence of at least two distinct microenvironments in the same tumor, which were both infiltrated by TAM subsets. TAM residing in avascular regions are sessile, have a high phagocytic capacity, and express high levels of many prototypical M2 markers such as MMR. In contrast, perivascular TAM are migratory, are not able to ingest dextran, have a less pronounced M2-profile and produce epidermal growth factor

(EGF), which attracts M-CSF-producing cancer cells, resulting in migration and intravasation of cancer cells (90–94). In line with these findings, differentially activated macrophages within the same tumors, residing in distinctively oxygenated tumor regions, could be discriminated based on the expression of MHC-II. MHC-II^{high} TAM are excluded from hypoxic avascular areas and more M1 oriented, while hypoxic MHC-II^{low} TAM express higher levels of M2-associated markers and are more angiogenic (25–27, 95). However, increasing the oxygenation of neoplastic lesions by vessel normalization in *PHD2*-haplodeficient mice was recently found not to alter the expression of the most prominent M2 markers, such as MMR, IL-4R α , and Arginase-1. Rather, reduced hypoxia down-regulated a subset of genes and proteins involved in glycolysis, angiogenesis, and metastasis, thereby lowering their angiogenic functions, specifically and solely in the hypoxic MHC-II^{low} TAM subset (27). Hence, hypoxia is not the main driver of TAM differentiation, but M2-like TAM preferentially home to hypoxic areas where the pro-tumoral activities of these cells are promoted. The importance of the intratumoral TAM location in shaping the phenotype of TAM subpopulations was further confirmed by a study showing that Neuropilin-1 deficiency in macrophages prohibits their migration to hypoxic tumor areas, resulting in an increased inflammatory phenotype and the initiation of an anti-tumor immune response (96).

EFFECTS OF M-CSFR SIGNALING ON NUMBERS AND PHENOTYPE OF TAM AND OTHER TIM

The critical role of M-CSF in the turn-over of TAM is reflected in the drastic reduction in macrophages in the primary tumor at different stages of tumor progression to malignancy that has been observed in the absence of M-CSF in osteopetrotic *op/op* mice (97, 98). Conversely, restoration of M-CSF signaling via transgenic expression of M-CSF in the mammary epithelium led to enhanced numbers of macrophages in primary mammary tumors (97). Similarly, strong reductions in the number of TAM have been reported in various tumor models upon blocking of M-CSF/M-CSFR signaling to TAM using either blocking monoclonal antibodies (mAbs) targeting M-CSF (66, 99) or the extracellular domain of M-CSFR (100–102) or small molecule inhibitors of the M-CSFR tyrosine kinase activity in order to block the downstream signaling (102–105) (**Figure 2**).

Despite numerous reports of the differential effects of M-CSF versus GM-CSF on macrophage polarization (44, 57), only few studies directly addressing the effect of M-CSFR blockade on the M1/M2 activation state and/or subpopulation heterogeneity of TAM have recently been documented (**Table 1**).

One study in a mouse model based on subcutaneously inoculated colon carcinoma cells was aimed at evaluating whether cytokine signaling could induce reprogramming of the TAM phenotype *in vitro*. The authors reported that GM-CSF treatment in conjunction with suppression of M-CSF signals using siRNA against the M-CSFR resulted in an altered signal transduction pathway of TAM, whereby expression of STAT1, STAT5, and STAT6 was increased. In this study, treatment of TAM with GM-CSF alone, or in conjunction with suppression of M-CSFR signals,

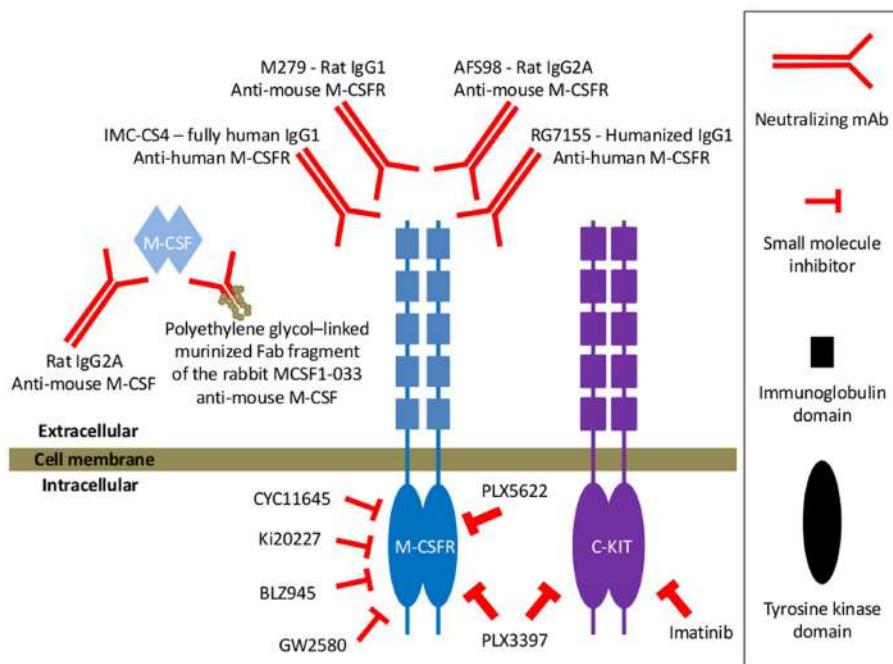


FIGURE 2 | Examples of various types of M-CSFR signaling blocking agents mentioned throughout this manuscript. In some studies, neutralizing anti-mouse M-CSF mAb has been used for blocking M-CSF/M-CSFR signaling (66). One study also reported on the use of a murinized, polyethylene glycol-linked recombinant Fab fragment of the MCSF1-033 neutralizing rabbit anti-mouse M-CSF antibody (99). Yet, blocking mAbs targeting the extracellular domains of the M-CSFR have more frequently been documented for blocking the M-CSF/M-CSFR signaling axis. Typical examples of the latter that have been used in mouse tumor models are the rat IgG1 M279 (100) and the rat IgG2A AFS98 (101, 102). A recent report documented the generation of RG7155, a humanized anti-human M-CSFR IgG1 mAb that inhibits M-CSFR activation (106). And also the fully human IgG1 anti-human M-CSFR mAb IMC-CS4 is currently in clinical trials (107). M-CSFR signaling has also been inhibited via

pharmacological, small molecule inhibitors targeting the intracellular catalytic domains of the receptor involved in signal transduction. A number of these tyrosine kinase inhibitors, such as CYC11645, Ki20227, GW2580, or BLZ945, have been screened for highly selective inhibition of M-CSFR signaling, very potent IC₅₀ values for M-CSFR and at least a 100-fold lower inhibitory activity for other tested receptor tyrosine kinases (66, 108–110). Also the PLX3397 tyrosine kinase inhibitor has been used, which has higher M-CSFR inhibitory activity as compared to GW2580, but which is less specific since it inhibits the c-Kit receptor tyrosine kinase with similar potency as the M-CSFR tyrosine kinase (105). In one study, the actual contribution of M-CSFR blockade in the effect of PLX3397 has been assessed by comparing it with the specific cKit tyrosine kinase inhibitor imatinib and PLX5622, an M-CSFR-specific inhibitor of equal potency to PLX3397 that does not appreciably inhibit Kit (111).

did not alter the TAM expression pattern of M1/M2 marker molecules (112).

In a study, whereby the tyrosine kinase inhibitor PLX3397 was used as a combination treatment with adoptive cell therapy of melanoma-targeted T cells in a syngeneic mouse model of BRAFV600E-driven melanoma, PLX3397 as single or combination treatment resulted in a dramatic reduction of TAM and a skewing of the subpopulation balance in the remaining TAM from predominant M2-oriented MHC-II^{low} to predominant M1-oriented MHC-II^{high} macrophages (113). A similar shift in the relative amount of TAM subpopulations was documented in the transgenic mouse MMTV-Neu model, in which mammary carcinogenesis is driven by the mammary epithelial restricted expression of the ErbB2/Neu oncogene. Blocking M-CSFR in this model by using the M-CSFR inhibitor GW2580, led to a significant reduction in the amount of M2-like F4/80^{high} TAM, which had moderate levels of MHC-II, and not in the MHC-II^{high} F4/80^{low} TAM, elucidating a role for M-CSFR signaling in the maintenance or expansion of the M2-like TAM subset (95). A recent

study in a mouse model of pancreatic ductal adenocarcinoma confirmed this notion. In this model, treatment with PLX3397 or a neutralizing anti-M-CSF mAb resulted in a drastic reduction in TAM (114). Thereby, the authors demonstrated that blocking M-CSF/M-CSFR signaling resulted in preferential depletion of M2-like MMR^{high} TAM, whereas M1-like MMR^{low} TAM were much less affected. The observation that the MMR^{high} TAM subset had significantly higher M-CSFR expression levels as compared to the MMR^{low} TAM subset further supports the notion that these M2-like cells may be more dependent on the M-CSF signal. As a consequence, the gene-expression profile of TAM upon M-CSFR signaling blockade featured a reduced expression of M2 markers and an increased expression of M1 markers and MHC-II. In parallel, the TAM phenotype shifted from predominant immuno-suppressive properties to improved antigen presentation capacity (114). In a mouse glioblastoma model, *in vivo* M-CSFR inhibition using the small molecule M-CSFR inhibitor BLZ945 was reported not to result in TAM depletion. Instead, glioma-secreted factors, including GM-CSF and IFN-γ, facilitated TAM survival in the

Table 1 | Documented effects of M-CSFR inhibition on TAM abundance and activation state.

Mouse tumor model	Tool used to inhibit M-CSFR signaling	Amount of TAM	TAM M1/M2 activation state	Effect/outcome	Reference
Colon carcinoma	<i>In vitro</i> : siRNA against M-CSFR + GM-CSF	Unaltered	Unaltered	Increased expression of STAT1, STAT5, STAT6 in TAM	(112)
Melanoma	M-CSFR inhibitor: PLX3397 + adoptive cell therapy	Reduced	Skewing from M2 MHC-II ^{low} to M1 MHC-II ^{high}	Improved adoptive cell therapy; increased amount and activation of tumor-infiltrating lymphocytes; reduced tumor growth	(113)
Mammary carcinoma	M-CSFR inhibitor: GW2580	Reduced (only M2-like MHC-II ^{low} TAM)	Not assessed	Role of M-CSFR in maintenance of M2-like TAM	(95)
Pancreatic ductal adenocarcinoma	M-CSFR inhibitor: PLX3397/neutralizing α-M-CSF MAb	Reduced (mainly M2-like MMR ^{high} TAM)	Remaining TAM are less immunosuppressive, better Ag presenting M1	Increased anti-tumor T cell activity; enhanced response to immunotherapy	(114)
Glioblastoma	M-CSFR inhibitor: BLZ945	Unaltered	Repolarization from pro-tumoral M2 to phagocytic M1 TAM	Reduced tumor growth	(108)
Cervical and mammary carcinoma	M-CSFR inhibitor: BLZ945	Reduced (both MHC-II ^{low} and MHC-II ^{high} TAM)	Not assessed	Increased amount of CD8 ⁺ T cells; reduced tumor growth	(104)
Pancreatic ductal adenocarcinoma	M-CSFR inhibitor: PLX3397 or GW2580	Reduced (mainly M1-like immunosuppressive MHC-II ^{high} TAM)	Remaining TAM are less immunosuppressive	Enhanced response to chemotherapy; increased CTL response; reduced metastases	(105)

context of M-CSFR inhibition and resulted in a repolarization from pro-tumoral M2 to a highly phagocytic M1 phenotype, with a decreased expression of M2 markers (108).

Despite the above examples indicating that M-CSFR blockade can shift the balance in TAM subpopulations from tumor-promoting M2-oriented MHC-II^{low} or MMR^{high} to anti-tumoral M1-oriented MHC-II^{high} or MMR^{low} macrophages, conflicting reports also exist. For example, the M-CSFR inhibitor BLZ945 was reported to result in a decrease in the level of TAM in cervical and breast carcinomas by attenuating their turn-over rate. Hereby, similar kinetics of depletion and recovery were observed for both MHC-II^{low} and MHC-II^{high} TAM subpopulations (104). And in mice bearing transplantable pancreatic ductal adenocarcinomas, the M-CSFR inhibitors GW2580 or PLX3397 were even reported to significantly deplete macrophages expressing high levels of MHC-II, but not the more M2-oriented MHC-II^{low} or Tie2⁺ TAM (105). Yet, in the latter case and in contrast to the examples above, the MHC-II^{high} TAM were found to constitute the predominant TAM subpopulation and to exert pro-tumoral activities by suppressing anti-tumoral CD8⁺ T cell responses (105). Therefore, also in that case, the observed reduction in the level of the predominant tumor-promoting TAM subpopulation, occurring upon M-CSFR blockade, resulted in attenuation of cancer malignancy (**Table 1**).

Further studies will be required to obtain better insights into the extent and the underlying mechanisms by which M-CSFR signaling and blockade thereof can contribute to shaping the phenotypic and subpopulation heterogeneity of TAM, thereby re-educating TAM toward anti-tumoral effector populations, thus contributing

to combating disease progression. It will also be of importance to assess to what extent the remaining TAM populations detected after M-CSFR signaling blockade in various cancer types and tumor models are actually M-CSF-dependent macrophages for which the depletion was incomplete or the M-CSF dependence has been (partially) compensated for by other factors. Or do these remaining cells in some instances represent M-CSF-independent cells with a distinct lineage origin (such as for example certain dendritic cell types) for which the lineage surface markers and morphological analysis used in the current studies have not allowed to discriminate them from macrophages?

Of note, a number of recent publications evaluating the effect of M-CSFR inhibitors such as GW2580 or PLX3397 on various populations of tumor-infiltrating myeloid cells (TIM) have documented a reduction of not only mature CD11b⁺Ly6G⁻Ly6C^{low}F4/80^{high} TAM, but also of CD11b⁺Ly6G⁻Ly6C^{high}F4/80^{mid} cells, resembling the surface receptor phenotype and morphology of inflammatory (classical) monocytes or monocytic myeloid-derived suppressor cells (MO-MDSC) (105, 114–116). Taking into account the diversity of cell populations that can express these combinations of surface markers and the fact that an actual suppressive activity of the cells has not been demonstrated by the authors of most of these studies, we will term these cells MO-MDSC-like cells in the current review. It makes sense that, as monocyte-lineage cells, these tumor-infiltrating MO-MDSC-like cells are dependent on M-CSFR signaling to a similar extent as mature TAM and these MO-MDSC-like cells may in fact very well represent precursors of mature TAM

(117). In contrast to MO-MDSC-like cells, documented effects of M-CSFR signaling inhibitors on CD11b⁺Ly6G^{high}Ly6C^{low} cells, resembling the surface receptor phenotype and morphology of immature granulocytes/neutrophils or polymorphonuclear myeloid-derived suppressor cells (PMN-MDSC) and which we will term PMN-MDSC-like cells have been more variable. Most studies revealed no reduction (and sometimes even a limited increase) in the number of PMN-MDSC-like cells in response to PLX3397 or GW2580 treatment, for example in mice bearing murine pancreatic ductal adenocarcinoma (105, 114) or in the 3LL lung carcinoma model (115). In contrast, PLX3397 was found to reduce both MO-MDSC-like cells and PMN-MDSC-like cells in one study in the RM-1 and Myc-CaP prostate cancer models (116). The variable effect of M-CSFR blockade on PMN-MDSC-like cells suggests that this effect is most likely indirect and may depend on other (growth) factors in the tumor microenvironment that are affected indirectly via the M-CSFR blocking.

EFFECTS OF M-CSFR SIGNALING BLOCKADE ON CANCER PROGRESSION AND THE ROLE OF TAM THEREIN

Depending on the tumor type/model and the blocking agents used to impede M-CSFR signaling (**Figure 2**) (variable) effects of M-CSFR blockade on different aspects of cancer progression have been reported (**Figure 1B**).

EFFECTS ON TUMOR INCIDENCE AND PRIMARY TUMOR GROWTH

To assess the role of M-CSF in tumor development and progression, *csf1^{op/op}* mice have been crossed with transgenic mice in which mammary tumors develop due to mammary epithelial restricted expression of the Polyomavirus middle T oncogene (PyMT). In these experiments, the drastic reduction in TAM numbers in the absence of M-CSF was reported neither to affect the incidence nor the growth of the primary tumors but rather to delay their development to invasive, metastatic carcinomas (97). In fact, the PyMT model is characterized by the development of a single primary tumor focus on the ducts emanating from the nipple, after which other tumors arise in the ducts distant to the nipple. Although the development of multiple foci on the distal ducts was reduced in the *csf1^{op/op}* PyMT mammary glands, the growth rate of the primary tumor size and the proliferation rate of the cancer cells were comparable to those in M-CSF sufficient mice.

Similarly, treatment of AE5MG mesothelioma or LLC lung carcinoma bearing mice with the M-CSFR blocking mAb M279 was described not to result in a significant effect on tumor growth or final tumor burden, despite a strong reduction in the number of TAM (100). In contrast, publications reporting on the use of another mAb, AFS98, for M-CSFR blockade and ensuing TAM inhibition, documented inhibition of primary tumor growth in different mouse tumor models including the implanted AX osteosarcoma model (102) and later also in the EL4 transplanted lymphoma model, the PyMT transgenic breast carcinoma model and the MDA-MB231 breast cancer metastasis-induced osteolysis model (101). It has been suggested that the effect of the rat IgG1 M279 may represent the biological response to blocking CSF-1R signaling *per se*, whereas the isotype of the rat IgG2A AFS98 may result in additional effector functions such as direct macrophage depletion upon recognition by and/or aggregation

with other macrophages through binding of the IgG2A antibody to the high affinity IgG receptor CD64 on mouse macrophages (52).

In human, MCF-7 mammary carcinoma cell xenografts in immunodeficient mice, M-CSF blockade by antisense oligonucleotides and small interfering RNAs has been shown to reduce host macrophage infiltration and suppress tumor growth (118). Concerning the effect of pharmacologic M-CSFR blockade on primary tumor growth, the M-CSFR tyrosine kinase inhibitor Ki20227 was described to reduce TAM content of tumors and retard tumor cell proliferation in osteosarcoma (102) and similar results were reported for GW2580 in papillary thyroid cancer (103). Yet, the reduction in intratumoral proliferation in GW2580-treated papillary thyroid cancers was most evident within the stromal compartment (103). These results suggest that the observed effects of M-CSF blockade may in that case at least partially reflect inhibition of stromal cells such as TAM rather than cancer cell proliferation *per se*. In murine prostate cancer models, the M-CSFR inhibitors GW2580 or PLX3397 as a single treatment were reported to have little effect on tumor growth compared with the control group, despite effective TAM ablation.

A recent study clearly illustrates that the specificity of the applied inhibitors for M-CSF as compared to other tyrosine kinases and the relative contribution of the effect on TAM as compared to direct effects on the cancer cells should be carefully considered when interpreting the effect of M-CSFR blockers on tumor growth. In this study, PLX3397 was found to result in effective reduction of tumor weight and cellularity in both the Kit^{V558del/+} transgenic murine gastrointestinal stromal tumor (GAST) model and in human GAST xenografts (111). These GAST cells are known to be strongly dependent on signaling via the oncogene cKit for their survival and the growth inhibitory effect of PLX3397 was even stronger than that of the cKit tyrosine kinase inhibitor imatinib, correlating with a superior capacity of PLX3397 as compared to imatinib to decrease the viability of two human GAST cell lines *in vitro*. On the other hand, TAM were deleted to a much greater degree in mice treated with PLX3397 than with imatinib, correlating with a more potent M-CSFR inhibition by PLX3397 as compared to imatinib. Therefore, one could hypothesize that the superior effect of PLX3397 on tumor growth inhibition could at least in part be related to superior inhibition of M-CSF signaling and consequent TAM attenuation, acting synergistically to the Kit inhibition. To address this possibility, the authors combined imatinib with PLX5622, an M-CSFR-specific inhibitor of equal potency to PLX3397 that does not appreciably inhibit Kit. Despite comparable levels of TAM reduction as PLX3397 therapy, treatment with PLX5622 did not enhance the effect of imatinib on tumor weight, cell number, or histology, suggesting that inhibition of cKit signaling but not M-CSFR signaling is the main factor determining the capacity of tyrosine kinase inhibitors for GAST growth inhibition (111).

Overall, despite consistent reduction in TAM content in primary tumors in the various tumor models discussed above, the effects of M-CSF or M-CSFR blockade and consequent TAM attenuation on primary tumor growth seem to be quite variable, depending on the tumor model and the blocking agents used, and thus do not seem to correlate with TAM depletion *per se*.

EFFECTS ON TUMOR ANGIOGENESIS

Crossing PyMT and *csf1^{OP/OP}* mice revealed that a low density of macrophages in the primary tumors correlated with a delay in the angiogenic switch, identified as the formation of a high-density vessel network. Genetic restoration of macrophage numbers in the tumors of these mice by the transgenic expression of M-CSF specifically in the mammary epithelium thereby rescued the vessel phenotype (119). Similarly, crossing *csf1^{OP/OP}* mice to the RIP1-Tag2 (RT2) mouse model of pancreatic islet cancer was documented to decrease TAM by approximately 50% during all stages of RT2 tumor progression and to generate a substantial reduction in cumulative tumor burden, which resulted from a significant decrease in angiogenic switching and the number of tumors, rather than an evident effect on the growth of established tumors or on the cancer cell proliferative capacity (98).

In a mammary tumor model based on xenografts of human MCF-7 breast cancer cells in athymic nude mice, mouse (host) M-CSF expression was found to be induced as the tumors progressed. In these mice, treatment with a murinized, polyethylene glycol-linked recombinant Fab fragment of the MCSF1-033 neutralizing rabbit anti-mouse M-CSF antibody reduced the density of both macrophages and proliferating endothelial cells, the latter reflecting decreased levels of angiogenic activity in the mammary tumor xenografts (99). In an immunocompetent mouse model of osteosarcoma, in which mice were subcutaneously transplanted with the mouse AX osteosarcoma cell line, the M-CSF inhibitor Ki20227 or the AFS98 rat anti-murine M-CSFR mAb dramatically decreased peritumoral and perivascular TAM, suppressed tumor angiogenesis and lymphangiogenesis, disorganized extracellular matrices and concomitantly dramatically suppressed metastasis and improved prognosis (102). In contrast to VEGF blockade, interruption of M-CSF signaling did not promote rapid vascular regrowth. In addition, continuous M-CSF inhibition did not affect healthy vascular and lymphatic systems outside tumors (102). The notion that M-CSFR⁺ TIM, including both TAM and MO-MDSC-like cells, contribute significantly to tumor angiogenesis, was supported by Priceman et al. (115), showing that depletion of M-CSF-dependent TAM and MO-MDSC-like cells in the 3LL lung carcinoma model, using either the M-CSFR inhibitor GW2580 or a transgenic approach in chimeric mice, resulted in significant reduction in angiogenesis in TIM-ablated tumors (without a concomitant decrease in tumor growth). The authors confirmed that, also in the orthotopic RM-1 prostate tumor model, M-CSF blockade resulted in reduced levels of TAM, and MO-MDSC-like cells, associated with reduced angiogenesis and, to a lesser extent, lymphangiogenesis, as reflected by vessel density in these tumors.

In the 3LL lung carcinoma model, GW2580 was in addition shown to attenuate tumor evasion of anti-angiogenic therapy. In combination with DC101, a specific blocking antibody against VEGFR-2, GW2580 resulted in greater inhibition of tumor angiogenesis along with synergistic tumor growth reduction compared with anti-angiogenic therapy alone. In search for a hypothesis on the mechanism underlying the reversal of anti-angiogenesis in the combination therapy, the authors provided histological data revealing more abundant MMP9 expressing cells with heterogeneous myeloid cell morphology in viable areas of

DC101-treated tumors, which were reduced in the combination group (115).

At a mechanistic level, M-CSF was also shown to induce VEGF production in human monocytes through the MAPK/ERK pathway via Sp1 and was reported to enhance angiogenesis *in vivo*, as evidenced in an angiogenesis assay using an *in vivo* polymerized MatrigelTM plug in mice (120). Recently, the mechanistic basis of the tumor angiogenesis-promoting effect of M-CSF was further expanded by showing that M-CSF augments differentiation of the subpopulation of M2 macrophages expressing the endothelial cell tyrosine kinase receptor, Tie2. Hereby, M-CSF-mediated upregulation of Tie2 on these Tie2-expressing monocytes/macrophages (TEM) increased branching of human umbilical vein endothelial cells (HUVECs) *in vitro* and enhanced angiogenesis in PyMT tumor-bearing mice. This M-CSF-stimulated Tie2 receptor expression was found to be dependent on a synergistic contribution from the PI3 kinase and HIF-1 α pathways. (121).

As a final remark, it should be realized that high levels of angiogenesis, driven by M2-like TAM, often lead to dysfunctional blood vessels in tumors, resulting in more malignant cancer cells under the influence of tumor hypoxia and an easy access of these cells to the blood circulation. TAM depletion or the conversion of M2-like TAM to M1-like TAM, thereby results in vessel normalization and reduced metastasis (122). In addition, normalized vessels allow a more efficient administration of therapeutic agents to the tumor microenvironment.

EFFECTS ON CANCER CELL INVASION AND METASTASIS

Accumulating evidence in the first decade of this century has supported the tenet that delayed development of invasive, metastatic carcinomas in PyMT *csf1^{OP/OP}* mice is reflective of a role for M-CSF in promoting cancer cell invasion by regulating the infiltration and function of TAM. Indeed, at the PyMT tumor site, expression of M-CSFR was reported to be restricted to macrophages. Moreover, restoration of macrophage infiltration upon transgenic expression of M-CSF in the mammary epithelium restored progression of primary tumors to the stages of invasive carcinoma (97). In fact, macrophages and tumor cells in mammary tumors were documented to be comigratory and to be mutually dependent for invasion and for cancer cell intravasation (90, 123). Hereby, M-CSF produced by carcinoma cells promotes the expression of EGF by macrophages, which in turn promotes the formation of elongated protrusions and cell invasion by carcinoma cells. In addition, EGF promotes the expression of M-CSF by carcinoma cells, thereby generating a positive feedback loop. Disruption of this paracrine amplification loop by blockade of either EGF receptor or M-CSFR signaling was found to be sufficient for inhibiting both macrophage and tumor cell migration and invasion (91).

A similar EGF/M-CSF paracrine interaction with macrophages, resulting in enhanced cancer cell invasion as reported for murine carcinoma cells, was confirmed in a mouse xenograft model of human breast tumor derived cancer cells. Yet, for these human breast carcinoma cells, the EGF/M-CSF paracrine feedback loop was found to be complemented by autocrine M-CSF signaling in the cancer cells (124). These data correlated with the expression of M-CSFR by human but not mouse breast carcinoma cells. The possibility of macrophage-independent effects of M-CSF on

human cancer cell invasion is also supported by a direct stimulation of *in vitro* invasive capacity, but not proliferation, of human adenocarcinoma cell lines by recombinant human M-CSF (125).

The studies in *csf1^{op/op}* mice also indicated a role of M-CSF in enhancement of metastatic growth of cancer cells. In particular, M-CSF was shown to be required for the recruitment of a population of CD11b⁺F4/80⁺Gr1⁺ host macrophages to extravasating pulmonary metastatic cells in the PyMT model. This recruited CD11b⁺F4/80⁺Gr1⁺ macrophage population displayed a distinct phenotype as compared to CD11b⁻, CD11c⁺ lung resident macrophages and also did not express Tie2, rendering them distinct from the M-CSF-induced pro-angiogenic Tie2-expressing monocytes/macrophages. The recruited macrophages enhanced cancer cell metastasis through effects on cancer cell metastatic seeding, extravasation, survival, and subsequent growth (126). The authors confirmed that the reduced metastasis in *csf1^{op/op}* PyMT mice could be recapitulated in wild-type PyMT mice via macrophage ablation using clodronate-containing liposomes. Importantly, even after metastatic growth had been established, macrophage ablation using clodronate-containing liposomes inhibited subsequent metastatic growth (126). This effect also seems to be M-CSF-specific since transgenic expression of M-CSF in the mammary epithelium of both *csf1^{op/op}* and wild-type tumor-prone mice led to an acceleration to the late stages of carcinoma and to a significant increase in pulmonary metastasis. The clinical significance of these findings is illustrated by the observation that the density of close tripartite interactions between cancer cells, macrophages, and endothelial cells (tumor microenvironment of metastasis or TMEM) is predictive of metastasis formation in breast cancer patients (127).

Since M-CSF signaling not only plays a critical role in the turnover of TAM, but is also crucial for osteoclasts, blocking M-CSFR signaling may not only attenuate metastasis via effects on TAM, but may have additional beneficial effects on metastatic disease via inhibitory effects on osteoclasts. As an example of this, the AFS98 M-CSFR blocking mAb was recently documented to potently block the differentiation of osteoclasts and their bone destruction activity in a breast cancer model of bone metastasis (101).

A recent study placed a cautionary note on blocking M-CSFR signaling as a therapeutic modality in cancer. In that study, mice bearing two independently derived mammary cancer cell lines (4T1.2 and EMT6.5) injected orthotopically into the mammary gland, were treated with the AFS98 neutralizing anti-M-CSFR mAb, with a neutralizing anti-mouse M-CSF mAb, or with two different small molecule inhibitors of M-CSFR (GW2580 or CYC11645). The authors observed variable effects on reduction of TAM in the primary tumors or metastatic lung tissue, whereby TAM could be reduced using GW2580 or high dose of AFS98, but were not reduced when using lower dose of AFS98. Yet in all these cases, not only did these various modalities for blocking M-CSFR not reduce primary tumor growth, but the intended treatment actually increased metastasis to the lung and spine (66). The authors found that the increased spontaneous metastasis upon blocking of M-CSFR or M-CSF was associated with increased levels of serum granulocyte-colony-stimulating factor (G-CSF), increased numbers of neutrophils and Ly6C^{high} monocytes in the

peripheral blood and increased frequency of neutrophils in the primary tumor and in the lung. It is currently unclear why M-CSFR blockade resulted in increased G-CSF levels in this model, but the authors did observe that blood neutrophil numbers were proportional to the metastatic capacity of the different mammary carcinomas evaluated, suggesting that certain carcinomas may be more prone to mobilize neutrophils, and leading to increased metastasis. Interestingly, combining blockade of M-CSFR signaling with a neutralizing antibody against the G-CSF receptor (G-CSFR), which regulates neutrophil development and function, reduced the enhanced metastasis, and neutrophil numbers that resulted from M-CSFR blockade. In fact, the combined blocking of M-CSFR and G-CSFR resulted in significantly reduced metastasis as compared to the control condition (66).

POTENTIATION OF RADIO-, CHEMO-, AND IMMUNOTHERAPY

Whereas the M-CSFR inhibitors GW2580 or PLX3397 on their own were reported to have little effect on tumor growth in murine prostate cancer models, when added to radiotherapy, the M-CSFR inhibitors suppressed tumor growth more effectively than radiation alone (116). The synergistic effect of M-CSF blockade on the efficacy of radiotherapy was explained by the observation that irradiation resulted in increased M-CSF levels due to recruitment of the DNA damage-induced kinase ABL1 into cell nuclei where it bound the *csf1* gene promoter and enhanced *csf1* gene transcription. Consequently, enhanced recruitment of TIM, including TAM and MO-MDSC-like and PMN-MDSC-like cells, was detected and this enhanced TIM recruitment was counteracted via the M-CSFR inhibitors (116). These results suggest that blockade of the M-CSF/M-CSFR axis can be a promising approach for developing more effective combination cancer therapies. The authors supported the human relevance of these findings by reporting that also in prostate cancer patients, serum levels of M-CSF were increased after radiotherapy.

Such potential synergistic effects in combination therapy are not only restricted to radiotherapy, but also extend to chemotherapy. Indeed, combination therapy with a murinized, polyethylene glycol-linked antigen-binding fragment against mouse (host) M-CSF reportedly reversed chemoresistance in athymic nude, immunodeficient mice bearing human, and chemoresistant MCF-7 breast cancer xenografts (99). Also treatment with the AFS98 anti-M-CSFR monoclonal antibody in mice already bearing established PyMT tumors was reported to prolong their survival and potentiate the effect of chemotherapy with Paclitaxel (101). Finally, GW2580 or PLX3397 were found to improve chemotherapeutic efficacy in mice bearing murine pancreatic ductal adenocarcinoma cell lines. In this tumor model, gemcitabine chemotherapy was documented to increase M-CSF levels and consequently enhance the tumor infiltration of T-cell suppressive TAM (and MO-MDSC-like cells). This effect was blunted when chemotherapy was combined with M-CSFR blockade, resulting in increased anti-tumor CD8⁺ T-cell responses and improved inhibition of tumor growth and metastasis as compared to chemotherapy as monotherapy. Accordingly, the higher therapeutic efficacy of combined treatment with GW2580 plus gemcitabine compared with the effects of gemcitabine alone was shown not to occur upon depletion of CD8⁺ T lymphocytes (105).

Considering the above, it comes as no surprise that, in the same mouse model of pancreatic ductal adenocarcinoma, M-CSFR signaling blockade using PLX3397 or GW2580 was shown to enhance the therapeutic efficacy of so-called T-cell checkpoint immunotherapy using PD1 and CTLA4 antagonists in combination with gemcitabine (114). In this case, M-CSFR signaling blockade was reported to result in preferential depletion of MMR^{high} M2-like TAM and reprogramming of the phenotype of the remaining TAM, with alleviated immunosuppressive activities and enhanced antigen presentation capacity and which in turn correlated with enhanced CD4⁺ and CD8⁺ T cell responses. Here also, the increased therapeutic efficacy of the combination treatment was shown to be blunted upon depletion of CD4⁺ and CD8⁺ T cells (114). As another example of a synergistic effect of M-CSFR blockade on immunotherapy, PLX3397 has been reported to improve the efficacy of adoptive cell therapy of melanoma-targeted T cells in a syngeneic mouse model of BRAFV600E-driven melanoma. Mice receiving the combined treatment produced superior anti-tumor responses and exhibited improved overall survival compared with single treatments, correlating with a dramatic reduction of TAM (but in this setting no significant change in already low numbers of MO-MDSC-like or PMN-MDSC-like cells), a skewing of the subpopulation balance in the remaining TAM from predominant M2-oriented MHC-II^{low} to predominant M1-oriented MHC-II^{high} macrophages and an increase in tumor-infiltrating lymphocytes and T cells. The authors conclude that macrophages are the targets of PLX3397 by confirming that PLX3397 and macrophage-depleting clodronate-containing liposomes have the same effect on tumor growth and that this effect is not further increasing when combining both depletion methods (113).

CONCLUDING REMARKS AND CLINICAL PERSPECTIVES

Macrophage-colony-stimulating factor receptor inhibitors are currently in clinical development as cancer therapeutics. Plexxicon has, for example, initiated several clinical trials of the cKit and M-CSFR inhibitor PLX3397, either as a stand-alone cancer treatment (128–130) or in an adjuvant setting with chemo- and/or radiotherapy (131–134). Phase I clinical trials of anti-M-CSFR mAbs in patients with advanced solid tumors are currently being conducted by Eli Lilly and Company for the fully human IgG1 IMC-CS4 (107) and by Roche for the humanized IgG1 RG7155 (135). For the latter, it was mentioned in a recent publication that, based on preliminary results of an ongoing clinical trial, administration of RG7155 to diffuse-type giant cell tumor patients led to significant reductions of M-CSFR⁺CD163⁺ macrophages in tumor tissues, which correlated with at least partial clinical objective responses (106). The ultimate value of these M-CSFR targeted therapies will need to be assessed in follow-up studies aimed at demonstrating effects that go beyond reduction in the primary tumor burden, but extend to attenuation of metastasis and prolongation of patient survival.

In this context, it is encouraging that numerous studies in preclinical tumor models have revealed that blocking M-CSFR signaling, despite variable effects on primary tumor growth *per se*, has the potential to attenuate tumor-promoting effects of TAM on tumor angiogenesis and cancer cell invasion and metastasis.

And especially, synergistic effects of M-CSFR blocking agents in diminishing TAM-dependent resistance to anti-angiogenic therapy, radiotherapy, chemotherapy, or immunotherapy offer promising perspectives for effective combination therapy. Recent studies thereby suggest that intratumoral M-CSF levels and their balance with GM-CSF levels are not only critical for TAM differentiation and maintenance, but can also contribute to shaping the M1/M2 phenotypic and subpopulation heterogeneity of TAM. Hence, M-CSFR blocking agents may not only have the potential to counteract cancer progression by reducing TAM content in tumors and metastatic lesions, but also by re-educating TAM from tumor-promoting toward anti-tumoral effector populations.

Recently, more attention is in addition being given to better characterize other tumor-infiltrating myeloid cell populations such as MDSC-like cells that are also affected by M-CSFR blockade and to evaluate whether these contribute to the observed effects of M-CSFR blockade on various aspects of cancer progression. Additional effects on other cells are not necessarily a disadvantage in the context of anti-cancer therapeutic activity, as exemplified in reported attenuation of metastatic disease via dual inhibitory effects on TAM and osteoclasts (101). Yet, the data recently reported by Swierczak and colleagues on neutrophil-dependent enhanced metastasis upon M-CSFR blockade (66) indicate that blocking M-CSFR signaling may have variable effects according to the tumor model and may in some cases exhibit unwanted side effects. These cautionary findings are testaments to the notion that successful clinical translation will be critically dependent on proper patient stratification to focus on those patient groups in which high M-CSF or M-CSFR expression is linked to disease pathophysiology and correlates with worse prognosis and in which M-CSFR/macrophage-targeted therapies are thus most likely to exert a beneficial effect.

AUTHOR CONTRIBUTIONS

All authors have made substantial contributions to the conception of the manuscript. Geert Raes and Damya Laoui have drafted the initial version of the manuscript. All other authors have critically reviewed the manuscript for important intellectual content. All authors approve of the version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

ACKNOWLEDGMENTS

The authors thank FWO-Vlaanderen, IWT-Vlaanderen, the “Stichting tegen Kanker,” and the “Vlaamse Liga tegen Kanker” for their support.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 13 August 2014; accepted: 22 September 2014; published online: 07 October 2014.

Citation: Laoui D, Van Overmeire E, De Baetselier P, Van Ginderachter JA and Raes G (2014) Functional relationship between tumor-associated macrophages and macrophage colony-stimulating factor as contributors to cancer progression. *Front. Immunol.* **5**:489. doi: 10.3389/fimmu.2014.00489

This article was submitted to Molecular Innate Immunity, a section of the journal *Frontiers in Immunology*.

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Depletion of tumor-associated macrophages slows the growth of chemically induced mouse lung adenocarcinomas

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Chronic inflammation is a risk factor for lung cancer, and low-dose aspirin intake reduces lung cancer risk. However, the roles that specific inflammatory cells and their products play in lung carcinogenesis have yet to be fully elucidated. In mice, alveolar macrophage numbers increase as lung tumors progress, and pulmonary macrophage programming changes within 2 weeks of carcinogen exposure. To examine how macrophages specifically affect lung tumor progression, they were depleted in mice bearing urethane-induced lung tumors using clodronate-encapsulated liposomes. Alveolar macrophage populations decreased to $\leq 50\%$ of control levels after 4–6 weeks of liposomal clodronate treatment. Tumor burden decreased by 50% compared to vehicle treated mice, and tumor cell proliferation, as measured by Ki67 staining, was also attenuated. Pulmonary fluid levels of insulin-like growth factor-I, CXCL1, IL-6, and CCL2 diminished with clodronate liposome treatment. Tumor-associated macrophages expressed markers of both M1 and M2 programming in vehicle and clodronate liposome-treated mice. Mice lacking CCR2 (the receptor for macrophage chemotactic factor CCL2) had comparable numbers of alveolar macrophages and showed no difference in tumor growth rates when compared to similarly treated wild-type mice suggesting that while CCL2 may recruit macrophages to lung tumor microenvironments, redundant pathways can compensate when CCL2/CCR2 signaling is inactivated. Depletion of pulmonary macrophages rather than inhibition of their recruitment may be an advantageous strategy for attenuating lung cancer progression.

Keywords: macrophage, programming, lung tumor, clodronate, inflammation

INTRODUCTION

Lung cancer is responsible for 29% of all cancer deaths in North America, making it more lethal than breast, colon, prostate, and pancreatic cancer combined (1). Approximately 85% of lung cancer cases are smoking-related (2, 3), and tobacco smoke contains both direct carcinogens and agents that promote the growth of nascent tumors. Non-small cell lung cancer (NSCLC) constitutes >75% of lung cancer cases, with adenocarcinoma (AC) being the most frequently diagnosed subtype, regardless of smoking status (4, 5). Lung cancer has long been associated with chronic inflammatory disease. Limiting chronic inflammation may halt the rapid growth and progression of this disease (6, 7) since long-term, low-dose aspirin use reduces the risk of death from lung AC by 45%. However, patients with non-AC subtypes of lung cancer were not protected by aspirin use, suggesting that chronic inflammation may be uniquely important for AC progression (7). In addition, increased numbers of pulmonary macrophages correlate with poor prognosis in NSCLC patients (8–10), and alveolar

macrophage numbers also increase during lung tumor progression in mouse models of AC (11, 12). Macrophage depletion early in tumor formation decreases tumor multiplicity (12, 13) indicating a role for inflammatory cells in tumor development even before increased macrophage numbers are detected. Macrophages have been described as obligate partners for breast cancer metastasis to the lung in animal models, and activation of PPAR γ in pulmonary macrophages promotes lung cancer progression and metastasis in a murine orthotopic model (14). Prolonged lung inflammation increases tumor multiplicity by promoting clonal expansion of previously initiated cells (15), and chronic anti-inflammatory drug therapy during chemical promotion decreases tumor multiplicity (13, 16). Chronic inflammation drives lung tumor growth and progression in mouse models and human disease, and alveolar macrophages facilitate much of this effect.

Alveolar macrophages produce numerous epithelial growth factors in response to tissue damage, including insulin-like growth factor-I (IGF-I) (8, 17). IGF-I receptor (IGF-IR) is required

for anchorage-independent growth of epithelial cells and has been studied in neoplastic proliferation for over 20 years. IGF-IR inhibitors are an area of interest for lung cancer therapy (17, 18). Macrophage IGF-I production is highly induced in response to environmental insult (19). While resident alveolar macrophages are a likely physiological source of lung IGF-I, this growth factor is undetectable in undifferentiated human peripheral blood monocytes (20–22). Alternative macrophage programing occurs early in lung tumorigenesis, corresponding with elevated IGF-I production (23–26). Consistent with this association of tumor growth and enhanced macrophage IGF-I production, transgenic mice that produce twice as much IGF-I in bronchoalveolar lavage fluid (BALF) compared to wild-type controls develop spontaneous lung hyperplasias and adenomas after 12 months (27). Despite the evidence linking lung inflammation, macrophage function, IGF-I production, and tumor progression, the relationship between macrophage-derived IGF-I and lung tumor cell proliferation *in vivo* has not been fully explored. We previously showed that BALF from lung tumor-bearing mice contains 3.5-times more IGF-I than that from naïve mice, and macrophage-produced IGF-I enhances neoplastic proliferation *in vitro* (26), indicating that macrophage IGF-I production may play a major role in early lung tumor progression.

Macrophages are selective targets for liposomal clodronate-induced apoptosis because they aggressively phagocytize liposomes (28). Their increased expression of phospholipases facilitates rapid release of clodronate from the liposome vehicle into the phagocyte upon liposome engulfment (29–31). When administered intratracheally (IT), liposomes do not enter the systemic circulation and deplete only alveolar macrophages (29, 31). Conversely, liposomes given intravenously (IV) systemically deplete myeloid cells in the bone marrow, liver, spleen, and other tissues, and reduce the number of circulating cells available for recruitment to the lungs (32). Herein, we use a combination of IT and IV administration of clodronate liposomes to deplete macrophages from the lungs of tumor-bearing mice, and measure the resulting changes in lung pathophysiology by assessing primary lung tumor growth, macrophage depletion, programing of remaining macrophages, and BALF cytokine contents.

MATERIALS AND METHODS

MOUSE LUNG TUMORIGENESIS

Male A/J mice (6–8 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME, USA), maintained on hardwood bedding with a 12-h light/dark cycle, and given Teklad-8640 standard laboratory chow (Harlan Teklad; Madison, WI, USA) and water *ad libitum*. CCR2^{+/−} breeding pairs on a BALB/cJ background were kindly provided by Cara L. Mack, M.D. Department of Pediatrics, School of Medicine, University of Colorado, Anschutz Medical Center. BALB/cJ and CCR2^{−/−} mice were bred in the Center for Comparative Medicine (CCM) at the University of Colorado, Anschutz Medical Center. A/J lung tumors were initiated by a single 1 mg/g body weight intraperitoneal (IP) injection of urethane dissolved in 0.9% NaCl (Alfa Aesar; Heysham, Lancashire, UK) as described previously (33). CCR2^{−/−} and wild-type BALB/cJ mice were given six weekly 1 mg/g IP injections of urethane, a regimen shown to reproducibly induce lung tumors in this moderately

resistant strain (34). At the times indicated, mice were euthanized by IP injection of sodium pentobarbital (Sigma Aldrich; St. Louis, MO, USA). All animal procedures were performed in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Colorado, Anschutz Medical Campus.

BRONCHOALVEOLAR LAVAGE

Primary alveolar macrophages and lung protein exudates were isolated by bronchoalveolar lavage (BAL), as previously described (23, 35). The BALF fractions were separated by centrifugation, BAL cells counted, and inflammatory macrophages, lymphocytes, and neutrophils differentiated by Wright Giemsa (Fisher Scientific) staining (23). BAL cell populations from both naïve and lung tumor-bearing mice were composed predominantly of alveolar macrophages (routinely >95%) (10).

MACROPHAGE DEPLETION BY CLODRONATE-ENCAPSULATED LIPOSOMES

Dichloromethylene diphosphonate (clodronate, 2.5 g; Sigma) was encapsulated in liposomes formed by a 25:1 w/w ratio of phosphatidylcholine:cholesterol (Sigma) as described (31), and the resulting liposomes resuspended in 4 ml sterile PBS. Only 1–2% of clodronate becomes encapsulated, yielding an estimated dose of 0.7–1.0 mg clodronate per 100 µl of liposome suspension. Saline (vehicle) liposomes and clodronate-encapsulated liposomes were synthesized in parallel <2 weeks before use, stored at 4°C, and gently resuspended immediately before instillation or injection (31). Clodronate liposomes for syngeneic transfer experiments were synthesized or purchased from ClodronateLiposomes.org (The Netherlands).

A/J mice bearing urethane-induced lung tumors were anesthetized by a single 50 µl IP injection containing 100 mg/kg ketamine and 10 mg/kg xylazine (CU Clinical Pharmacy; Aurora, CO, USA). Fifty microliters of vehicle or clodronate-containing liposomes were instilled into the lungs via a ball-tip gavage needle bent to a 30° angle and guided by a rodent laryngoscope (Penn Century, Inc.). Follow-up liposome treatments were administered by IV in all mice starting 2 days after IT instillation (100 µl of vehicle or clodronate liposomes administered IV via the tail vein) and repeated once weekly for 5 weeks. IT administration of clodronate is necessary to deplete resident alveolar macrophages, which are not exposed to IV administration, but recruitment of bone marrow macrophages to replenish the alveolar macrophage population can be prevented by IV clodronate liposome ablation of bone marrow monocytes (31).

TISSUE COLLECTION

Plasma was obtained by retro-orbital bleeding with heparin-lined capillary tubes (Fisher Scientific) following administration of terminal anesthesia, and stored at −80°C. Lungs were removed following BAL, the lobes dissociated, and tumors dissected from adjacent unininvolved lung under a dissection microscope, as described (33). Tumor diameters were measured by digital calipers, and tumors pooled in pre-tared microfuge tubes (one tube/mouse) and weighed (tumor burden). Tumor dissection and evaluation were conducted in a blinded fashion.

IMMUNOHISTOCHEMISTRY, TUMOR GRADING, AND ASSESSMENT OF PROLIFERATION

In a similarly treated group of mice, lungs were perfused via the pulmonary artery with 0.9% NaCl, then gently inflated with formalin through the cannulated trachea for 1 h. Lungs were separated into individual lobes and dissected into 14 similarly sized portions and fixed in formalin overnight (33). Lung pieces were embedded in paraffin and sectioned (4 μ m). Sections were processed as previously described, and incubated with anti-Ki67 primary antibody (1:200; Fisher Scientific) (33). Incubation with a biotinylated goat anti-rabbit secondary (1:100; Vector Laboratories) was followed by incubation with horse-radish peroxidase conjugated avidin, detection with 3,3-diaminobenzidine (Vector Laboratories), and counterstain with hematoxylin. Sections were evaluated at 400 \times magnification under an upright microscope (BX41 Olympus), using Spot Advanced software (v4.0.1) to determine tumor area (33). The Ki-67 staining index was calculated by dividing the number of positively staining cells in each tumor by the corresponding tumor area (Ki-67 $^{+}$ /cm 2). This Ki-67 index was averaged per animal and then per group. Serial lung sections were stained with hematoxylin and eosin (Fisher Scientific), and lesions graded as hyperplasia (Hyp), atypical adenomatous hyperplasia (AAH), adenoma (AD), adenoma containing a focus of adenocarcinoma (ADwAC) or AC following the guidelines established by Nikitin et al. (36) and using images found at the digital atlas of virtual histological slides as examples (37). Grading was performed in a blinded fashion by three individuals at a multiheaded microscope and evaluated by a board certified pathologist (Daniel T. Merrick). Particular attention was paid to nuclear morphology, density of the lesion, and vessel involvement. We found that adenomas and AC comprised the majority of lesions in this mouse model, and squamous cell and neuroendocrine carcinomas were not observed.

DETERMINATION OF MACROPHAGE PROGRAMMING BY IMMUNOFLUORESCENCE

Sections were deparaffinized and rehydrated prior to antigen retrieval as described (23). Tissue sections were then incubated overnight at 4°C with 1:50 dilution of anti-arginase I (ArgI, Santa Cruz Biotechnology) primary antibody followed by 1:1000 dilution of Alexa 568-conjugated anti-goat secondary antibody. A mixture of anti-NOS2 (BD Transduction Labs; 1:50 dilution) and anti-F4/80 (ABD-Serotec; 1:50 dilution) primary antibodies were then applied for 1 h at 37°C, followed by 20 min incubations with Alexa 488-conjugated anti-rabbit and Alexa 680-conjugated anti-rat secondaries. Nuclei were stained with DAPI-containing mounting media (Vector Laboratories). Images were obtained with a digital deconvolution microscopy imaging system attached to a Zeiss AxioPlan 2 epi-Fluorescence upright microscope. Macrophages were identified by positive F4/80 staining and morphology. Total pixel counts/macrophage were calculated for ArgI, NOS2, and F4/80 immunofluorescence (~50/animal) using ImageJ software (38), and ArgI and NOS2 values were normalized to F4/80 staining. To confirm ArgI $^{+}$ M2 programming, adjacent sections were subjected to a similar IF protocol substituting an antibody against M2 marker phosphoTyr 641 STAT6 (Cell Signaling, 1:50 dilution) for NOS2.

Fluorescence intensity was calculated similarly and phospho-STAT6/ArgI ratios determined.

BALF IGF-I AND CYTOKINE DETERMINATION

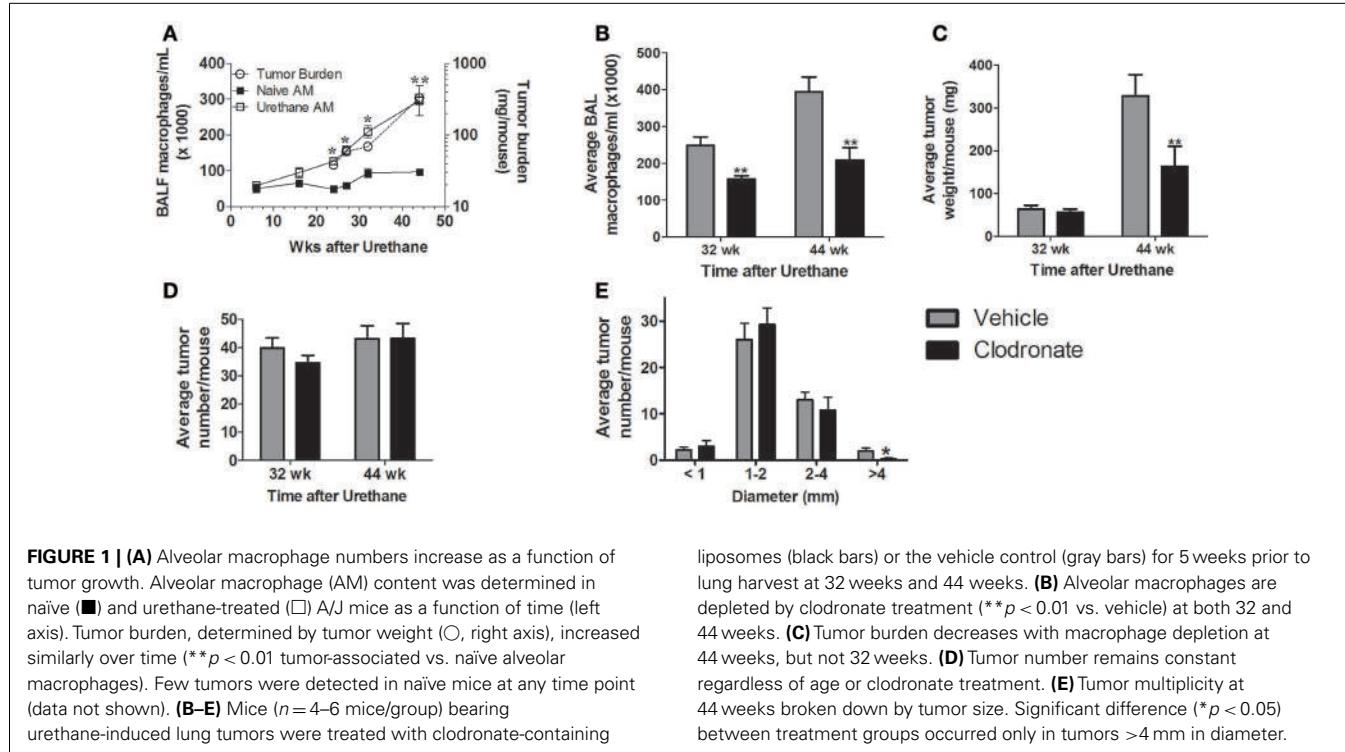
Insulin-like growth factor-I concentrations in BALF were determined by enzyme-linked immunosorbent assay (ELISA) in a 96-well format as directed (R&D Systems, Inc.). For lung cytokine levels, BALF samples were concentrated 5 \times by centrifugation in 3 kDa molecular weight cut off YM-3 microcon spin-columns (Millipore), and applied to Quantibody® mouse cytokine array slides (Raybiotech, Inc.). Fluorescent Cy3-equivalent antibody signal was read by the CU Cancer Center Microarray Core Facility using a Perkin Elmer Scan Array Ex glass slide laser scanner (Perkin Elmer). Analytes were quantified by regression of log-transformed data sets against within-run standard curves.

CCL2/CCR2 INVOLVEMENT IN LUNG TUMOR PROGRESSION

Tumorigenic mouse lung epithelial E9 cells (39) were maintained in CMRL media (Invitrogen) supplemented with 10% fetal bovine serum. For syngeneic transplant studies, 1 \times 10 6 log-phase E9 cells were suspended in 100 μ l of serum-free CMRL media (Life Technologies) and injected subcutaneously (s.c.) into the shaved right flanks of syngeneic male and female BALB/c (WT) or CCL2 receptor null (CCR2 $^{-/-}$) mice, a protocol previously shown to generate rapidly growing tumors in nearly 100% of recipient animals (40). Wild-type and CCR2 $^{-/-}$ mice received vehicle or clodronate-encapsulated liposomes by IV injection 1 day prior to tumor inoculation (day -1), and once/week thereafter. Tumor size was determined twice/weekly for 24 days, and tumor volume was calculated using the equation for an elliptical cone (as recommended due to the non-spherical growth pattern of the implants): $V = (d^2 \times l \times \pi)/6$, in which "d" is the smallest diameter, and "l" the largest. Tumor-bearing mice were euthanized and flank tumors removed and weighed. The experiment was performed twice, with 5–6 mice/group/repetition. Previous studies showed that both macrophage conditioned media (MΦCM; 1:1 mixture of fresh media with media harvested after 24 h incubation with MH-S murine alveolar macrophage cells) and/or IGF-I stimulated *in vitro* proliferation of cultured mouse lung epithelial cells. To determine if this was also true for E9 cells, subconfluent cultures were incubated with MΦCM or 50 ng/ml IGF-I for 48 h, harvested, and relative cell numbers were compared using CellTiter96® proliferation assays (Promega).

STATISTICAL ANALYSIS

Continuous variables were analyzed by two-way ANOVA with Bonferroni *post hoc* comparison to determine significant differences between groups and account for multiple inter-group comparisons. One-way ANOVA with student Newman–Keuls *post hoc* analysis was used to determine significant differences between three or more groups while Student's two-tailed independent *t*-test was used when only two groups were compared, with Welch's correction for unequal variances when appropriate. All statistics including Spearman correlations were performed using Prism 5.0 software (Graphpad; La Jolla, CA, USA). Data are presented as mean \pm SEM, unless otherwise indicated. In all analyses, $p < 0.05$ was considered to be statistically significant.



RESULTS

Tumor burden and alveolar macrophage numbers (obtained by lavage) increased similarly over time in urethane-treated A/J mice (Figure 1A), while alveolar macrophage numbers changed little over the same time course in naïve mice. Few tumors are detected in naïve mice (data not shown). Liposomal clodronate significantly depleted alveolar macrophages in tumor-bearing mice [harvested 32 (37%) and 44 (48%) weeks after urethane injection compared to vehicle liposome treated, tumor-bearing mice (Figure 1B)]. Tumor burden at the 44-week time point decreased by ~50% with clodronate treatment (Figure 1C) while tumor number did not change (Figure 1D). Comparing tumor weight at 44 weeks to that of mice sacrificed at 32 weeks suggests that tumors did not regress with clodronate treatment, but simply did not grow as rapidly (Figure 1C). A significant decrease in tumors with diameter >4 mm was detected, indicating that clodronate preferentially slowed the growth of larger tumors (Figure 1E). Immunohistochemical staining of Ki67, a marker of cell division, decreased by $>50\%$ in tumors from 44-week clodronate-treated mice compared to the vehicle liposome-treated controls (Figures 2A,B), affecting tumors of varying size (Figure 2D). Similar numbers of hyperplasias, AAH, adenomas, adenomas with AC-like foci, and AC (Figures 2C,E) were detected in the lungs of both vehicle and clodronate-treated mice.

Programming in tumor-associated macrophages (TAMs) was examined by immunofluorescence to determine whether clodronate liposomes targeted a specific subset of macrophages in mice bearing 44-week lung tumors. TAMs in this study exhibited a mixed M1/M2 phenotype characterized by both NOS2 (an M1 programming marker) and Arg1 (an M2 programming marker)

liposomes (black bars) or the vehicle control (gray bars) for 5 weeks prior to lung harvest at 32 weeks and 44 weeks. (B) Alveolar macrophages are depleted by clodronate treatment (** $p < 0.01$ vs. vehicle) at both 32 and 44 weeks. (C) Tumor burden decreases with macrophage depletion at 44 weeks, but not 32 weeks. (D) Tumor number remains constant regardless of age or clodronate treatment. (E) Tumor multiplicity at 44 weeks broken down by tumor size. Significant difference (* $p < 0.05$) between treatment groups occurred only in tumors >4 mm in diameter.

expression (Figure 3A). Because earlier studies showed predominantly NOS2⁺ macrophages in this model at this late time point, a second M2 marker, phosphoSTAT6, was analyzed confirming the presence of M2 markers (Figure 3B). Previously, we determined that human lung TAMs express similar mixed (NOS2⁺CD206⁺) programming (23). We saw no large-scale differences in programming marker expression between vehicle and clodronate exposed macrophages (Figure 3C), and Arg1/phosphoSTAT6 ratios were also similar between groups (Figure 3C) suggesting that the TAMs in this model are similarly programmed, and this programming is not affected by clodronate exposure.

Cytokine and IGF-I levels were measured in BALF from vehicle and clodronate-treated, tumor-bearing mice 44 weeks after urethane. BALF levels of GM-CSF, IFN- γ , IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-9, IL-10, IL-12, IL-17, M-CSF, and RANTES were unchanged by clodronate treatment (data not shown). IL-3, IL-13, and TNF- α contents were below the limit of detection in all samples (data not shown). Clodronate treatment significantly decreased levels of IL-6, CCL2, CXCL1, and IGF-I, while VEGF levels increased 1.5-fold (Figures 4A,B). Serum levels of IGF-I did not change with clodronate exposure (Figure 4B). Levels of IGF-I, CXCL1, IL-6, and CCL2 were higher in BALF from tumor-bearing mice than naïve mice 32 weeks after urethane treatment, but VEGF levels were unchanged (Figure 4C). IGF-I levels correlate significantly with BAL macrophage numbers in naïve and tumor-bearing animals (Figure 4D, $p < 0.0001$) as well as in animals exposed to vehicle and clodronate-containing liposomes (Figure 4E, $p < 0.03$). Activated macrophages produce IL-6, CCL2, CXCL1, and IGF-I, but concomitant production of all four signaling molecules also indicates a “mixed phenotype” of macrophages since IL-6 and IL-8

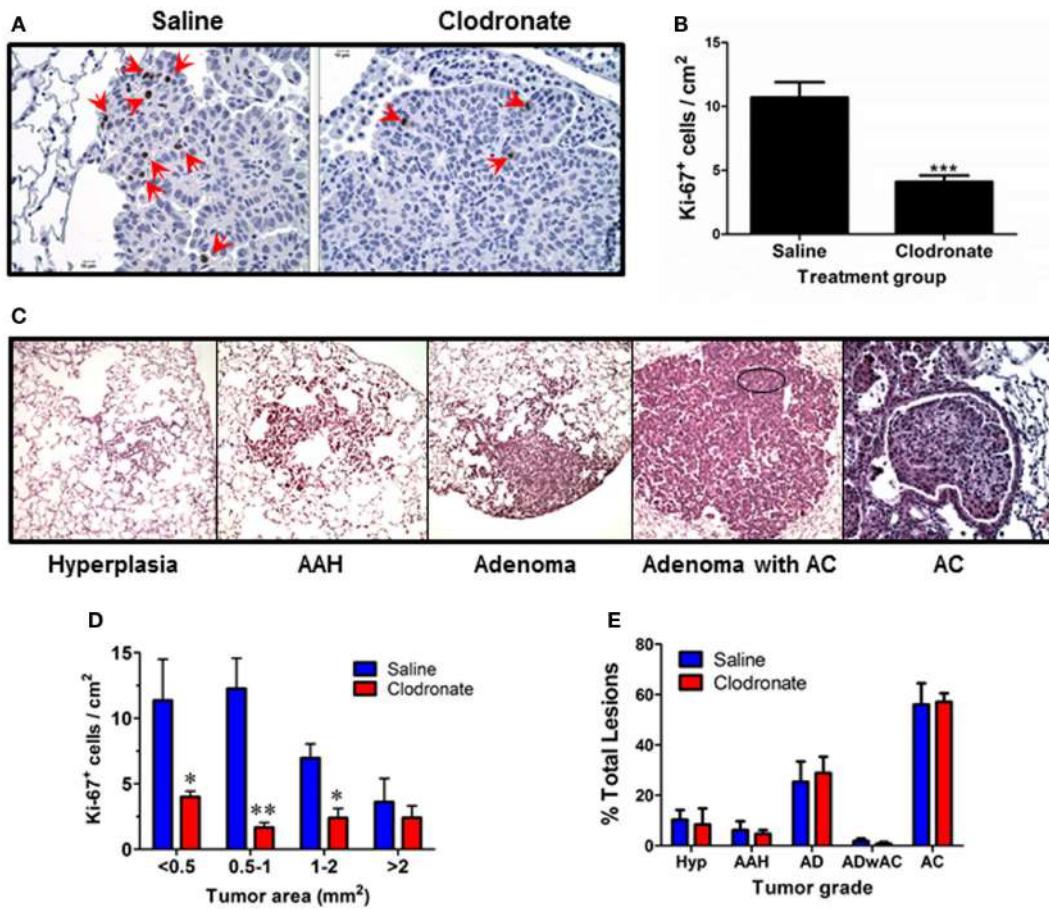


FIGURE 2 | Effects of clodronate treatment on tumor growth and progression. (A) Representative Ki67 IHC on tissue sections from similar sized tumors from vehicle (left) and clodronate (right) treated mice (400 \times final magnification). Red arrows point to positively stained tumor cells. **(B)** Ki-67 index was calculated as the average number of Ki-67⁺ cells/cm² tumor area for each tumor in each group (mean \pm SEM, ** p < 0.01 vs.

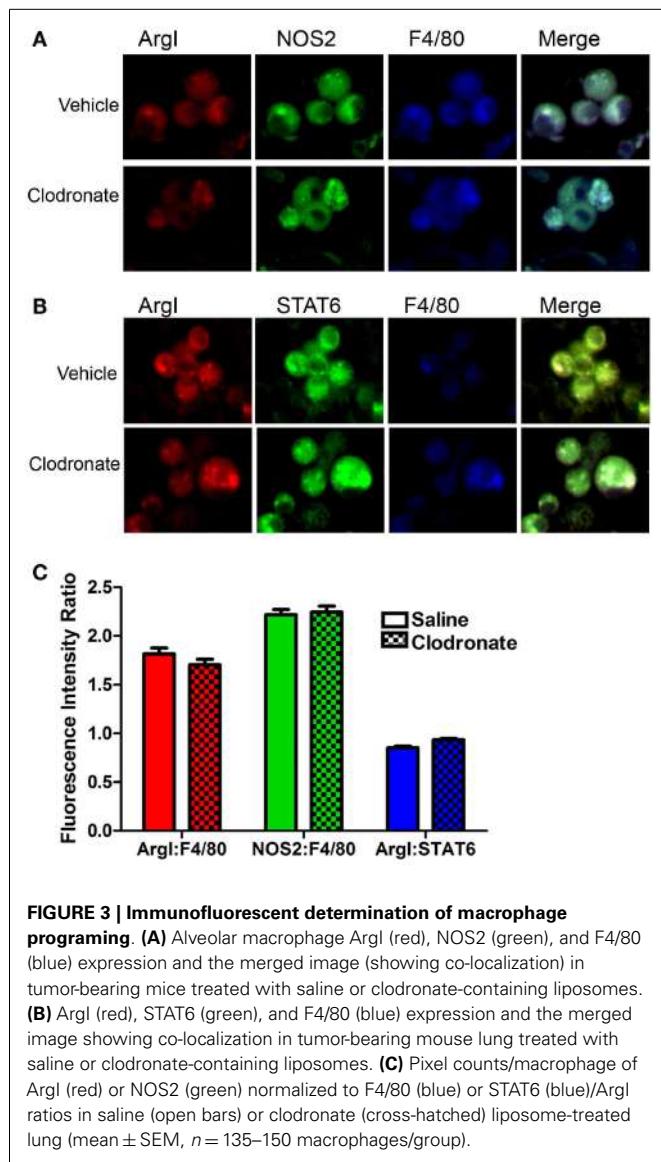
vehicle). **(C)** Representative examples of each tumor grade (400 \times final magnification). **(D)** Ki67 index as a function of tumor size at 44 weeks in saline and clodronate-treated mouse lung tumors. Significant differences were seen in the number of Ki67⁺ cells in the smaller lung tumors. **(E)** Percent of each lesion type was calculated/mouse. No significant differences were detected.

(human ortholog of murine CXCL1) are most often associated with M1 programming and IFG-I and CCL2 with M2 programming.

Vehicle treated mice secreted 6.5-fold more CCL2 into BALF compared to clodronate-treated mice (**Figure 4A**). As CCL2 is a chemotactic factor for monocytes and could be integral in the recruitment of macrophages to the site of tumors development, we tested whether ablation of CCL2/CCR2 signaling affected tumor growth in a syngeneic transplant model. Transformed mouse lung epithelial E9 cells were injected into the flanks of immunocompetent syngeneic BALB/cJ wild-type or CCR2^{-/-} mice. After tumors were established, mice were subjected to systemic liposome-based macrophage depletion. There were no significant differences in tumor growth among the vehicle treated wild-type, vehicle treated CCR2^{-/-}, or clodronate-treated CCR2^{-/-} mice. However, flank tumors in wild-type mice receiving clodronate liposomes grew significantly slower (**Figure 5A**). We previously showed that macrophage conditioned media (MΦCM) increases proliferation of mouse lung epithelial cells *in vitro*, and IGF-I is the major

component in MΦCM that contributes to this proliferative effect (**26**). Both MΦCM and IGF-I increased E9 proliferation by more than threefold over control media suggesting that the inhibition of syngeneic tumor growth, resulting from systemic macrophage depletion may be due to decreased macrophage production of IGF-I (**Figure 5B**).

Ablation of CCL2/CCR2 signaling did not affect the growth of syngeneically transplanted E9 cells, and although there was a slight, but not significant trend of slower growth of the E9 tumors in the CCR2^{-/-} mice, the effect was not as dramatic as that seen with clodronate treatment. In addition, tumor growth in CCR2^{-/-} mice was not significantly slowed by clodronate exposure. To test whether CCL2/CCR2 signaling is required for the *de novo* development of tumors in the lung, wild-type and CCR2^{-/-} mice were initiated with six weekly urethane injections. Lung tumors were harvested, counted, and tumor burden (by weight) assessed at 20, 32, 38, and 42 weeks after the initial urethane exposure. No significant decreases in tumor number, tumor burden, or alveolar



macrophage numbers were detected in $CCR2^{-/-}$ mice at any time point (data not shown). Results from the 42-week time point are shown in Figures 5C–F. Not only did $CCR2^{-/-}$ mice show similar number of alveolar macrophages and lung tumors at this late time point, there was a significant increase in lung tumor diameter in the $CCR2^{-/-}$ mice. As CCL2/CCR2 signaling was ablated in the $CCR2^{-/-}$ mice, either a redundant pathway compensates for macrophage recruitment to the lung or resident macrophages proliferate to compensate for the lack of CCL2/CCR2-mediated macrophage recruitment.

DISCUSSION

Depletion of pulmonary macrophages by nearly 50% decreased the growth of the largest lung ACs in the A/J urethane model. Decreased Ki67 staining indicated that cell division slowed as a result of macrophage depletion *in vivo*, an observation complementing previous results showing that macrophage co-culture and

exposure to macrophage conditioned media increased tumor cell proliferation *in vitro* (26). Macrophage depletion did not cause tumor regression as tumor number and stage did not change after clodronate treatment. TAMs produce signals that support tumor growth and promote tumor cell survival. When TAMs are depleted, production of these signals decreases causing a reduction in tumor cell proliferation. Clodronate exposure decreased BALF levels of IGF-I, IL-6, CXCL1, and CCL2 and increased VEGF levels while not affecting most of the other factors examined (largely $T_{H}1$ and $T_{H}2$ -associated cytokines), suggesting that alveolar macrophages may not be their primary source in murine lungs. IGF-I involvement in lung tumorigenesis is well established, and the positive correlation of pulmonary BAL IGF-I levels with macrophage numbers (Figures 4C,D) suggests that macrophage production of IGF-I is important in maintaining tumor growth. The decrease in IGF-I levels caused by clodronate-induced macrophage depletion may be partially responsible for slowing lung tumor growth *in vivo*, which is consistent with our previous observations of IGF-I mediation of a significant portion of MΦCM induced lung tumor cell proliferation *in vitro*. The increased BALF VEGF levels detected in the clodronate-treated animals may occur in response to clodronate-induced macrophage apoptosis, as VEGF expression is induced in macrophages that clear apoptotic cells by efferocytosis (41, 42). The remaining healthy alveolar macrophages may express more VEGF as they clear the apoptotic macrophages, resulting from clodronate depletion.

The attenuation of CCL2 production caused by clodronate liposome exposure suggested that monocyte recruitment might also play a role in tumor progression. CCL2 (MCP-1; monocyte chemotactic protein-1) is involved in recruitment of monocytes (43), T cells (44), and dendritic cells (45) to areas of inflammation induced by tissue injury or infection. High levels of CCL2 are associated with poor prognosis in breast cancer (46) and pancreatic cancer (47). However, Zhang et al. (48) found that CCL2 over-expression is associated with improved survival in NSCLC patients indicating that CCL2/CCR2 signaling may have different roles in different tissues. To determine whether CCL2 signaling affected lung tumor growth in mice, syngeneic mouse lung tumor cells were transplanted into wild-type and $CCR2^{-/-}$ mice. Ablation of CCL2/CCR2 signaling had no effect tumor growth, and the lack of CCL2/CCR2 signaling actually nullified the growth inhibitory effects of clodronate liposomes for reasons, which remain to be determined. Because E9 cells are already transformed and do not require a progression phase for tumor growth and because alveolar macrophages differ in function and response from macrophages present near subcutaneous tumors (49), we tested whether CCL2/CCR2 signaling was required for *de novo* lung tumor formation. BAL macrophage content and tumor numbers from urethane-exposed $CCR2^{-/-}$ and wild-type mice were similar between genotypes at each time point. A slight, but significant increase in tumor size in the $CCR2^{-/-}$ mice compared to wild-type littermates was detected at the 44-week time point, which is consistent with the poorer survival observed in human NSCLC patients with low-CCL2 expression (48). Macrophages are known to produce CCL2 in response to IL-4/IL-13 stimulation (50), and the increase in CCL2 production we detected during lung tumorigenesis indicates that there is a Th2 response occurring

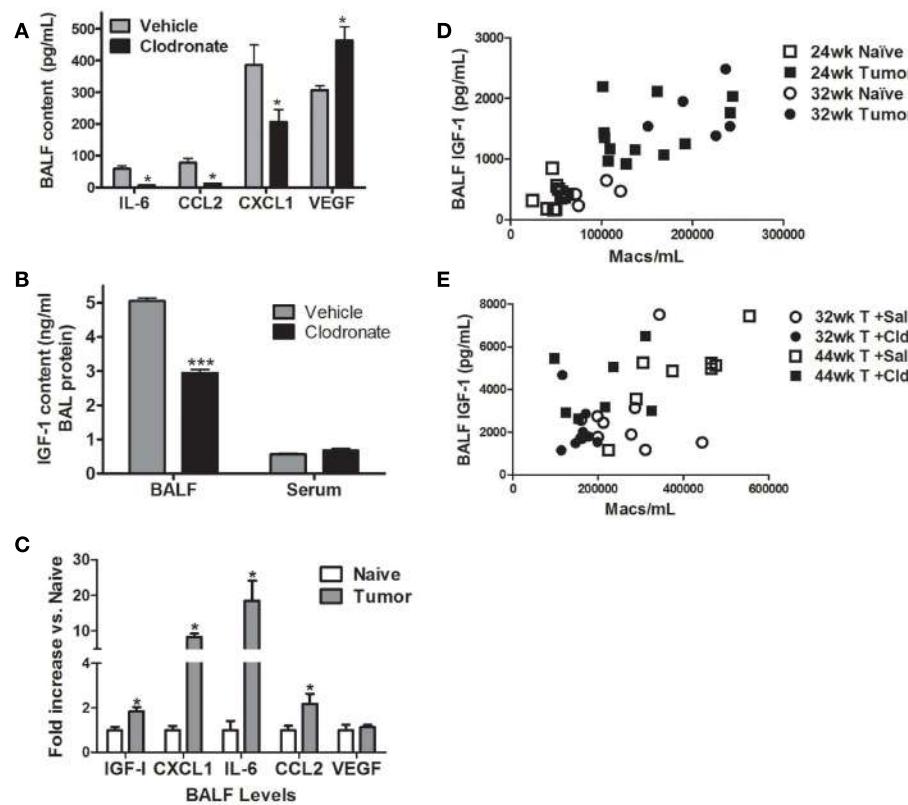


FIGURE 4 | Effects of macrophage depletion on BAL cytokine content. Cytokine levels were determined in BALF from naïve (white bars), vehicle liposome (gray bars), or clodronate liposome (black bars) treated mice 44 weeks after urethane treatment. **(A)** Clodronate administration decreased IL-6, CCL2, and CXCL1 in BALF, while VEGF increased 1.5-fold. Data presented as mean \pm SEM, $n=4\text{--}6$ mice/group (* $p < 0.05$ vs. vehicle treated mice). **(B)** BALF IGF-I levels decreased with clodronate treatment in AC-bearing mice (** $p < 0.001$ vs.

vehicle). **(C)** Fold change in BALF IGF-I, IL-6, CXCL1, CCL2, and VEGF levels in age-matched naïve and tumor-bearing mice 32 weeks after urethane exposure (* $p < 0.05$) **(D)** Correlation between IGF-I and BAL macrophage number in naïve and urethane-treated mice 24 and 32 weeks after urethane treatment (Spearman $p = 0.8055$, $p < 0.0001$). **(E)** Correlation between IGF-I and BAL macrophage number in 44-week urethane mice treated with saline and clodronate liposomes (Spearman $p = 0.4990$, $p < 0.0031$).

in lungs during tumor formation. The CCL2 receptor (CCR2) is expressed only in monocyte-lineage cells and T lymphocytes, so CCL2 is unlikely to directly affect neoplastic epithelial cells (9). CCL2 may regulate macrophage recruitment following lung injury, as CCL2 levels in BALF are elevated just prior to the macrophage influx that follows chemically induced pneumotoxicity (51). The similar alveolar macrophage content in tumor-bearing lungs from both wild-type and CCR2^{-/-} mice suggests that either there are redundant macrophage recruitment pathways at play in the lung tumor microenvironment or that the increased number of TAMs results from proliferation of resident macrophages rather than recruitment as has been reported (52).

High-serum IL-6 levels correlate with poor survival and poor response to chemotherapy in NSCLC patients (53, 54). IL-6 is an inflammatory cytokine produced primarily by T cells and M1-programmed macrophages to stimulate immune response to injury and trauma. IL-6 may be required for M1 programming in some macrophages, but IL-6 production can induce M2 programming in certain systems to limit existing inflammation (55). Recently, Fernando et al. showed that M2 programming is enhanced by IL-6

exposure in cultured macrophages and suggested that IL-6 augments cytokine expression during both M1 and M2 programming (56). Similar to our findings, Karnevi et al. report that human pancreatic tumor-educated macrophages display mixed M1/M2 programming and produce increased IL-6 and IL-8 (57). Pine et al. report that increased IL-6 and IL-8 production are associated with increased lung cancer risk (58), and IL-6 and IL-8 are both associated with increased NSCLC cell proliferation (59) although neither directly induces increased cell division. Human IL-8 (CXCL8) and murine keratinocyte-derived chemokine CXCL1 (KC) are orthologous in function. CXCL1 production is high in murine alveolar macrophages (60, 61), but not in peritoneal macrophages (62–65). The specific combination of cytokines and surfactant proteins intrinsic to the lung make alveolar macrophages functionally unique compared to macrophages from other tissues. Lung production of IL-8/CXCL1 is induced by inflammatory stimuli through NF-κB and AP-1 activation (63, 65), and both NSCLC cells and macrophages express IL-8/CXCL1. IL-8/CXCL1 is a pro-angiogenic factor as well as a chemotactic factor for neutrophil recruitment to the lungs during emphysema and lung cancer. Few

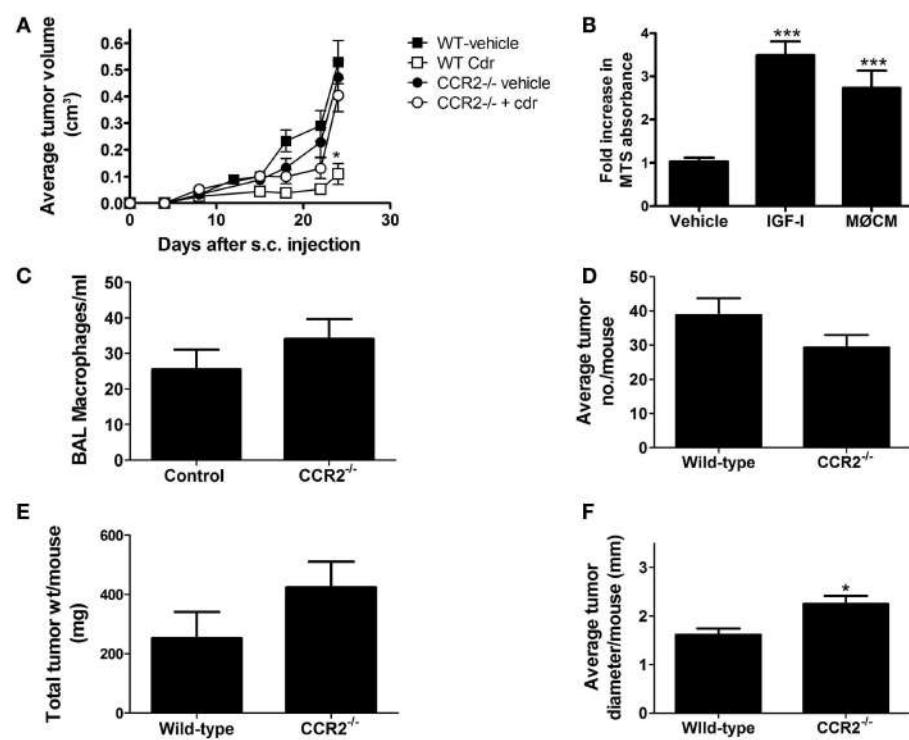


FIGURE 5 | Clodronate reduces syngeneic implant growth in wild-type (WT), but not CCR2^{-/-} mice. (A) E9 cells were injected into the flank of female BALB/cJ (WT) or CCR2^{-/-} mice. After tumors were established (8 days post injection), clodronate-containing or vehicle liposomes were injected IV once/week until sacrifice. Tumors were measured 2×/week and volumes calculated. Clodronate liposome treatment (Cdr) decreased tumor growth in wild-type mice (* $p < 0.05$, $n = 10\text{--}12$ mice/group from two independent experiments). **(B)** E9 cells

were cultured in serum-free media (vehicle), 50 ng/ml IGF-I containing media, or macrophage conditioned media (MΦCM). Cell density was determined by MTS and graphed as fold-increase over control (mean \pm SEM, *** $p < 0.001$ vs. control). **(C-F)** Wild-type BALB/cJ and CCR2^{-/-} mice were injected with 1 mg/g urethane once/week for 6 weeks. BALF and tumors were harvested 42 weeks after the first injection and BAL macrophages ($\times 1000$) counted, and lung tumors counted, measured, and weighed (mean \pm SEM, * $p < 0.05$ vs. wild-type, $n = 5$).

neutrophils were detected in the lungs of vehicle or clodronate liposome-treated tumor-bearing mice (data not shown), so we could not determine whether clodronate treatment altered their numbers further.

Macrophage programming in mouse lung is homogeneous, probably due to the small size of the organ. As urethane-exposed A/J mice form >30 tumors/mouse and these tumors are spread evenly throughout the lobes, alveolar macrophages are exposed to tumor produced factors throughout the lung. The A/J urethane model of lung cancer is unique among most murine lung tumor models in that TAMs surrounding late-stage ACs express high levels of NOS2 and little ArgI, indicating that they are primarily M1-programmed (23, 24, 34). However, BALF cytokine levels in these mice indicate that macrophage programming might be more complex. Although ArgI expression decreases as tumors progress, production of certain M2-associated signaling molecules (i.e., IGF-1, CCL2) increases. When these mice were exposed to either vehicle or clodronate-containing liposomes, we detected an increase in both ArgI and phosphoSTAT6 expression (M2 programming markers) in the same macrophages that maintained high-NOS2 expression. The M1/M2 classification represents a continuum of plasticity and does not encompass the functional diversity of

macrophages (66), and the measurement of biomarker expression rather than activity and/or function may not yield an accurate picture of macrophage programming. In our study, we detected NOS2⁺ArgI⁺ macrophages, but we did not measure NO levels or determine arginase activity, so although both enzymes were present, we do not know that both were active in the same cells. The presence of phosphoSTAT6 in these TAMs indicates that they were more “M2-like” and the presence of CCR2 and IGF-1 in the BALF supports this. Although there were fewer macrophages in the clodronate-treated lungs, the ratio of ArgI to NOS2 remained constant in the remaining cells indicating that the remaining tumor microenvironment and not macrophage depletion continued to affect the programming of the remaining macrophages.

Our previous studies indicated that TAMs near human lung AC also expressed M1 and M2 markers simultaneously (24), and others have seen similar mixed phenotypes in TAMs in pancreatic (57) and gastric cancer (67). Mixed macrophage programming was also demonstrated in the resolution of liver fibrosis (68) and pulmonary fibrosis (69) and in the early stages of diet induced obesity (70). These mixed populations could be due to catching the macrophages as they change from one programming state to another as in the resolution of a disease, or as they respond

to conflicting microenvironmental signals. The detection of both inflammatory and anti-inflammatory cytokines in BALF from these mice indicates that these macrophages do not follow the canonical roles of M1 or M2 macrophages. Further research to determine phagocytic and efferocytic activity, proliferative capacity, and gene expression of TAMs from tumor-bearing mice before and after exposure to vehicle and clodronate liposomes is necessary to characterize this novel macrophage population. The ability of clodronate liposomes to deplete pulmonary macrophages may be enhanced by changing the composition of the liposome delivery vehicle to more effectively and specifically target TAMs that accumulate in the lungs of tumor-bearing mice. These mixed phenotype macrophages may express scavenger receptors such as the mannose receptor, so adding mannose to the liposome surface may increase their uptake, resulting in greater depletion (71). Also, M2 macrophages exhibit enhanced efferocytosis (72) while M1 macrophages are professional phagocytes, so the presence of phosphatidyl serine in the liposome membrane to mimic apoptotic cells and/or lipopolysaccharides on the exterior of the liposome to mimic bacterial cell walls may lead to enhanced uptake and possibly increased macrophage depletion. As IGF-I signaling activates survival factors such as AKT in tumor cells (26), macrophage depletion and the subsequent decrease in BALF IGF-I levels may make tumors more sensitive to therapy-induced death. Although macrophage depletion cannot eliminate lung tumors, it may sensitize tumors to classic chemotherapeutics and radiation by removing both macrophage-produced survival factors from the lung prior to cytotoxic therapies and growth promoting signals such as IGF-I during tumor recovery. Finally, by decreasing the permissive nature of the tumor microenvironment, macrophage depletion may allow the host's own defenses to recover and eliminate tumor cells.

ACKNOWLEDGMENTS

This paper is dedicated to the memory of our late mentor, colleague, and friend Dr. Alvin M. Malkinson whose work with inflammation and tumor promotion formed the basis for these studies. These studies were funded by NIH CA132552 and supported by NCI Cancer Center Support Grant P30CA046934 (Microarray Core Facility).

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Conflict of Interest Statement: The Guest Associate Editor Laurel L. Lenz declares that, despite being affiliated to the same institution as the authors Jason M. Fritz, Meredith A. Tennis, David J. Orlicky, Hao Yin, Cynthia Ju, Daniel T. Merrick, Alvin M. Malkinson and Lori D. Dwyer-Nield, the review process was handled objectively and no conflict of interest exists. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 15 August 2014; accepted: 03 November 2014; published online: 25 November 2014.

Citation: Fritz JM, Tennis MA, Orlicky DJ, Yin H, Ju C, Redente EF, Choo KS, Staab TA, Bouchard RJ, Merrick DT, Malkinson AM and Dwyer-Nield LD (2014) Depletion of tumor-associated macrophages slows the growth of chemically induced mouse lung adenocarcinomas. *Front. Immunol.* **5**:587. doi: 10.3389/fimmu.2014.00587

This article was submitted to Molecular Innate Immunity, a section of the journal *Frontiers in Immunology*.

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Phenotypic diversity and emerging new tools to study macrophage activation in bacterial infectious diseases

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Macrophage polarization is a concept that has been useful to describe the different features of macrophage activation related to specific functions. Macrophage polarization is responsible for a dichotomous approach (killing vs. repair) of the host response to bacteria; M1-type conditions are protective, whereas M2-type conditions are associated with bacterial persistence. The use of the polarization concept to classify the features of macrophage activation in infected patients using transcriptional and/or molecular data and to provide biomarkers for diagnosis and prognosis has most often been unsuccessful. The confrontation of polarization with different clinical situations in which monocytes/macrophages encounter bacteria obliged us to reappraise this concept. With the exception of M2-type infectious diseases, such as leprosy and Whipple's disease, most acute (sepsis) or chronic (Q fever, tuberculosis) infectious diseases do not exhibit polarized monocytes/macrophages. This is also the case for commensals that shape the immune response and for probiotics that alter the immune response independent of macrophage polarization. We propose that the type of myeloid cells (monocytes vs. macrophages) and the kinetics of the immune response (early vs. late responses) are critical variables for understanding macrophage activation in human infectious diseases. Explorating the role of these new markers will provide important tools to better understand complex macrophage physiology.

Keywords: macrophage, activation, polarization, infectious diseases, bacteria

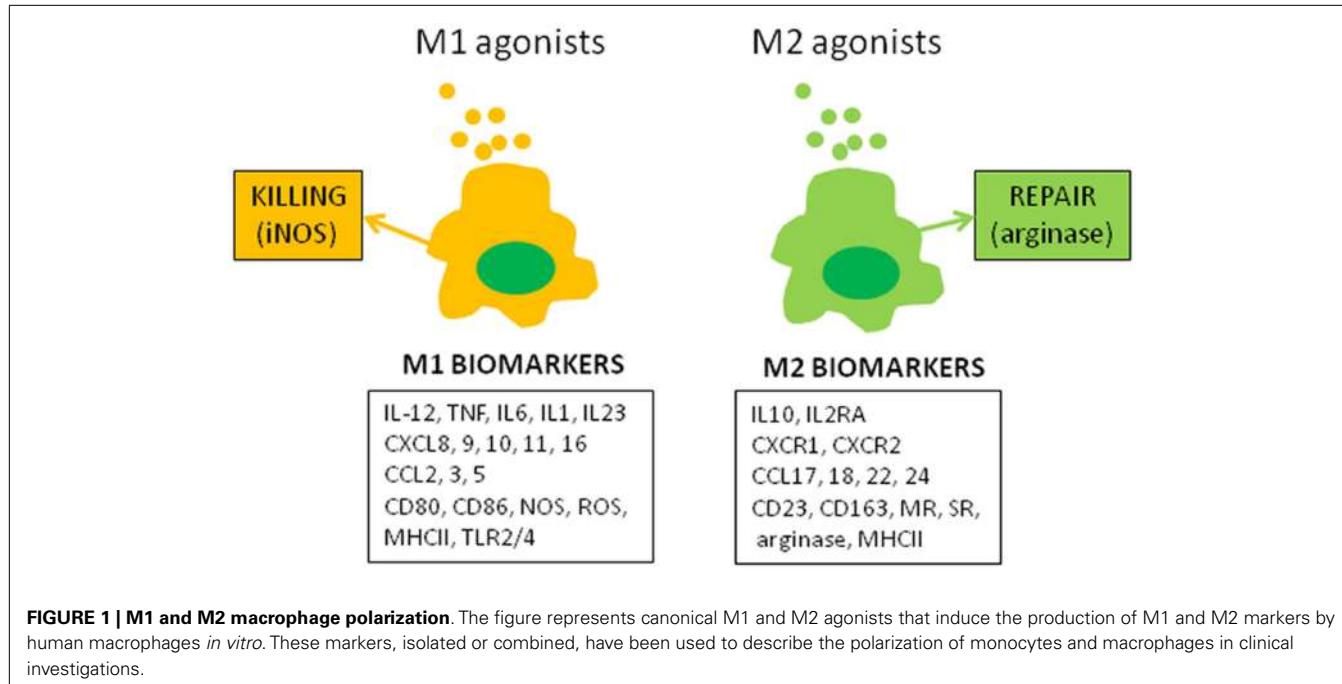
INTRODUCTION

Why a new review about macrophage polarization during bacterial infectious diseases? The initial analysis of macrophage activation, based on *in vitro* experiments and the use of animal models, suggested a dichotomous classification based on the production of canonical molecules associated with a specific function. The production of nitric oxide is associated with the killing of microorganisms or tumor cells and characterizes M1-type macrophage response whereas the expression of arginase (production of ornithine) is associated with the repair and characterizes M2-type macrophage responses (1). The concept of M1/M2 polarization has been largely popularized because macrophage polarization was considered the reflection of Th1 and Th2 polarization of lymphocytes, although the idea that activation by T cells is required for macrophage polarization is likely incorrect (1). As the Th1/Th2 paradigm has progressively been replaced by several functional statuses over the past years, the meaning of a similar dichotomy of macrophage activation is unknown. During the last years, numerous transcriptional and/or molecular markers associated with M1- or M2-type macrophage responses were found but they did not have a clear relationship with macrophage functions, which has been a source of controversies. We feel that these new markers could provide additional important tools to better understand complex macrophage physiology. In addition, recent advances suggest that monocytes readily available in humans are not able to polarize like mature tissue macrophages. As a consequence, the

increasing number of publications in which clinical cohorts are investigated with new tools of macrophage investigation allows a global analysis of the cell responses, which results in a more precise overview of the clinical data. It is likely that the concept of M1/M2 macrophages is likely insufficient to describe human infectious diseases. While M2-type infectious diseases such as leprosy and Whipple's disease represent a clinical exception; most acute (sepsis) or chronic (Q fever, tuberculosis) bacterial diseases do not exhibit polarized monocytes/macrophages. According to the analysis of Thomas Kuhn, the "paradigm" of macrophage polarization applied to human bacterial diseases suffers from abnormalities that could lead to a paradigm shift to a kinetic vision of macrophage activation.

THE MACROPHAGE POLARIZATION CONCEPT

The molecular concept of the polarization of human macrophages has been initially based on the selective expression of a few markers that have poor specificity when expressed alone. The development of high-throughput profiling technologies that enable the investigation of complex macrophage states (2) has increased the number of biomarkers associated with the M1 or M2 status (Figure 1). Among the papers reporting transcriptomic analysis of activated macrophages that of Martinez et al. was the most contributive (3). The authors showed that M1 and M2 polarization affect 5.2 and 0.3% of transcripts, respectively. The functional annotation reveals the enrichment with categories such as DNA transcription, protein



metabolism, G protein coupled-receptors, and lipid metabolism in addition to well-identified cytokine and chemokine families. Hence, the polarization of human macrophages has become more complex than the initial descriptions.

A recent transcriptomic analysis of human macrophages stimulated by a large panel of agonists allowed a description of macrophage activation as a spectrum. This spectrum of activation was more complex than the M1 vs. M2 model of activation because at least nine distinct activation programs were identified. The use of network analyses demonstrated a central transcriptional regulator present in all activation conditions that was complemented by regulators associated with the programs stimulated by each agonist (4). The authors used this model of activation to analyze human alveolar macrophages from patients who were smokers or from patients with chronic obstructive pulmonary disease (COPD). They found that the activation program of macrophages was more complex than predicted in smokers and in patients with COPD. They did not find enrichment with modules associated with interleukin (IL)-4/IL-13 activation in patients with COPD, as was expected, but did find a decrease in the modules associated with interferon (IFN)- γ (4). This report clearly demonstrates that the prominent, popular point of view that cigarette smoke and COPD increase M2-like characteristics (5) was not supported when high-throughput approaches were used.

A proteomics approach has also been used to investigate macrophage polarization. The MALDI-TOF mass spectrometry (MS) technique combined with gel electrophoresis permitted the identification of a large number of soluble or membrane proteins in activated macrophages. This double approach allowed the identification of an M1 signature in human macrophages stimulated with LPS and IFN- γ (6). Recently, we used MALDI-TOF MS to characterize whole eukaryotic cells (7) and the activation status of human macrophages (8). We found that whole-cell MALDI-TOF

MS analysis was able to discriminate macrophages according to the type of M1 or M2 agonists and allowed for the identification of different subtypes of M1 or M2 macrophages. The MALDI-TOF MS analysis of pathogen-stimulated macrophages also enabled the detection of pathogen-associated fingerprints that did not correspond to the standard M1/M2 polarization model (8). Taken together, the use of polarization markers other than iNOS and arginase has been controversial. Recently, we proposed guidelines for macrophage activation in which we favored an approach based on a combination of markers instead of isolated canonical markers of polarization (9).

The exploration of tissue macrophages, excepted alveolar macrophages, requires biopsies in infected patients even if it is possible to identify M1 and M2 macrophages in tissues using proteomic or immunohistochemical approaches. Recently, macrophage polarization was investigated in tissues from patients with diseases characterized by a Th1 or Th2 response. M1 macrophages were defined as those expressing CD68 or CD163 with phosphorylated STAT1 (pSTAT1), and M2 macrophages were defined on the basis of the co-expression of CMAF (macrophage activation factor) with CD68 or CD163 (10). The pSTAT1 and CMAF are preferentially associated with M1 and M2 macrophages, respectively. In contrast, CD163, which was considered by several authors as an M2 specific-marker (11), was unable to discriminate M1 and M2 macrophages within pathological tissues. These findings were confirmed by a recent study in which macrophages were differentiated by granulocyte macrophage-colony stimulating factor (GM-CSF) or macrophage-colony stimulating factor (M-CSF) and secondarily polarized by IFN- γ or IL-4/IL-13; CD163 was unable to discriminate the M1 status from the M2 status (12). The investigation of macrophage activation in infected patients concerns essentially circulating monocytes that are accessible after blood collection and purification from blood, but the situation

regarding their M1/M2 polarization is complex. Using a microarray approach, we showed that M1/M2 polarization, defined by comparison with the IFN- γ and IL-4 signatures of macrophages, was transient in human monocytes, and gene expression data from published reports showed that not even small signatures of polarized macrophages were found in monocytes (13). Hence, the study of activation in tissue macrophages or circulating monocytes suffers from the lack of convenient tools, suggesting that the concept of macrophage polarization is not convenient. Among the recommendations for reporting macrophage activation, the recommendation precising how macrophages are isolated and which marker combinations are used to measure macrophage activation is likely a solution for the investigation of monocytes *ex vivo* (9).

MACROPHAGE POLARIZATION AND MICROBIOTA

The microorganisms present at the surfaces of mucosa mainly consist of commensals that have developed mutualistic relationships with hosts such as human beings. Indeed, during steady-state conditions, the microbiota influences the efficiency of digestion, controls metabolism, and affects the differentiation and functions of intestinal immune cells, including macrophages. This coevolution has been illustrated by numerous reports based on studies on germ-free animals or antibiotic-treated hosts (14–16). It has been established that the intestinal microbiota maintains a tolerant environment that allows the development of M2-like intestinal macrophages. Indeed, the macrophages from lamina propria show down-regulated expression of innate response receptors and inflammatory functions, but they retain phagocytosis and bactericidal activities (17). It is likely that commensals may directly or indirectly shape the polarization status of intestinal macrophages. Hence, *Bacteroides fragilis* and intestinal *Clostridia* are known to stimulate regulatory T cells (Tregs) and polarization toward an M2 phenotype (14). The exopolysaccharide from *Bacillus subtilis* prevents the intestinal disease associated with *Citrobacter rodentium*, and protection is transferred by peritoneal macrophages (18). The probiotic *Clostridium butyricum* promotes the development of IL-10-producing macrophages that prevent inflammatory colitis (19). Some end-products of bacterial anaerobic fermentation, such as short-chain fatty acids (α -butyrate), inhibit the inflammatory response of macrophages via a mechanism based on the inhibition of histone deacetylase (20). In contrast, intestinal commensals such as *Enterococcus faecalis* polarize colon macrophages to an M1 phenotype in a murine model in which macrophages are depleted with clodronate (21). These findings suggest that the diversity of commensal bacteria accounts for the diversity of macrophage responses. Probiotics such as *Lactobacillus* sp. or *Bifidobacterium* sp. may benefit the host (14), but we ignore their effect on macrophage polarization. The strain G-101 of *Lactobacillus brevis* inhibits the inflammatory response of mice treated by trinitrobenzenesulfonic acid. This anti-inflammatory property is related to the ability of the bacteria to prevent the expression of M1 markers and to favor M2 markers, likely via the production of IL-10 (22). For other authors, probiotics have either no effect on the polarization of RAW 264.7 macrophages as a readout (23), or these bacteria promote an activation profile of the M1-like type in THP-1 cells stimulated with lipopolysaccharide (LPS) (24). It is noteworthy that all of these studies are limited

to *in vitro* experiments or animal models, and the extrapolation to human beings must be careful.

If the hypothesis that a breach of intestinal homeostasis is true, the presence of pathogenic bacteria would interfere with the polarization status of intestinal and systemic macrophages. Hence, an M1 profile would be found in patients with acute typhoid fever due to *Salmonella enterica* serovar Typhi, whereas an M2 signature would be observed in convalescent patients. The M2 response does not mean eradication of the pathogen because persistence of the M2 status favors re-infection, relapses, and development of a carrier state (25, 26). On the other hand, there is an increase in M1 and M2 markers in antrum from patients infected with *Helicobacter pylori* and uncomplicated gastritis. The presence of atrophic gastritis is associated with the expression of M1 polarization. It is predictable that shifting macrophage polarization from the M1 to M2 status is protective in chronic *H. pylori* infection. This may be reminiscent of the association of high levels of CCL18, a typical M2 marker, with prolonged survival of patients with gastric carcinoma (26, 27).

Imbalances in gut microbiota have also been associated with systemic diseases such as allergy. Recently, Kim et al. reported the induction of allergen-induced infiltration of inflammatory cells in mice treated with antibiotics. This treatment alters macrophage functions but reorients alveolar macrophages and circulating monocytes toward an M2 phenotype. This latter response is involved in allergic airway inflammation induced by allergens. Antibiotic treatment facilitates fungal overgrowth that exacerbates airway inflammation. The prostaglandin E2 produced by gut fungi is responsible for eosinophil-mediated inflammation and M2 polarization of macrophages (28). If the concept of macrophage polarization is useful for analyzing the host response to intestinal pathogens, there is no clear evidence that it is a convenient tool to measure the response to commensals and probiotics.

MACROPHAGE POLARIZATION AND ACUTE INFECTIOUS DISEASES

As sepsis is a consequence of the systemic inflammatory response to infectious aggression, it was tantalizing to consider sepsis as an M1-associated disease (25). Sepsis can also associate a secondary immunodeficiency in which the polarization of macrophages may be altered, as in LPS tolerance. Indeed, LPS-tolerant macrophages express M2 markers, but not M1 markers, and this phenotype can be reversed by IFN- γ (29). It is thought that the evolution of sepsis is characterized by a transition from an initial M1 response to a secondary M2 response. The interaction of macrophages with pathogens accounts for their initial polarization, and the M1-to-M2 transition should rather involve mechanisms of activation control such as suppressors of cytokine signaling (SOCS) proteins; SOCS1 and SOCS2 are associated with M2 macrophages whereas SOCS3 is overexpressed in M1 cells. A high SOCS1/SOCS3 expression ratio might be a biomarker of M2 cells *in vivo* (30). The fact that M2 bias is associated with the resistance of mice does not account for the poor prognosis of patients who exhibit secondary immune deficiency with an M2 phenotype. Indeed, this latter phase, named immune paralysis, is associated with increased susceptibility to nosocomial infections and late lethality (31). In patients with sepsis, the percentage of monocytes

expressing CD163 and CD206 is increased. The increase in monocytes expressing M2-like markers has been associated with a lower proportion of IFN- γ -producing T cells or with a higher proportion of Tregs in patients with sepsis. Nevertheless, enrichment with M2-type monocytes has no impact on sepsis prognosis (32). In others reports, the expression of CD163 by monocytes is accurate for discriminating patients with inflammatory presentation from those with sepsis (33), suggesting that CD163 may be a biomarker of prognosis and that the expression of CD163 by monocytes is higher in non-survivors than in survivors (34). Soluble forms of M2-type markers such as CD163 and CD206 are also increased in patients with sepsis, and their high levels are associated with poor prognosis in sepsis. Although membrane and soluble forms of CD163 share the ability to be biomarkers of prognosis in sepsis, circulating CD163 reflecting the polarization of monocytes or their activation independently of M1/M2 polarization tends to be ignored (34, 35). The measurement of monocyte activation is a partial reflection of the altered immune functions in tissues from patients with sepsis and does not assess the diversity of stimuli that they encounter from the initial pathological event. It is probably more pertinent to consider the level of monocyte activation and not the bias toward a polarized status as a biomarker.

INTERFERENCE WITH M1 POLARIZATION IN CHRONIC INFECTIOUS DISEASES: Q FEVER

As intracellular bacteria subvert host microbicidal effectors *in vitro*, we proposed that they have evolved specific strategies to interfere with M1 polarization (25). The example of Q fever is informative as we have assessed the concept of macrophage polarization in *in vitro* experiments, animal models, and patients. Q fever is a zoonosis caused by *Coxiella burnetii*, an intracellular bacterium related to *Legionellae* species, and for which the major targets are monocytes and macrophages. The severity of the infectious disease is chronic evolution with a risk of endocarditis or vascular infection (36).

The circulating monocytes exhibit a pro-inflammatory M1-type response, which is consistent with epidemiological data showing bacterial clearance in most infected patients when they are challenged by *C. burnetii* *in vitro*. More surprisingly, monocyte-derived macrophages are polarized toward an atypical M2-type in response to bacterial stimulation. This latter effect is characterized by the release of IL-10, transforming growth factor (TGF)- β , and CCL18 and the expression of the mannose receptor (MR) and of arginase-1, but macrophages also express IL-6 and CXCL8, two molecules that are associated with M1 polarization (37). These differences in monocyte/macrophage activation may account for the unexplained differences in bacterial survival: *C. burnetii* are unable to replicate in monocytes but replicate within macrophages (38). Similar findings were found *in vitro* with *Mycobacterium tuberculosis*, which prevents M1 polarization and activates peroxisome proliferator-activated receptor (PPAR)- γ , which is characteristic of macrophage M2 polarization (25, 39).

Nevertheless, we identified IL-10 as the only cytokine able to induce the replication of *C. burnetii* in monocytes and macrophages, suggesting that IL-10-associated M2 polarization is involved in bacterial replication and tissue persistence. The role of

IL-10 in the pathogenesis of chronic infection is strengthened by the correlation of the amount of IL-10 and the chronic evolution of Q fever with the restoration of the microbicidal competence of monocytes when IL-10 was neutralized (40, 41). The engulfment of apoptotic cells by monocytes and macrophages is associated with an M2 program induced by IL-10 and favors the intracellular replication of *C. burnetii*. In contrast, treatment of these M2 polarized myeloid cells with IFN- γ and the uptake of necrotic cells suggest that the M1 program is sufficient to clear *C. burnetii* (42). The role of IL-10 is demonstrated in transgenic mice that constitutively overexpress IL-10 in the macrophage compartment and exhibit sustained infection, as in chronic Q fever. Macrophages from IL-10-overexpressing mice are unable to clear *C. burnetii* infection and exhibit an M2-type transcriptional program in which arginase, MR and Yim1/2 are increased and inflammatory markers are down-modulated (43). The infection of mice over-expressing IL-10, which mimics tuberculosis reactivation, reveals features of M2 macrophages, as reported above in *C. burnetii* infection of mice (26).

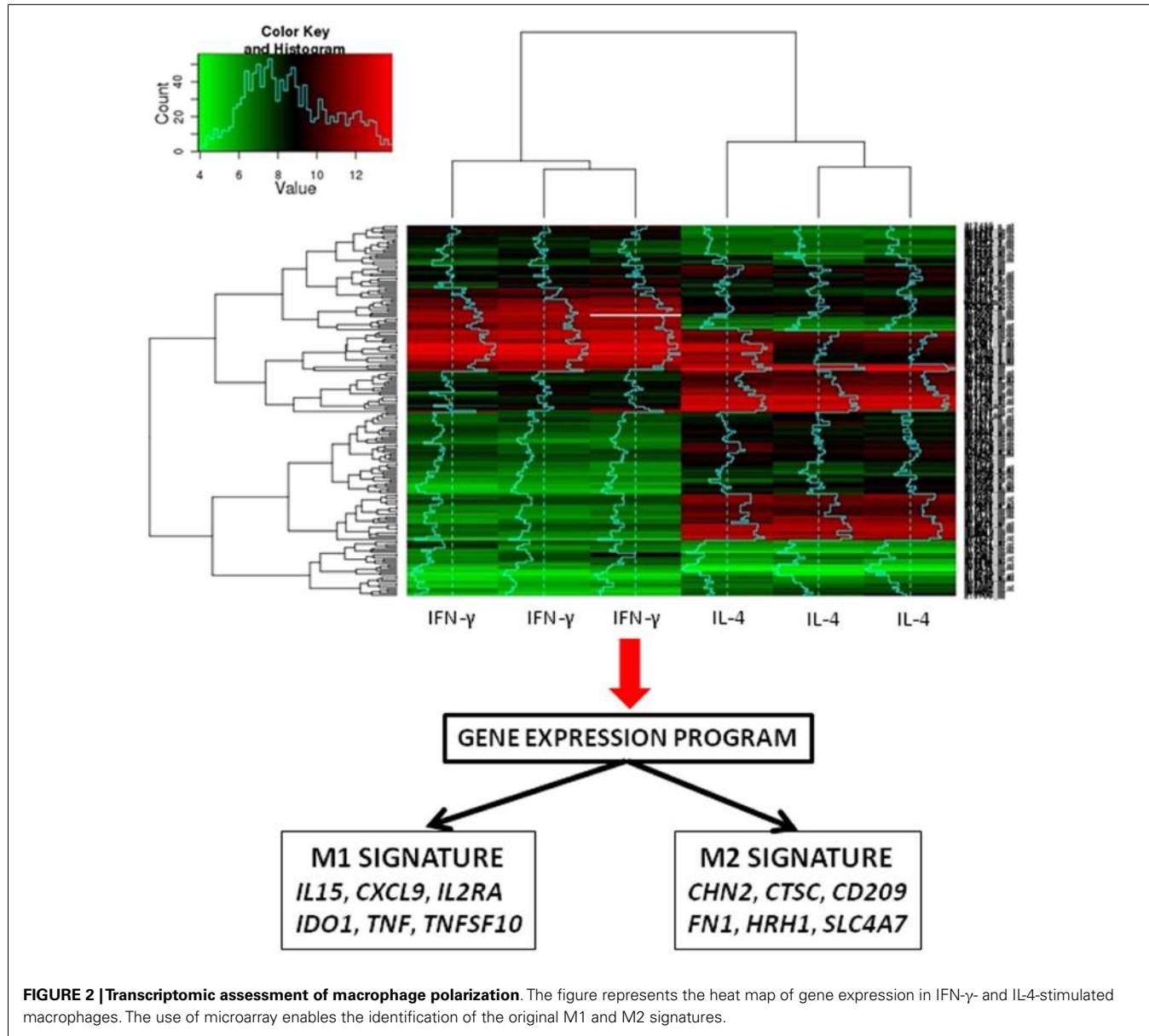
Concomitantly, we found that mice deficient for vanin-1, a membrane-anchored pantetheinase that controls tissue inflammation, are permissive for *C. burnetii* and exhibit an activation program in macrophages that is skewed toward an IL-10-associated M2 phenotype (44). Hence, IL-10-mediated polarization of macrophages is necessary for *C. burnetii* persistence in tissues.

To test the relevance of these findings in patients, we selected M1- and M2-related genes from the microarray analyses of IFN- γ and IL-4-stimulated macrophages (Figure 2). The expression of these genes was not different in patients with acute Q fever and healthy controls. These findings did not support the hypothesis that patients with acute Q fever, who are able to control the infection, should exhibit an M1-type phenotype. The expression of a minority of M1/M2 genes was increased in patients with Q fever endocarditis and who were unable to clear *C. burnetii* and who were expected to exhibit an M2-type phenotype (13). The analysis of the transcriptional profiles of patients with active tuberculosis shows the modulation of M1-related genes, but not that of M2 genes. Similar results were obtained in infants vaccinated with Calmette–Guerin bacillus (26, 45, 46).

In conclusion, the activation program of monocytes from patients with acute and chronic Q fever and tuberculosis cannot be reduced to an M1/M2 dichotomy. We cannot rule out that macrophages in tissues such as endocardium, lungs, or liver are polarized, as suggested by *in vitro* studies and animal models. This is illustrated by the example of pleural macrophages. Tuberculous pleural effusion, an extra-pulmonary form of tuberculosis, is associated with the M1 profile in pleural fluid that is characterized by an increase in M1 macrophages and inflammatory cytokines (47).

M2 POLARIZATION IN CHRONIC INFECTIOUS DISEASES: LEPROSY AND WHIPPLE'S DISEASE

Two infectious diseases, leprosy and Whipple's disease, which share several features such as the tropism for macrophages of *Mycobacterium leprae* and *Tropheryma whipplei*, and the role of the immune response into features of pathogenesis, are associated with M2 polarization (26). The overexpression of IL-10 is found in



lepromatous lesions and likely reflects M2 polarization. The transcriptional analysis of these lesions reveals an enrichment of M2 genes, which is in contrast to what occurs in tuberculoid lesions (48). The expression of CD163 by foamy macrophages in lepromatous lesions but not by macrophages from tuberculoid lesions has been considered strong evidence of M2 polarization in lepromatous leprosy (26). Whether this polarization is a consequence of the production of IL-10 or if it reflects a Th2 response is often ignored.

Whipple's disease is characterized by the presence of macrophages with periodic acid-Schiff inclusions within the lamina propria; these macrophages exhibit some features of macrophages from mycobacterial lesions. As described above for lepromatous leprosy, there is converging evidence that Th2 polarization of the immune response is critical for the pathophysiology

of Whipple's disease. An M2 macrophage signature was observed in duodenal biopsies from one patient with intestinal Whipple's disease (49). Moos et al. reported the increased expression of CD163 on duodenal macrophages and circulating monocytes, and this finding was strengthened by an increase in IL-10 and a decrease in inducible NO synthase expression in these cells, suggesting a functional polarization toward an M2 profile (50, 51). The conclusion that IL-10 may be critical for *T. whipplei* pathogenicity was not confirmed by *in vitro* studies, in which we found an increase in IL-1 β , IL-16, and type I IFN production, but not in IL-10 (52, 53). It is likely that type I IFN prevents the IFN- γ -protective effect, as reported for mycobacterial infections (54). This finding underlines the caution that must be taken regarding conclusions about polarization when based on a limited number of markers.

COMPLEXITY OF MACROPHAGE ACTIVATION IN INFECTIOUS DISEASES

The analysis of infectious disease literature (see above) reveals that modulation of monocyte/macrophage activation is frequently observed, whereas clear-cut M1/M2 polarization is rather a rare event. This observation is related to the history of infected patients. Indeed, the stage of the disease is a critical parameter. For instance, the activation of monocytes/macrophages is different in patients with initial sepsis and those with delayed complications. In addition, numerous patients are distributed between two extreme situations: between patients with acute Q fever and those with Q fever endocarditis, there is a population of patients with valvular disease and Q fever associated with a risk of chronic evolution, and these patients overproduce IL-10 in a sustained manner. However, the measurement of IL-10 at a given time of Q fever evolution is not sufficient to assess the prognosis of patients with Q fever (55). In patients with tuberculosis, the transcriptional signature is transient at the beginning of the disease and is finished 1 year later (45). Clearly, the analysis of the transcriptional pattern of patients with tuberculosis will be dramatically different according to the time of the inclusion, and such an analysis is often difficult to assess at the beginning of the disease. These different clinical and experimental situations drove us to propose a model of monocyte/macrophage activation in which the kinetic component of the disease was integrated. This model is based on the comparison of the transcriptomes from activated monocytes and macrophages. The responses of monocytes to polarizing ligands are characterized by two early and late phases of monocyte activation. The hallmarks of the M1/M2 status are found in the early phase but are absent from the late phase of activation. We selected a series of early and late genes and measured their expression in monocytes from patients with acute and chronic Q fever. Most of the early genes were found to be up-regulated in monocytes from patients with acute Q fever, two of them, NLRC5 and RTP4, were up-regulated by IFN- γ , suggesting that IFN- γ plays a role in the host response during acute Q fever. In contrast, the late genes were up-regulated in chronic Q fever, and some early genes were down-modulated. There was a specific association between late genes such as ALOX15, CLEC4F, CCL13, and CCL23 and chronic Q fever (13). It is noteworthy that some of them have been associated with the M2 program, which is a result that might lead to incorrect conclusions about monocyte activation. We are unable to assign a function to the modulated genes.

In conclusion, the analysis of macrophage polarization through clinical situations revealed that the mechanisms underlying the activation of monocytes and macrophages are distinct. This point is critical because most clinical investigations are based on monocytes and the conclusions are extrapolated on data obtained with macrophages. The second observation is the importance of activation kinetics in the assessment of infected patients who are at different stages of disease history. Therefore, early and late genes may be alternative biomarkers for analyzing infectious and inflammatory diseases. The lessons from the investigation of infected patients do not invalidate the functional model of M1/M2 polarization. They revealed the difficulty to relate a signature and a function. In addition, the finding of a role for these genes in the activation of macrophages will be useful to understand the

complexity of macrophage physiology in normal and pathological conditions.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 24 July 2014; accepted: 25 September 2014; published online: 10 October 2014.

*Citation: Ka MB, Daumas A, Textoris J and Mege J-L (2014) Phenotypic diversity and emerging new tools to study macrophage activation in bacterial infectious diseases. *Front. Immunol.* **5**:500. doi: 10.3389/fimmu.2014.00500*

This article was submitted to Inflammation, a section of the journal Frontiers in Immunology.

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The role of myeloid cell activation and arginine metabolism in the pathogenesis of virus-induced diseases

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When an antiviral immune response is generated, a balance must be reached between two opposing pathways: the production of proinflammatory and cytotoxic effectors that drive a robust antiviral immune response to control the infection and regulators that function to limit or blunt an excessive immune response to minimize immune-mediated pathology and repair tissue damage. Myeloid cells, including monocytes and macrophages, play an important role in this balance, particularly through the activities of the arginine-hydrolyzing enzymes nitric oxide synthase 2 (Nos2; iNOS) and arginase 1 (Arg1). Nitric oxide (NO) production by iNOS is an important proinflammatory mediator, whereas Arg1-expressing macrophages contribute to the resolution of inflammation and wound repair. In the context of viral infections, expression of these enzymes can result in a variety of outcomes for the host. NO has direct antiviral properties against some viruses, whereas during other virus infections NO can mediate immunopathology and/or inhibit the antiviral immune response to promote chronic infection. Arg1 activity not only has important wound healing functions but can also inhibit the antiviral immune response during some viral infections. Thus, depending on the specific virus and the tissue(s) involved, the activity of both of these arginine-hydrolyzing enzymes can either exacerbate or limit the severity of virus-induced disease. In this review, we will discuss a variety of viral infections, including HIV, SARS-CoV, LCMV, HCV, RSV, and others, where myeloid cells influence the control and clearance of the virus from the host, as well as the severity and resolution of tissue damage, via the activities of iNOS and/or Arg1. Clearly, monocyte/macrophage activation and arginine metabolism will continue to be important areas of investigation in the context of viral infections.

Keywords: arginase, iNOS, viral pathogenicity, macrophages, immunity, cellular

INTRODUCTION

Tissue -resident and monocyte-derived macrophages are innate immune cells that play a key role in normal tissue homeostasis, presentation of foreign and self antigens following infection or injury, pathogen clearance, and resolution of inflammation and wound healing. Depending on the microenvironment, macrophages can be programmed to adopt a variety of proinflammatory, regulatory, resolving, and immunosuppressive activation phenotypes, particularly *in vivo*. These activation states exist as a complex continuum of overlapping phenotypes; however, macrophage subsets with distinct functions have been defined (1). Macrophages are considered M1-polarized when stimulated by IFN- γ or Toll-like receptor (TLR) ligands, such as lipopolysaccharide (LPS), to express inducible nitric oxide synthase (iNOS; Nos2) and produce nitric oxide (NO). NOS enzymes metabolize L-arginine to citrulline and NO. NO is a short-lived gaseous messenger with physiological and pathological effects. Nanomolar concentrations of NO, generated by endothelial NOS and neuronal NOS, are important for maintaining homeostasis, regulating vasodilation, and for the aggregation, recruitment, and adhesion of platelets to the vascular endothelium. iNOS generates micromolar levels of NO that modulates

various pathophysiological processes and is important for killing intracellular pathogens (2).

In contrast, M2-polarized macrophages result following stimulation of cells with a variety of stimuli, including type 2 cytokines such as interleukin (IL)-4 or IL-13. M2-polarized macrophages express a distinct L-arginine-metabolizing enzyme, arginase 1 (Arg1), which hydrolyzes L-arginine to L-ornithine and urea. L-Ornithine can be further metabolized to polyamines, which participate in a variety of fundamental cellular functions (e.g., proliferation, cell membrane transport), and L-proline, which is an essential component of collagen. In addition to playing important roles in defense against extracellular parasites and tissue repair, Arg1 expression and activity in myeloid cells have emerged as a major regulator of innate and adaptive immune responses (3). Other M2-like suppressive or anti-inflammatory macrophages include myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs). MDSCs are considered to be an immature population of myeloid cells, including both monocyte-like (GR-1/Ly-6C $^{+}$) and neutrophil-like (GR-1/Ly-6G $^{+}$) populations, associated with tumors or infections that suppress proinflammatory responses (4, 5). Depending on the context, MDSCs have been shown to mediate their suppressive activity via NO- and/or

Arg1-dependent mechanisms. Importantly, macrophages are not permanently programed, but are considered “plastic” – that is, macrophages have been shown to change activation phenotypes depending on the local environment.

Although the M1/iNOS and M2/Arg1 division is generally appropriate, Arg1 can be induced in M1-like macrophages under certain conditions. Thus, due to the spectrum of activation states for macrophages, a framework for macrophage-activation nomenclature was recently suggested (6). In an attempt to avoid confusion in this review, we focused on the specific effects of the L-arginine metabolizing enzymes iNOS or Arg1 on the pathogenesis of viral infections, noting other activation markers where appropriate.

Increasing evidence suggests that myeloid cell programing, iNOS, and Arg1 contribute to the pathogenesis of numerous virus infections, suggesting that therapies that target these cells and pathways may be beneficial for the treatment of some virus diseases. In this review, we highlight recent studies of viral infections where myeloid cell polarization – resulting in expression of iNOS or Arg1 – contribute to viral control or the development of chronic virus infection and mediate the resolution of tissue damage or cause immunopathology.

NO PRODUCTION CAN BE BENEFICIAL DURING VIRUS INFECTION

NO has antimicrobial activity against a number of bacteria, parasites, and fungi (7, 8). Additionally, NO has been shown to have direct antiviral effects *in vitro* and/or *in vivo* against several viruses, including DNA viruses such as herpes simplex virus type-1 (HSV-1), ectromelia virus (EV), and vaccinia virus (VV) (9, 10), as well as some RNA viruses such as vesicular stomatitis virus (VSV) (11), Japanese encephalitis virus (JEV) (12), dengue virus (DENV) (13), and coxsackievirus (Table 1) (14–17). There are several advantages of using NO as an antiviral agent. For instance, unlike complement and antibody, NO can readily pass through cellular membranes into neighboring cells as well as some viruses. Additionally, NO is likely to act on a variety of both viral and virally exploited cellular targets, inhibiting viral replication as well as limiting the capacity of viruses to develop resistance. Lastly, the effect of NO is independent of immune recognition of the infected cell, in contrast to that of antiviral lymphocytes, which could be important in virus-infected cells where expression of MHC class I molecules may be downregulated and in some virally infected tissues such as the brain where expression of MHC class I and II molecules is limited.

In initial studies *in vitro*, inhibition of EV, VV, and HSV-1 replication in mouse RAW 264.7 macrophages and in primary mouse macrophages following IFN- γ treatment was shown to be largely dependent on NO production (9, 10). Additionally, pharmacologic inhibition of NOS or genetic deletion of *Nos2* resulted in increased viral titers and mortality following EV infection in mice (9, 18). Moreover, NO affects several events in the late stages of the life cycle of VV, including viral DNA replication, viral protein synthesis, and virion maturation *in vitro* (32). These studies provided some of the first evidence that macrophage-produced NO has direct antiviral effects.

In addition to inhibiting HSV-1 replication *in vitro*, macrophage-derived NO has been shown to have anti-HSV properties *in vivo*. In a mouse model of HSV-1-mediated corneal

disease, iNOS was highly induced in the trigeminal ganglion (TG) of HSV-1-infected mice, and its expression was markedly reduced in mice depleted of macrophages (22). Depletion of macrophages prior to HSV-1 infection resulted in markedly reduced iNOS expression and higher viral loads in the TG of infected mice (22, 23), suggesting that macrophages were the main source of iNOS expression in the affected tissues following HSV-1 infection and that NO had important anti-HSV-1 properties *in vivo*. Consistent with these data, inhibition of NOS activity resulted in increased viral loads in the TG (22). Additional studies showed that F4/80 $^{+}$ GR-1 $^{+}$ inflammatory monocytes were recruited to the eye via an IFN- α -driven CCL2 gradient and restricted HSV-1 replication in that tissue via NO production (24). It was further shown that NO production by F4/80 $^{+}$ macrophages in the brains of HSV-1-infected mice blocked viral replication in a partially TLR2- and TLR9-dependent mechanism (25). Finally, following footpad inoculation, HSV-1-infected *Nos2* $^{-/-}$ mice displayed a delayed clearance of virus from the dorsal root ganglia (DRG) and exhibited an increase in the frequency of virus reactivation in DRG (26).

The reactivity of NO and its higher oxides and nitrosothiol products (84) makes it likely that a variety of molecular targets are involved in its antiviral action. It has been shown that NO can inhibit ribonucleotide reductase (85, 86), a rate-limiting enzyme in DNA synthesis, and NO can lead to the deamination of mammalian and bacterial DNA (87, 88), which may be important antiviral mechanisms. Indeed, HSV-1 encodes its own ribonucleotide reductase and although it is not required for HSV-1 replication *in vitro*, it is necessary under conditions where the intracellular pool of deoxynucleotides is limited (89, 90). Thus, by inactivating this cellular and/or viral enzyme, NO may halt virus replication by directly inhibiting viral DNA synthesis.

In addition to HSV-1, treatment of primary human cells with an NO donor following infection with human cytomegalovirus (HCMV), a beta-herpesvirus, resulted in a significant reduction of early and late viral protein expression (28). Consistent with these *in vitro* data, *Nos2* $^{-/-}$ mice (129/Sv/Ev x C57BL/6 F1) exhibited increased viral titers and mortality following infection with murine CMV (MCMV; Smith VR194 strain) (29).

Nitric oxide has also been shown to have antiviral properties on a chicken herpesvirus, Marek’s disease virus (MDV), which can cause T cell lymphomas in chickens: Addition of NO-generating compounds inhibited viral replication in chicken fibroblasts (33). Additionally, the treatment of chickens with an inhibitor of iNOS increased the level of MDV replication *in vivo* (34). Further studies demonstrated that NO production was limited to chickens that were genetically resistant to tumor development following MDV infection or to chickens that were vaccinated before being inoculated with MDV (35). Thus, NO appeared to be produced in both types of resistance to tumor development in Marek’s disease, either acquired after vaccination or genetic. Together, these findings suggest a role of NO in the protective immune mechanisms against Marek’s disease, possibly through its activity on viral replication.

Finally, studies with HBV, a hepadnavirus associated with acute and chronic hepatitis, demonstrated that HBV replicated to higher levels in the livers of HBV-transgenic *Nos2* $^{-/-}$ mice than control transgenic mice, and transgenic *Nos2* $^{-/-}$ mice had increased

Table 1 | Differential roles for the L-arginine-hydrolyzing enzymes iNOS and Arg1 in virus-induced diseases.

	iNOS/NO	Reference	Arg1	Reference
Beneficial for host	Ectromelia virus (EV)	(9, 10, 18)	Tissue repair/regeneration	Respiratory syncytial virus (RSV) (19–21)
	Herpes simplex virus-1 (HSV-1)	(9, 10, 22–26)		Influenza (27)
	Cytomegalovirus (CMV) ^a	(28, 29)		Coxsackievirus B3 (CVB3) ^d (30, 31)
	Vaccinia virus (VV)	(9, 10, 32)		
	Marek's disease virus (MDV) ^b	(33–35)		
	Hepatitis B virus (HBV)	(36)		
	Vesicular stomatitis virus (VSV)	(11)		
	Japanese encephalitis virus (JEV)	(12)		
	Dengue virus (DENV)	(13, 37–41)		
	West Nile virus (WNV)	(42)		
	Sindbis virus (SINV)	(43)		
	Reovirus (T3A strain)	(44)		
	Coxsackievirus B3 and B4 (CVB3, CVB4)	(14–17, 91)		
Detrimental to host	Influenza	(45–55)	Immunopathologic	SARS-CoV (56, 57)
	HSV-1	(58–60)		Murine γ-herpesvirus-68 (MHV-68) ^e (61–63)
	Feline immunodeficiency virus	(64, 65)		
Promotes viral persistence	Cytomegalovirus (CMV) ^c	(66)	Promotes viral persistence	LCMV clone 13 (67)
	rJ2.2 strain of mouse hepatitis virus (neurotropic coronavirus)	(68)		Ross River virus (RRV), chikungunya virus (CHIKV) (69)
	Lymphocytic choriomeningitis virus (LCMV) clone 13	(70)		Marek's disease virus (MDV) ^f (35)
				Hepatitis C virus (HCV) (71)
				Human immunodeficiency virus (HIV) (72–77)
				Hepatitis B virus (HBV) (78–80)
				Influenza (81, 82)
				Human papillomavirus (HPV) ^g (83)

^aHCMV, *in vitro*; MCMV, *in 129/Sv/Ev x C57BL/6 F1 mice*.^bFor resistant chickens.^cMCMV, *in BALB/c mice*.^dIn female BALB/c mice.^eIn Ifngr^{-/-} mice.^fFor susceptible chickens.^gMouse model of HPV-induced cancer.

liver disease (36). It was further demonstrated that NO production by mononuclear cells, most likely macrophages, in the liver mediated most of the antiviral activity resulting from IFN-γ production by virus-specific T cells (36), suggesting an antiviral role for macrophage-derived NO following HBV infection in mice.

In addition to DNA viruses, macrophage-derived NO also exerts antiviral effects against a number of RNA viruses. Inhibition of JEV, a mosquito-transmitted flavivirus that causes encephalitis in humans, in IFN-γ-activated RAW 264.7 macrophages *in vitro*

correlated with NO production, and IFN-γ-activated RAW 264.7 macrophage-mediated inhibition of JEV replication in murine neuroblastoma N18 cells was NO-dependent (12). Moreover, inhibition of NOS activity led to increased mortality in JEV-infected mice (12).

In terms of its mechanism of action, NO was found to inhibit JEV RNA synthesis, viral protein accumulation, and virus release from infected cells *in vitro* (12). These data suggest that NO may be directly or indirectly inhibiting viral enzymes and/or other

cellular components required for viral replication, and this may subsequently block viral protein synthesis. Additionally, NO may interfere with the release and/or maturation of virions.

Monocyte/macrophage-derived NO may also block replication of DENV, another mosquito-transmitted flavivirus. Infection with DENV resulted in increased levels of NO in patients with dengue fever, the classic form of the disease (37). Additionally, iNOS expression was induced in CD14⁺ monocytes from a subset of acutely infected individuals (13). It was further shown that *ex vivo* infection of human monocytes with DENV-1 resulted in increased iNOS expression, and inhibition of iNOS activity led to increased DENV antigen detection in these cells (13). Moreover, treatment of C6/36 mosquito cells with an NO donor resulted in reduced DENV-positive cells (13). These data suggest that DENV replication is susceptible to NO-mediated inhibition. Consistent with this, *Nos2*^{-/-} mice were shown to be more susceptible to DENV infection, resulting in more severe disease and increased lethality in mouse models of DENV-2 and DENV-3 infection (38, 39). It was further demonstrated that, following DENV infection *in vivo*, IL-12 and IL-18 induced IFN- γ production, resulting in iNOS expression and NO production, which contributed to viral control (38, 39).

In addition to monocyte/macrophage-derived NO, a recent study demonstrated that platelets isolated from patients with dengue fever had increased L-arginine transport and increased NO production compared to platelets from healthy controls (40). However, NO has anti-aggregatory properties, and Mendes-Ribeiro et al. (40) found that dengue patients exhibited decreased collagen-induced platelet aggregation, consistent with the vascular leak and hemorrhagic manifestations of dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), thus establishing an association between reduced platelet aggregation, enhancement of the L-arginine–NO pathway, and DHF/DSS (41).

In contrast, Getts and colleagues showed that experimentally abrogating NO activity during West Nile virus (WNV) encephalitis, a related flavivirus, in NO-competent mice at a specific, relatively late time point prolonged survival of infected mice, while pharmacological inactivation throughout disease did not (42). Combined, these data suggest that although during DENV infection IFN- γ -induced NO production has a role in antiviral defense, it is likely that dysregulation of the IL-12/18–IFN- γ –NO axis leads to immune-mediated damage in certain flavivirus infections. Along these lines, it has also been shown that treatment of mice with a NOS inhibitor increased mortality rates following Sindbis virus (SINV) infection (43), suggesting a protective role for NO during this particular CNS infection. However, SINV replication in the brain was unaffected. Furthermore, treatment of neuroblastoma cells with NO donors had little effect on SINV replication but increased cell viability (43). These data suggest that NO protects mice from fatal SINV-induced encephalitis by a distinct mechanism that does not directly involve the inhibition of virus growth but rather may enhance survival of the infected neuron until the immune response can control virus replication.

Nitric oxide also plays an antiviral role during CNS infection with reovirus. Infection of neonatal mice with the prototypic neurotropic reovirus strain (T3A) induced iNOS expression in brain areas demonstrating reovirus antigen expression and associated

virus-induced injury (44). Reovirus also induced iNOS expression following *in vitro* infection of primary neuronal and glial cultures. Reovirus was shown to infect a subpopulation of microglial cells *in vitro* (44), suggesting that direct virus interaction may induce iNOS in this specialized population of macrophages. Treatment of neuronal cultures with an NO donor inhibited viral replication whereas a NOS inhibitor increased viral growth (44), suggesting iNOS has the potential to exert antiviral activity *in vivo*.

Finally, coxsackievirus infection has been shown to induce expression of iNOS in macrophages infiltrating the hearts of infected mice (17). Treatment of WT mice with a NOS inhibitor and infection of *Nos2*^{-/-} mice resulted in more severe coxsackievirus-induced pancreatitis and myocarditis, elevated viral loads in tissues, and decreased survival compared to WT mice following coxsackievirus B3 (CVB3) infection (14, 15, 17). Similarly, *Nos2*^{-/-} mice infected with coxsackievirus B4 exhibited decreased survival and delayed viral clearance compared to WT mice (16). These data suggest an antiviral effect of NO against coxsackievirus infection. Consistent with this, it was demonstrated that NO inhibits the 2A and 3C proteinases of CVB3 *in vitro* (91). Additionally, CVB3-infected outbred mice showed significantly reduced signs of myocarditis after treatment with NO donors (91).

NO PRODUCTION CAN BE DETRIMENTAL TO HOST MYELOID CELL PRODUCTION OF NO CAN BE IMMUNOPATHOLOGIC DURING VIRUS INFECTIONS

Despite its protective capacity during some viral infections, NO can also contribute to immunopathology. The pathological effects of NO are likely due, at least in part, to oxidative damage caused by the interaction of NO with oxygen radicals such as the superoxide anion radical (O_2^-) and hydrogen peroxide (H_2O_2).

For example, although addition of an NO donor to virus-infected MDCK cells reduced influenza A and B viral burden *in vitro* (45), treatment of mice with inhaled NO (iNO) did not decrease the viral load of influenza A (mouse-adapted H1N1 strain)-infected mice; in fact, prophylactic treatment with iNO resulted in enhanced weight loss and decreased survival following infection (46), suggesting a pathogenic role for NO. Consistent with this, chickens, which show a high level of mortality and associated pathology following avian influenza infection, had higher levels of iNOS expression in the lungs compared with H5N1 influenza-infected ducks, which show relatively minor symptoms following influenza infection (47). Additionally, Akaike and colleagues (48) found evidence of the production of peroxynitrite, which is generated through the reaction of NO and O_2^- , in the lungs of influenza A (mouse-adapted H2N2 strain)-infected mice. Moreover, inhibition of NOS resulted in enhanced survival and decreased pneumonia, but not decreased viral loads, in influenza-infected mice (48, 49), suggesting that NO was contributing to pathogenesis rather than having direct antiviral effects. *Nos2*^{-/-} mice also survived a lethal dose of influenza A virus (PR/8/34 strain) infection with little histopathologic evidence of pneumonia; however, in these studies no infectious virus was detected in *Nos2*^{-/-} mice at day 6 after infection (49). The enhanced viral control in *Nos2*^{-/-} mice was shown to require the activity of IFN- γ (51), with *Nos2*^{-/-} mice also producing increased virus-specific IgG2a antibody titers (50). Additionally, genetic deletion

of *Nos2* or pharmacologic inhibition of NOS enhanced survival of mice inoculated with the highly pathogenic (non-mouse-adapted) 1918 influenza virus strain, although mice exhibited similar viral loads to control mice in lung tissue at the peak of viral replication (51). Influenza infection *in vitro* was shown to induce apoptosis, and a reduction in influenza-mediated apoptosis was noted in cells treated with a NOS inhibitor (52). Similarly, fewer apoptotic cells were found in the lungs of influenza-infected *Nos2*^{-/-} mice, suggesting that NO mediates cell death following influenza infection (52). The cellular source of iNOS/NO following influenza infection in mice was shown to be CCR2⁺ inflammatory monocytes that accumulate in the lungs: CCR2^{-/-} mice survived a lethal challenge of influenza infection (PR/8/34 strain) and had significantly reduced accumulation of iNOS-expressing macrophages in the lung, with no associated increase in viral titers or dissemination (53).

It was also recently shown that a subset of monocyte-derived dendritic cells (DCs), described as TNF- α /iNOS-producing DCs (tipDCs), accumulate in greater numbers during the course of lethal versus sublethal influenza infections, suggesting a pathogenic role for this subpopulation of myeloid cells (54). Interestingly, though, Aldridge et al. (54) found that the tipDCs also stimulated a local, protective CD8⁺ T cell response in the virus-infected respiratory tract, indicating both protective and pathogenic roles for these cells in influenza infection. It was further shown that partially compromising tipDC recruitment via treatment with pioglitazone, a synthetic agonist of the peroxisome proliferator-activated receptor- γ (PPAR- γ), was protective against lethal influenza challenge (54). Pioglitazone treatment led to a reduction in the levels of CCL2 (MCP-1) and MCP-3 in the BAL fluid of influenza-infected mice (54). Pioglitazone has also been shown to reduce the production of a wide range of proinflammatory molecules, including iNOS (55), providing further evidence for the importance of NO production by monocyte-derived cells in the pathogenesis of influenza infection.

Pharmacologic inhibition of NOS using L-NMMA also decreased pneumonitis and increased survival following intranasal infection of CBA/J mice with HSV-1, despite a 17-fold increase in viral titers in the lung at day 3 after inoculation (58). In contrast, treatment of BALB/c mice with a different NOS inhibitor [aminoguanidine (AG), administered intranasally] resulted in enhanced pneumonitis, viral titers, and mortality following infection with a different strain of HSV-1 (59). Thus, the precise role of NO in HSV-1 pneumonitis remains to be determined. NO and other ROS/RNS were also shown to be pathogenic in the brains of mice with herpes encephalitis: iNOS was induced in CD11b⁺ resident microglia following intranasal infection with HSV-1, and oxidative and nitrative damage was found in the brains of infected animals (60).

A common neurological complication of HIV infection in the developed world is sensory neuronal injury accompanied by inflammation, which is clinically manifested as disabling pain and gait instability. Feline immunodeficiency virus (FIV) infection of cats, which causes similar neuroinflammation together with immunosuppression in cats, resulted in induction of iNOS and STAT-1, which were predominantly produced by macrophages, in DRG (64). Additionally, inhibition of NOS resulted in reduced

nitrotyrosine and prevented neuronal injury in FIV-infected DRG cultures *in vitro* (64). These data suggest that lentivirus infection contributes to axonal and neuronal injury through a mechanism involving M1 macrophage immune activation mediated by STAT-1 and iNOS activation. In addition to these studies, infection of mice with the retrovirus LP-BM5, which causes profound immunodeficiency, induces CD11b⁺GR-1⁺Ly-6C⁺ MDSC-like cells that inhibit both T- and B-cell responses in an iNOS/NO-dependent but arginase-independent fashion (65). This study identified an important – and only recently appreciated – role for iNOS-expressing myeloid cell-mediated suppression of B cell responses in retrovirus infection.

MYELOID CELL PRODUCTION OF NO CAN INHIBIT VIRAL CLEARANCE

The oxidative effects of NO have also been shown to inhibit immune cells, particularly T cells. This phenomenon has been appreciated for a number of years in the context of tumors (92), where myeloid suppressor cells can inhibit the anti-tumor T cell response via the effects of NO in addition to other mechanisms (2, 4). In a similar manner, it has been shown that NO can inhibit the antiviral immune response.

MCMV clearance from BALB/c mice is predominantly CD8⁺ T cell-mediated. A recent report showed that MCMV infection in BALB/c mice induced CD11b⁺Ly-6C^{hi} inflammatory monocyte recruitment from the bone marrow to infected tissues that was dependent on CCR2 signaling (66). This recruitment was shown to inhibit antigen-specific CD8⁺ T cell activation, expansion, and cytotoxic activity via NO production, thus facilitating viral persistence (66).

In a similar fashion, NO may contribute to a defective immune response following infection of mice with an attenuated neurotropic coronavirus (rJ2.2 strain of mouse hepatitis virus). rJ2.2-infected WT mice exhibited mild acute encephalitis, followed by a non-lethal, chronic demyelinating disease (68). In marked contrast, rJ2.2 infection of mice that transgenically express CCL2 in the brain (CCL2 Tg) ineffectively cleared virus and rapidly succumbed to the infection (68). CCL2 Tg mice mounted a dysregulated immune response, characterized by increased accumulation of iNOS-expressing macrophages and microglia as well as regulatory T cells, but decreased Arg1 expression (68). These data suggest that persistent CCL2 overexpression establishes and sustains an immunological milieu that may predispose mice to a defective immune response to a typically minimally virulent virus.

ARGINASE ACTIVITY CAN BE BENEFICIAL FOR TISSUE REPAIR FOLLOWING VIRUS INFECTION

Arginase activity is important for wound healing and tissue regeneration through the production of polyamines and proline (2). In the context of some viral infections, arginase activity and M2 macrophage activation have been shown to be beneficial for tissue repair following virus-induced damage. For instance, resolution of severe respiratory syncytial virus (RSV)-induced bronchiolitis in mice is mediated by M2 macrophages that counteract cyclooxygenase (COX)-2-induced lung pathology (19, 20). Arg1 was induced in the lungs of RSV-infected mice, and its induction was shown to be IL-4R α -dependent (19). Additionally, WT macrophages adoptively transferred into RSV-infected IL-4R α ^{-/-} mice restored the

M2 phenotype in the lungs and decreased lung pathology (19). It was further shown that the lipoxigenase pathway was important for M2 macrophage activation and lung resolution following RSV infection (20). Most recently it was demonstrated that treating mice with agents that sustain Arg1 expression (e.g., IL-4/anti-IL-4 immune complexes) limited RSV-induced lung pathology (21).

Consistent with a pathogenic role for iNOS/NO following influenza infection (described above), it was recently shown that the presence of airway bacteria polarize alveolar macrophages into a M2 phenotype, thus limiting influenza-mediated lethal lung inflammation. Wang and colleagues (27) demonstrated that priming with *Staphylococcus aureus*, which commonly colonizes the upper respiratory mucosa, attenuated influenza-mediated lung injury via TLR2 signaling that recruited peripheral CCR2⁺CD11b⁺ monocytes into the alveoli (27). These monocytes polarized alveolar macrophages into a M2 phenotype characterized by high Arg1 as well as Ym1, FIZZ1, and IL-10 expression (27). It was further shown that *S. aureus*-primed M2 alveolar macrophages inhibited inflammatory cell recruitment to the lung, including neutrophils, NK cells, and CD8 T cells (27). *S. aureus*-primed M2 alveolar macrophages also expressed higher levels of the inhibitory ligand PD-L1 (27), suggesting that expression of a combination of anti-inflammatory cytokines and inhibitory ligands could be the mechanisms by which *S. aureus*-primed M2 alveolar macrophages limit influenza-mediated lung inflammation.

As discussed above, coxsackievirus B3 (CVB3) infection causes myocarditis in human beings as well as in male BALB/c mice. Although female mice do not develop severe myocarditis, both male and female mice have comparable numbers of infiltrating macrophages and viral titers in the heart following CVB3 infection (30). The macrophages infiltrating the heart in male mice were skewed toward a M1 phenotype characterized by high expression of iNOS (17) as well as M1-associated cytokines such as IFN- γ and IL-12 (30). Additionally, inhibition of NOS resulted in increased viral titers and higher mortality in CVB3-infected mice (17), consistent with an antiviral role for NO during CVB3 infection (see above). However, in contrast to male mice, the heart-infiltrating macrophages in female mice were skewed toward a M2 phenotype characterized by high expression of Arg1 as well as IL-4 and IL-10 (30). Moreover, adoptive transfer of *ex vivo*-programmed M1 macrophages significantly increased myocarditis in both male and female mice. Strikingly, transfer of M2-programmed macrophages into susceptible male mice alleviated myocardial inflammation by modulating the local cytokine profile from a M1 to M2 phenotype and promoting peripheral regulatory T cell (Treg) differentiation (30). Using different variants of CVB3, one that caused myocarditis in C57BL/6 mice and one that did not, it was additionally shown that the myocarditic variant induced a M1 macrophage phenotype (31). In contrast, the amyocarditic variant induced a M2 macrophage phenotype, which was also associated with the activation of NKT cells that promoted a Treg response (31). The ability of NKT cells to suppress myocarditis was shown by adoptive transfer of purified NKT cells into NKT knockout ($J\alpha 18$ knockout) mice infected with the myocarditic CVB3 variant, which inhibited cardiac inflammation and increased Treg response (31). Cardiac virus titers were equivalent in all mouse strains indicating that

NKT cells did not participate in control of virus infection (31). Thus, although NO appears to have antiviral properties against CVB3, these data indicate an important role for Arg1-expressing M2 macrophages in controlling CVB3-induced myocarditis.

ARGINASE ACTIVITY CAN PROMOTE VIRAL PERSISTENCE AND/OR EXACERBATED IMMUNOPATHOLOGY

ARGINASE ACTIVITY CAN INHIBIT VIRAL CLEARANCE

As a consequence of their co-evolution with their hosts, viruses have developed numerous strategies to evade the host immune system and ensure their own replication and survival. Recent studies have identified a new evasion strategy for viruses: exploitation of the host's anti-inflammatory, wound repair response to promote chronic infection.

Two strains of LCMV – Armstrong (Arm) and clone 13 (C13) – have been studied for decades as models for acute and chronic infections (93). Infection of mice with the Arm strain leads to a robust CD8⁺ T cell response that rapidly clears the virus (94), whereas infection with C13 results in T cells with impaired functionality, enabling the virus to persist (95). It was recently demonstrated that C13 infection led to an enhanced and sustained expansion of cells that resembled MDSCs (70). These suppressive myeloid cells inhibited T cell proliferation *ex vivo* via an iNOS/NO-dependent but Arg1-independent mechanism. Another study, however, found that Arg1-expressing immunoregulatory antigen presenting cells induced during C13 infection suppressed T cell responses (67). Most recently, it was demonstrated that T cell responses were improved – resulting in clearance of the normally chronic C13 infection – when either myeloid cells or T cells lacked IL-10 production (96). Overall, these data demonstrate the importance of iNOS/Arg1-expressing myeloid cells in viral persistence.

Similar to LCMV C13 infection, it was recently demonstrated that infection of mice with the arthritogenic alphaviruses Ross River virus (RRV) and chikungunya virus (CHIKV) resulted in the induction of Arg1 in macrophages in the infected and inflamed musculoskeletal tissues (69). It was further shown that genetic deletion of myeloid cell Arg1 resulted in enhanced viral control in inflamed muscle tissue and reduced tissue pathology following RRV infection in mice (69), suggesting an important role for Arg1-expressing macrophages in the persistence of these chronic viruses.

Infection of mice with Theiler's murine encephalomyelitis virus (TMEV) results in persistent virus infection in the CNS, which contributes to the development of a demyelinating disease that has similarities with multiple sclerosis. Bowen and Olson (97) showed that CD11b⁺Ly-6C⁺ cells infiltrated the CNS following infection and were the dominant cell type during the innate immune response. Depletion of the CD11b⁺Ly-6C⁺ cells via administration of an anti-GR-1 Ab resulted in reduced development of demyelinating disease and enhanced virus-specific CD4⁺ and CD8⁺ T cell responses (97). Additionally, TMEV-infected, anti-GR-1 Ab-treated mice had decreased myelin-specific CD4⁺ T cell responses compared to control Ab-treated mice during the demyelinating disease at a later time post-infection (97). Although the expression of Arg1 was not investigated in this study, TMEV-infected mice had elevated expression of IL-10 in the brain and

spinal cord (97), suggesting a role for this cytokine in the suppression of antiviral T cell responses, potentially through the effects of Arg1.

Interestingly, a role for the modulation of arginine metabolism in viral control versus persistence along with associated disease has recently been demonstrated for the tumor-inducing, chicken-specific herpesvirus MDV. We mentioned above that MDV was vulnerable to the antiviral properties of NO, with iNOS being induced in genetically resistant chickens and in vaccinated chickens (35). In contrast, MDV induced strong macrophage arginase activity in cell extracts from adherent monocytes from genetically susceptible chickens, but not in chickens that were resistant to Marek's disease, either genetically or acquired after vaccination (35). Together, these data suggest that in the case of Marek's disease, the state of resistance versus sensitivity to disease was correlated with a reciprocal balance of NOS versus arginase activities in macrophages.

This phenomenon of Arg1-mediated T cell suppression has also been recognized in human viral infections. Arg1 mRNA and protein levels were elevated in HCV-infected liver cell lines *in vitro* and in HCV-infected liver samples compared with paired hepatocellular carcinoma samples from the same patients or with uninfected liver tissues (71). Additionally, the number of MDSCs in chronic HCV patients correlated with levels of plasma HCV-RNA (98). Cai et al. (98) also found that MDSCs from patients with chronic HCV infection suppressed T cell function via an Arg1-dependent mechanism. An additional study found that more PBMCs from chronic HCV patients expressed the phenotypic markers of MDSCs than PBMCs from healthy controls, and these cells expressed increased levels of p47^{phox}, a component of the NADPH oxidase complex (99), suggesting a role for ROS in MDSC-mediated suppression. Consistent with this, CD33⁺ mononuclear cells co-cultured with HCV-infected hepatocytes or HCV core protein suppressed T cell proliferation in a ROS-dependent manner (99). Overall, these data suggest that multiple mechanisms – including arginine metabolism and ROS – may be at play in myeloid cell-mediated suppression of anti-HCV T cell responses.

It has been suggested that prolonged immune activation during chronic virus infections, such as HCV and HIV, provides an environment that drives viral replication and disease progression (100, 101). Moreover, immune activation can drive an anti-inflammatory response to limit immunopathology, which can be characterized by the presence of M2-like macrophages. Indeed, similar to HCV infection, a role for arginase and M2-polarized MDSC-like cells has been identified in the suppression of antiviral T cell responses following HIV infection. Individuals with detectable HIV-1 infection showed an increase in the frequency of CD163⁺CD16⁺CD14⁺ monocytes, which are thought to be precursors of M2 macrophages, when compared to seronegative or HIV-1-infected persons with undetectable viral loads, and monocyte frequency correlated positively with HIV-1 viremia and negatively with CD4⁺ T cell counts (in patients with counts <450 cells/ μ l) (72). Furthermore, Qin and colleagues (73) observed elevated levels of MDSCs, defined as HLA-DR^{-/low} CD11b⁺CD33^{+/high}CD14⁺CD15⁻ cells, in the peripheral blood of HIV-1-seropositive subjects compared with healthy controls, and these MDSCs suppressed T cell responses

in an Arg1-dependent manner. Moreover, PBMCs from HIV-seropositive patients exhibited increased levels of arginase activity (73). Cloke and colleagues (74) found that increased arginase activity correlated with lower CD4⁺ T cell counts, and this association was abrogated following antiretroviral treatment (75). Additionally, exposure of PBMCs to HIV gp120 expanded T cell-suppressive MDSCs *in vitro* (76). These data point to a direct role for arginase-expressing MDSC-like cells in the suppression of anti-HIV T cell responses. Consistent with that, individuals co-infected with HIV and *Leishmania* parasites had increased arginase activity in PBMCs and plasma compared with *Leishmania*-only infected individuals, even though *Leishmania* infection alone results in increased arginase activity (77). In addition, the parasite load in the spleen was significantly higher in co-infected patients (77). The arginase-expressing cells were identified as low-density granulocytes (77). These results suggest that increased arginase might contribute to the poor immune responses and disease outcome characteristic of patients with *Leishmania* and HIV co-infection.

Hepatitis B virus (HBV) infection is another common chronic viral infection, with estimates as high as 350 million chronically infected humans (102). Bility and colleagues (78) recently developed a humanized mouse model with both a human immune system and human liver cells, named the A2/NSG-hu HSC/Hep humanized mouse model, to study the pathogenesis of HBV infection. Following HBV infection, the mice developed persistent HBV infection as well as chronic hepatitis and liver fibrosis (78). The liver disease was associated with a high level of infiltrating human macrophages with a M2-like activation phenotype (78). Similarly, M2-like macrophage accumulation was seen in chronic HBV-infected patients, and M2-like macrophage induction in the liver was associated with accelerated liver fibrosis and necrosis in patients with acute HBV-induced liver failure (78), suggesting a role for M2 macrophages in persistent HBV infection. Additionally, patients with acute HBV infection had increased serum levels of arginase, and this serum inhibited IFN- γ production by CD8⁺ T cells (79). Das et al. (80) also found decreased L-arginine levels in the circulation of chronic HBV patients with marked liver inflammation (>100 ALT) and increased arginase activity in liver extracts taken directly *ex vivo* from patients with chronic HBV compared with those from patients with other types of liver pathology (80). They further showed that CD8⁺ T cells from chronic HBV patients, regardless of their antigen specificity, exhibited less IL-2 but not IFN- γ or TNF- α production and impaired proliferation following TCR-dependent stimulation, indicating an aberrant antiviral T cell response in chronic HBV infection (80). In the A2/NSG-hu HSC/Hep humanized mouse model, HBV-infected mice had impaired liver T cell responses, and M2 macrophages were associated with T cells in the liver (78). Expression of the TCR signaling molecule CD3 ζ was reduced in both peripheral and intrahepatic CD8⁺ T cells from chronic HBV patients; similarly, CD28 was also downregulated on CD8⁺ T cells from high viral load HBV patients (80). Downregulation of the CD3 ζ molecule has previously been shown to occur in the arginine-depleted tumor microenvironment. Consistent with this, *in vitro* transfection of CD3 ζ and CD28 restored IL-2 production and supplementation of L-arginine partially restored CD3 ζ expression and T cell proliferation (80). These data suggest a role

for arginase activity and arginine depletion in the impairment of anti-HBV T cells functions.

In the absence of iNKT cells, influenza A (PR/8 strain) infection was shown to induce the expansion of CD11b⁺GR-1⁺ MDSCs in the lungs of mice, which suppressed influenza-specific T cell and antibody responses through the activity of both arginase and NOS, resulting in higher viral titers and increased mortality (81). Adoptive transfer of iNKT cells reversed this phenotype; mice had an increased survival rate, reduced viral titers, and increased virus-specific immune responses, suggesting a novel immunomodulatory role for iNKT cells during influenza virus infection (81). Moreover, these authors identified that influenza infection in humans induced the expansion of CD11b⁺ myeloid cells with suppressive activity that could be reduced by iNKT cell activation or the inhibition of arginase and NOS activity. Similarly, it was recently shown that highly pathogenic H5N1 and H1N1 influenza virus infection induced the accumulation of CD11b⁺GR-1⁺ cells and the expression of Arg1 in the lungs (82), further supporting a role for M2-polarized MDSC-like cells in promoting viral persistence and immunopathology.

Helminth infection induces the expression of type 2 cytokines and is associated with M2 macrophage activation, as determined by Arg1, FIZZ1, and Ym1 expression. Indeed, Osborne and colleagues (83) found that Arg1, FIZZ1, and Ym1 were highly induced in the ileum of mice infected with the helminth *Trichinella spiralis* (Ts). Interestingly, they further showed that co-infection of mice with Ts and murine norovirus (MNV) resulted in decreased frequencies and numbers of MNV-specific CD8⁺ and CD4⁺ T cells within the small intestine and spleen as well as decreased polyfunctionality of these T cells, compared to Ts-only infected mice (83). Additionally, the defective T cell responses were associated with increased viral loads in the double-infected mice compared to the mono-infected controls (83), suggesting that Ts-elicited M2-activated macrophages inhibited the antiviral T cell response to MNV. Lastly, neutralization of Ym1, a chitinase-like molecule, in co-infected mice partially restored antiviral immunity and was associated with enhanced control of viral replication (83). These data point to a new mechanism by which Arg1-expressing macrophages inhibit antiviral responses.

Cumulatively, these data are reminiscent of macrophages found in tumors (e.g., MDSCs, TAMs) that have been shown to suppress anti-tumor T cell responses via a variety of NO- and/or Arg1-dependent mechanisms (4, 5). Indeed, in a mouse model of human papillomavirus (HPV)-induced cancer, Arg1-expressing CD11b⁺F4/80⁺ macrophages infiltrated the tumors and inhibited T cell responses, including virus-specific T cells, by suppressing T cell proliferation and promoting a regulatory phenotype (103). Moreover, depletion of the tumor-infiltrating macrophages resulted in reduced tumor growth and increased tumor infiltration by virus-specific CD8⁺ T cells (103). Thus, increasing evidence points to a direct role for arginase-expressing M2-polarized cells in the suppression of antiviral T cell responses and the persistence of a variety of important pathogenic viruses. In addition to the actions of iNOS and Arg1, MDSC-like cells can employ other mechanisms to promote chronic viral infections, which were recently reviewed by Goh and colleagues (104).

M2 MACROPHAGE ACTIVATION CAN PROMOTE IMMUNOPATHOLOGY

In contrast to some parasitic infections where M2 macrophages limit Th2 cell-mediated immunopathology, M2-polarized macrophages have been shown to promote immunopathology in some viral infections. For example, it was recently demonstrated that SARS-CoV infection of mice induced suppressive alveolar macrophages that inhibited the induction of antiviral T cell responses, a phenotype that was reversed by the adoptive transfer of activated bone marrow-derived DCs into mice prior to virus infection (56). Additionally, SARS-CoV-infected mice lacking hematopoietic STAT-1 expression were shown to have greater weight loss and lung pathology, and this was associated with the activation of M2 macrophages (57). To further test the role of M2 macrophages in enhanced pathogenesis following SARS-CoV infection, the authors generated STAT-1/STAT-6 double knockout mice due to the established role for STAT-6 in driving M2 macrophage activation in response to IL-4/IL-13 stimulation. STAT-1/STAT-6 double knockout mice, which reversed the upregulation of M2 macrophages observed in STAT-1-deficient mice, had reduced lung disease and prefibrotic lesions (57). These data support the notion that M2 macrophages contribute to SARS-CoV pathogenesis.

In another example, mice deficient in the IFN- γ R exhibit more severe disease following infection with murine gamma-herpesvirus-68 (MHV-68), including interstitial and intra-alveolar fibrosis that is reminiscent of idiopathic pulmonary fibrosis (IPF) in human beings. In this model, alveolar macrophages were recruited to the lungs of MHV-68-infected IFN- γ R^{-/-} mice, were associated with areas of fibrosis, and exhibited a M2-polarized phenotype characterized by the expression of FIZZ1, Ym1, and Arg1 (61). Additionally, lung tissue from patients with IPF showed increased expression of Arg1 in alveolar macrophages compared with normal lung (61). These results suggest that virus-induced upregulation of Arg1 could be mediating lung fibrogenesis. MHV-68 infection in IFN- γ R^{-/-} mice also resulted in fibrosis in lymphoid tissues such as the spleen, which is a site of latent MHV-68 infection, and the liver (62, 63). Similar to the lung, MHV-68 infection in the absence of IFN- γ R signaling induced a M2 macrophage response in the spleen, characterized by high Arg1 expression along with FIZZ1 and M2/Th2 cytokines such as IL-13, resulting in fibrotic disease in the spleen (105). Moreover, depletion of T cells prevented MHV-68-mediated fibrosis in IFN- γ R^{-/-} mice (62), suggesting that M2 macrophages were further driving Th2 activation to possibly create a M2/Th2 cytokine-induced cycle, resulting in the exaggerated pathology. In contrast to IFN- γ R^{-/-} mice, iNOS was induced in the spleen of MHV-68-infected WT mice (105), indicating an important role for IFN- γ in inducing a M1-associated immune response to control gamma-herpesvirus infection and limiting Arg1-mediated immunopathology.

CONCLUSION

Macrophages and other myeloid cells have marked phenotypic heterogeneity, as a result of distinct cellular differentiation programs, distribution in tissues, and responsiveness to various endogenous and exogenous stimuli. Indeed, macrophages have well-established roles in development, tissue homeostasis, coordinating the adaptive immune response and inflammation, as well as directing

tissue resolution and repair following damage – processes that are often modulated via the actions of the arginine-hydrolyzing enzymes Nos2 and Arg1. We have highlighted a number of viral infections in which these enzymes have a beneficial effect: NO has antiviral properties against a variety of viruses, and arginase activity can mediate tissue repair and regeneration following a viral insult (**Table 1**). However, NO production can also result in immunopathology in some virus infections, and the suppressive functions of Arg1-expressing macrophages can promote immunopathology. Additionally, some viruses have exploited the immune-suppressive properties of iNOS- and/or Arg1-expressing macrophages to evade the immune response, particularly the antiviral T cell response, resulting in chronic viral infections.

Clearly, iNOS- and/or Arg1-mediated responses are important in many viral infections. Thus, there is the potential to develop the means to selectively stimulate or inhibit either M1 or M2 responses to mediate viral clearance or repair tissue damage. Due to the overlap in immunosuppressive mechanisms of iNOS- and/or Arg1-expressing suppressor cells, therapeutic strategies under development to limit the immunosuppressive effects of myeloid cells in cancer may be beneficial in treating persistent/chronic virus infections. However, as described above, iNOS and Arg1 activity can be both beneficial and detrimental during certain viral infections. Therefore, further research is needed to define the molecular and tissue-specific mechanism(s) by which iNOS and Arg1 influence the clearance of viral pathogens as well as the injury and repair of tissues. In addition, a better understanding of the pathways regulating macrophage polarization (specifically iNOS and/or Arg1 induction and activity), macrophage trafficking, and the precise effects of iNOS and Arg1 activity on other immune cells following different virus infections will inform the development of therapeutics that target critical effector molecules to promote viral control and limit immunopathology.

ACKNOWLEDGMENTS

Work in Dr. Morrison's laboratory is supported by NIH-NIAID grants U19 AI109680 and R01 AI108725. Kristina S. Burrack was supported by NIH-NIAID training grant T32 AI052066.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 29 July 2014; paper pending published: 17 August 2014; accepted: 22 August 2014; published online: 08 September 2014.

Citation: Burrack KS and Morrison TE (2014) The role of myeloid cell activation and arginine metabolism in the pathogenesis of virus-induced diseases. *Front. Immunol.* **5**:428. doi: 10.3389/fimmu.2014.00428

This article was submitted to Antigen Presenting Cell Biology, a section of the journal *Frontiers in Immunology*.

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Classical and alternative activation and metalloproteinase expression occurs in foam cell macrophages in male and female ApoE null mice in the absence of T and B lymphocytes

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Background: Rupture of advanced atherosclerotic plaques accounts for most life-threatening myocardial infarctions. Classical (M1) and alternative (M2) macrophage activation could promote atherosclerotic plaque progression and rupture by increasing production of proteases, including matrix metalloproteinases (MMPs). Lymphocyte-derived cytokines may be essential for generating M1 and M2 phenotypes in plaques, although this has not been rigorously tested until now.

Methods and results: We validated the expression of M1 markers (iNOS and COX-2) and M2 markers (arginase-1, Ym-1, and CD206) and then measured MMP mRNA levels in mouse macrophages during classical and alternative activation *in vitro*. We then compared mRNA expression of these genes *ex vivo* in foam cells from subcutaneous granulomas in fat-fed immune-competent ApoE knockout (KO) and immune-compromised ApoE/Rag-1 double-KO mice, which lack all T and B cells. Furthermore, we performed immunohistochemistry in subcutaneous granulomas and in aortic root and brachiocephalic artery atherosclerotic plaques to measure the extent of M1/M2 marker and MMP protein expression *in vivo*. Classical activation of mouse macrophages with bacterial lipopolysaccharide *in vitro* increased MMPs-13, -14, and -25 but decreased MMP-19 and TIMP-2 mRNA expressions. Alternative activation with IL-4 increased MMP-19 expression. Foam cells in subcutaneous granulomas expressed all M1/M2 markers and MMPs at *ex vivo* mRNA and *in vivo* protein levels, irrespective of Rag-1 genotype. There were also similar percentages of foam cell macrophages (FCMs) carrying M1/M2 markers and MMPs in atherosclerotic plaques from ApoE KO and ApoE/Rag-1 double-KO mice.

Conclusion: Classical and alternative activation leads to distinct MMP expression patterns in mouse macrophages *in vitro*. M1 and M2 polarization *in vivo* occurs in the absence of T and B lymphocytes in either granuloma or plaque FCMs.

Keywords: atherosclerosis, macrophages, lymphocytes, cytokines, plaque rupture

INTRODUCTION

Atherosclerotic plaque rupture underlies most myocardial infarctions and thromboembolic strokes, which are principal causes of mortality and morbidity worldwide (<https://apps.who.int/infobase/mortality.aspx>). Macrophages play a key role in atherosclerosis progression, as demonstrated by their abundance in human plaques as foam cells and by the dramatic reduction in atherosclerosis in mice after genetic (1) or pharmacological (2) deletion of macrophages. Furthermore, production of mediators from activated macrophages is believed to be important in making plaques vulnerable to rupture (3). Reactive oxygen species, cytokines, and cell surface modifying proteinases produced by macrophages promote apoptosis, thereby contributing to the cellular rarefaction of vulnerable plaques (4). Moreover, extracellular proteinases, in particular matrix metalloproteinases (MMPs) can

directly degrade the extracellular matrix and promote plaque instability (5). These proposed mechanisms are supported by the histological appearance of vulnerable plaques, which contain an abundance of macrophages expressing MMPs but a relative lack of smooth muscle cells (SMC) and extracellular matrix proteins, importantly collagens, which contribute tensile strength to the plaque cap (5). Intervening to diminish the production of these harmful mediators is therefore a rational approach to new therapies and this motivates efforts to understand the cellular and molecular mechanisms involved.

Early work highlighted the functional diversity of macrophages (6) and the existence of distinct phenotypes has become increasingly discussed (7). Polarization of macrophage into a so-called classically activated or M1 phenotype was recognized, possibly reflecting the context of infection where pathogen associated

molecular patterns (PAMPs) and pro-inflammatory lymphocyte-derived cytokines, particularly interferons (IFNs) and interleukins (ILs) occurred in the same microenvironments. Consistent with this, the M1 phenotype is simulated *in vitro* by the combined action of PAMPs acting through Toll-like receptors (TLRs) and IFN γ (8), with some evidence for synergy. Mechanisms underlying synergy include the ability of IFN γ to prime responses to PAMPs by inducing expression of TLRs and their co-activators (9). Synergy also results from the combined activation of differing signaling pathways for TLRs through nuclear factor- κ B (NF- κ B) (9) and IFN γ through signal transducer and activator of transcription (STAT-1) (10).

The actions of IFN γ have led to the hypothesis that Thelper1 (Th1)-lymphocytes may be essential for, or at least prominent contributors to, M1 polarization *in vivo*, a concept that has been acknowledged in papers dealing with atherosclerosis (11–13). It is consistent with studies showing that knockout (KO) of IFN γ and its receptors reduces atherosclerosis progression (3), although the impact on M1 polarization was not measured directly in any of these IFN γ KO investigations. On the other hand, it is well recognized that other cytokines and combinations of cytokines that activate the pathways leading to M1 states could bypass the requirement for IFN γ (9, 14). Conversely, macrophages can polarized to a variety of alternatively activated or M2 states. The so-called M2a phenotype occurs after treatment with IL-4 or IL-13, which are potentially produced by Thelper2 (Th2) lymphocytes (15). Overall, this leads to the idea that T-lymphocytes, and, by implication, adaptive immunity play an essential part in M1/M2 macrophage polarization in atherosclerotic plaques. On the other hand, data from other areas of inflammation support the conclusion that M1/M2 polarization is primarily a function of innate immunity and that lymphocytes play a minor, at best modulatory role (16). Resolving this controversy is important for human atherosclerosis since a variety of immunotherapies have been proposed and some have already entered clinical trials (17).

Our present aim was formally to test the requirement for T and B lymphocytes in M1 and M2 macrophage activation and MMP production using an established ApoE-KO mouse model of atherosclerosis formation. To do this, we compared the expression of M1 and M2 markers, MMPs and the endogenous tissue inhibitors of MMPs (TIMPs) in foam cell macrophages (FCMs) from subcutaneous granulomas and atherosclerotic plaques in ApoE KO and ApoE/Rag-1 double-knockout (DKO) mice, which lack all T and B lymphocytes. As it is already known that there are differences in inflammation and plaque development between genders (18, 19), we used both male and female mice in this study. The mice were fed on a high-fat diet because it was previously shown that these conditions produced similar sized plaques in both genotypes (20). Hence, we could be sure that measurements of the prevalence of macrophages phenotypes would not be an artifact of different stages of atherosclerosis progression or plaque sizes.

MATERIALS AND METHODS

BONE MARROW MONOCYTE ISOLATION AND DIFFERENTIATION TO BONE MARROW-DERIVED MACROPHAGES

Mouse femurs and tibias were excised from C57BL/6 mice (Charles River, UK) on a normal diet. The bone ends were cut and bone

marrow was flushed out with sterile phosphate-buffered saline (PBS). Erythrocytes were removed using ACK lysis buffer (Life Technologies, UK) and the pelleted white blood cells resuspended in PBS and counted, giving an average yield of 40×10^6 cells per mouse. Cells were plated into 24-well plates at 0.5×10^6 cells/well and grown in RPMI 1640 media (Life Technologies) supplemented with antibiotics, glutamine, and 20% fetal calf serum (FCS) (Life Technologies) in the presence of 20 ng/ml recombinant human macrophage colony-stimulating factor (M-CSF, R&D Systems, USA). The media was changed every 3–4 days. After 7–10 days, when the cells had differentiated into macrophages, M-CSF was removed and the cells exposed to selected cytokines for 18 h in serum-free media (SFM), as specified in the text. These included mouse (m) IFN γ at 20 ng/ml (Miltenyi Biotec, USA), human (h) tissue necrosis factor (TNF)- α at 10 ng/ml (R&D Systems, USA), mIL-4 at 10 ng/ml (PreproTech, UK), and lipopolysaccharide (LPS) at 10 ng/ml (Sigma-Aldrich, USA, L2654). Human cytokines were used only when their efficacy on mouse cells had been previously documented by the suppliers.

PERIPHERAL BLOOD MONONUCLEAR CELL ISOLATION AND DIFFERENTIATION

Pooled peripheral blood was collected by cardiac puncture from C57BL/6 mice with heparin as anti-coagulant. Monocytes were then isolated density gradient separation on Ficoll Paque Plus and differentiated to macrophages as previously described (21).

RNA EXTRACTION, REVERSE TRANSCRIPTION, AND QUANTITATIVE PCR

RNA lysates from cultured macrophages were collected using RLT solution (Qiagen Ltd, UK) with β -mercaptoethanol and the total RNA extracted using the Qiagen RNeasy kit (Qiagen Ltd), according to the manufacturer's instructions. The quantity and quality of resulting RNA was assessed using a NanoDrop ND-1000 spectrophotometer (LabTech International, UK). Samples of cDNA were generated using QuantiTect Reverse Transcription Kit (Qiagen Ltd), according to the manufacturer's instructions and the resulting cDNA was diluted 1:1 in 10 mM TrisHCl, pH 8.0. Real time quantitative PCR was performed in a Roche Light Cycler 1.5 (Roche, UK) to quantify the steady-state concentration of RNA, using the QuantiTect SYBR Green PCR Kit (Qiagen Ltd). The primers used are listed in Table 1. Each reaction contained 2.5–7 ng RNA and 0.5 μ M primers. Initial denaturation (15 min at 95°C) was followed by 55 cycles of denaturation (15 s at 95°C), annealing (20 s at 60°C), and extension (25 s at 72°C). Copy numbers of gene transcripts per total nanogram RNA input were calculated using standard curves constructed as recommended by from purified amplicon (Bioline, USA).

IN VIVO STUDIES

Rag-1 KO mice that do not produce mature T or B cells (B6.129S7-Rag-1^{tm1Mom}/J) and ApoE KO mice on a C57BL/6 background (B6.129P2-Apoe^{tm1Unc}/J) were purchased from The Jackson Laboratories (USA), and bred together to create ApoE \pm /Rag-1 \pm mice. Breeding stocks of ApoE KO and ApoE/Rag-1 DKO mice were obtained by crossing the resulting F1 generation. Mice were kept in scintainers and given sterile food and water *ad libitum*. All animal work was in accordance with the Home Office Guidance

Table 1 | Primer sequences used for quantitative RT-PCR.

Gene	Primer sequence
ARG-1	AGTCTGGCAGTTGAAGCATCTCT TTCCTTCAGGAGAAAGGCACAGG
COX-2	ATACTGGAAGCCGAGCACCTTGG ATGGTGGCTGTTGGTAGGCTGT
CD206	CCATTATCATTCCCTCAGCAAGC AAATGTCAGTGGGTTCCATCACT
FIZZ1	AGAGGTGGAGAACCCAGCTTGAT TTCAAGAACAGGGTAATGGGCA
IL-12p35	CCACAACAAGAGGGAGCTGCC AGTGTGCGTTGATGGCTGGAACT
IL-12p40	AGACCAGGCAGCTCGCAGCAAAGCA GACACATCCCACCTCCACGCTGCC
iNOS	CTCATGACATCGACCAGAACGCT TATATTGCTGGCTCCATGTTG
MMP-2	GGCTGACATCATGATCAACTTGG GCCATCAGCCGTTCCATACTTAC
MMP-3	GCATCCCCTGATGTCCTCGTGG TCCCCGGAGGGTGCTGACTG
MMP-8	TGCCTCGATGTTGGAGTGCTGA GCCCTTGACAGCTGTGGCGT
MMP-9	AGAGAGGGAGTCTGGGCTGGTTT GAGAACACCACCGAGCTACCACT
MMP-12	AATTACACTCCGGACATGAAGCGT GGCTAGTGTACCACTTGCATC
MMP-13	ATGATGATGAAACCTGGACAAGCA ATAGGGCTGGTCACACTCTCTG
MMP-14	ACCACAAGGACTTGCCTGAG CACCGAGCTGTGAGATTCCCTGA
MMP-19	GATGAAGTGGCCAGAACGACTGACCTT GTCCCCGGTTGATGAGTTAGTGTC
MMP-23	CAAGGTTGGTGAGAGAGGGTAGGA AGGAGTAGGTGCTGAGAACACGCT
MMP-25	CTCTGAGTGGCAGTGGAGAAG TGATGTCAGGCTCTGGTACTGAG
TIMP-1	AGGAACGAAATTGACATCAGT CAAAGTGACGGCTGGTAGTCCT
TIMP-2	GACTCCCCCTCAGACTCTCCCTAC CATATTGATACCACCGCACAGGAA
TIMP-3	CACATCAAGGTGCCATTCAAGGTAG GTTCTCTCTCTCAACCCAAACA
Ym1	CAGGTCTGGCAATTCTCTG GTCTTGCTCATGTTGTAAGTG

on the operation of the Animals (Scientific Procedures) Act 1986 and conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). Genotyping of the mice was performed on ear or tail pieces, using the Direct PCR kit (Bioquote, UK or Viagen Biotech, USA) after a Proteinase K digestion (Sigma, UK). PCR was performed using Crimson Taq Pol (New England Biolabs, UK), with dNTPs from Bioline (UK), using primers designed by The Jackson Laboratories. Male and

female mice commenced a sterile high-fat diet (21–23% fat, Special Diet Services, UK) at 5 weeks of age, and were sacrificed with an anesthetic overdose 12 weeks later. Blood was taken via cardiac puncture and heparinized plasma was subsequently analyzed for total, HDL, and LDL/VLDL cholesterol (Cholesterol/Cholesterol Ester Quantification Kit, Abcam, UK), after a minor adaptation of the manufacturer's instructions. The levels of selected M1 and M2 cytokines was assessed in additional samples of mouse plasma using a Bio-Plex Pro Mouse Cytokine Th1/Th2 Panel 8-plex (Bio-Rad, USA). After the cardiac puncture, fresh tissue samples were taken (tail tip, spleen, liver lobe) and the animals perfused via the heart with PBS, then 10% formalin, at a constant pressure of 100 mmHg, with outflow through the left jugular vein. The brachiocephalic artery (BCA) (with a small piece of aortic arch), heart and remaining ascending and descending aorta were cleaned and removed from each mouse. Other tissues harvested included thymus and remaining liver. Tissue blocks of spleen less than 0.5 cm thick were post-fixed in 10% formalin for 24 h for subsequent histological examination, as described below.

SPONGE IMPLANTATION AND FOAM CELL MACROPHAGE ISOLATION

ApoE KO or DKO mice were fed the same high-fat diet as above from 6 weeks of age. Two weeks later these mice had 0.5 cm³ sterile polyurethane sponges containing ~50 µl of Matrigel (VWR, UK) placed under the dorsal skin under halothane anesthesia to generate FCMs as described previously (22, 23). The mice were fed the high-fat diet for a further 4 weeks. Recovered sponges were either fixed and embedded for immunohistological examinations as described below or the FCM were isolated and studied *ex vivo*. Fresh sponges were treated with 0.75 ml undiluted Dispase (VWR, UK or BD Biosciences, USA) and then squeezed to obtain a cellular exudate. FCM were then purified, as previously described (22, 24) by flotation after centrifugation on a metrizamide gradient (1.3507 refractive index, Sigma) followed by differential adherence. Only foam cells (validated by Oil-red-O staining) float because of the relatively low buoyant density of lipid.

Samples were taken from each preparation immediately for protein or RNA isolation, and mRNA levels quantified as described above. Other cell preparations were cultured for a short period, to allow adherence to coverslips. Oil-Red-O (2% Oil-Red-O in isopropanol; Sigma) staining was performed to confirm lipid content, and immunocytochemistry performed to confirm cell purity. Cells were also assessed for their proliferative capacity [BrdU (Sigma) incorporation, 8 h pulse] or *in situ* zymography (25). In this assay, the gelatinolytic capacity of the macrophages isolated from the sponges was determined using the EnzChek gelatinase/collagenase assay kit (Invitrogen, USA). Controls included cells treated with EDTA, 1,10-phenanthroline (Sigma) or GM6001 (Millipore, UK), to prevent MMP activity. Cells were fixed in paraformaldehyde and mounted in Vectorshield + DAPI (Vector Labs, USA). Several fields were photographed on each coverslip and the proportion of cells with gelatinase activity as indicated by the loss of fluorescence of the DQ-gelatin substrate determined.

HISTOLOGICAL METHODS

The proximal aorta and BCA from each mouse were embedded in paraffin and 3 µm sections cut at 3 µm intervals from the

atherosclerosis-prone areas of these vascular beds, as described previously (23, 26). The first section after the bifurcation of the BCA from the aorta was cleared and rehydrated and then stained using Miller's elastin/van Gieson (EVG) and plaque dimensions were measured using image analysis software (Image Pro, DataCell, Maidenhead, UK), as described previously (23). The aortic sinus from each mouse was treated and examined in a similar fashion, with the first leaflet section (from the aorta) stained using EVG, with subsequent image analysis being performed (26). For immunohistochemistry, 3 μm sections were brought to water and antigen retrieval performed using citrate buffer. Non-specific binding blocked with 10% goat serum (Sigma) in PBS. Primary antibodies for SMC (α -smooth muscle actin), macrophages [*Griffonia simplicifolia* Lectin II (GSL)], iNOS, COX-2, CD206, arg-1, Ym-1, MMP-12, MMP-13, MMP-14, and TIMP-3 (see Table 2) were added to the sections and incubated either overnight at 4°C or for 1 h at room temperature. After washing and further incubations with goat anti-rabbit-biotin (Dako or Sigma) and ExtrAvidin-HRP (Sigma) staining was visualized using 3,3'-diaminobenzidine (DAB, Sigma). A negative control where the primary antibody was replaced with the relevant species IgG at the same dilution was always included. The percentage of the

plaque area stained with each cell-specific or phenotypic marker or MMP/TIMPs antibody was determined using the same image analysis software detailed above. The number of buried layers was assessed manually on sections stained with EVG and on sections using antibodies that recognize SMC. Paraffin-embedded sponge sections were treated similarly, and the presence of markers of macrophage activation examined. Oil-Red-O staining was performed *en face*, and the percentage of fatty deposits in each aorta was measured using NIH ImageJ v1.43.

STATISTICAL METHODS

All analyses were performed using GraphPad InStat v3.05 (GraphPad Software, Inc. USA) or SPSS v21 (IBM, USA) software. Data were checked for normality (Kolmogorov and Smirnov normality test), and logarithmic transformation of data performed if necessary. Regression analyses were performed using Pearson's correlation co-efficient. Statistical analyses of data were performed using Students *t*-test, a Mann–Whitney *U*-test or 1- or 2-way ANOVAs, with the 1-way ANOVA followed by a Bonferroni or Tukey-Kramer post-test. Data are expressed as arithmetic mean \pm SEM or geometric mean and 95% confidence limits, and statistical significance defined as $P < 0.05$.

RESULTS

IN VITRO STUDIES IN MOUSE BONE MARROW MACROPHAGES

Bone marrow proved a convenient source of large quantities of mouse monocytes that were converted to bone marrow-derived macrophages (BMDM) using M-CSF. BMDM were 97% F4/80 and CD11b double positive by flow cytometry (results not shown). We used mRNA expression for established M1 and M2 marker genes as positive controls for classic or alternatively activation. As expected from previous literature (15), classical activation with LPS alone or LPS plus IFN γ increased mRNA levels of inducible NO synthase (iNOS, NOS-2) and cyclooxygenase-2 (COX-2) (Figure 1A), whereas alternative activation with IL-4 increased mRNA expression of arginase-I (arg-1), Ym-1, and CD206. We then investigated the concomitant regulation of a wide spectrum of MMPs and TIMPs, many of which have been previously implicated in atherosclerosis (27). The most abundant mRNAs under unstimulated conditions were MMP-12 > MMP-8 = MMP-19 = MMP-14 = TIMP-2 > other MMPs and TIMPs (Figure 1B). Among the MMPs studied, MMP-13 showed the most dramatic 121-fold stimulation by LPS + IFN γ (Figure 1B, note the scale is logarithmic). LPS + IFN γ treatment also increased expressions of MMP-14 (11.3-fold) and MMP-25 (14.6-fold); and decreased expressions of MMP-19 (4.5-fold) and TIMP-2 (9.0-fold) (Figure 1B). Classical activation with a different mediator, tumor necrosis factor α (TNF α), significantly increased expressions of MMP-2 (77-fold), MMP-9 (3.5-fold), and MMP-14 (3.5-fold) but not of MMP-13 (Figure 1B). Classical activation with TNF α also increased MMP-9 and MMP-14 expression in blood derived macrophages, similar to BMDM, but did not affect any of the other MMPs or TIMPs (results not shown). Treatment with IFN γ did not increase mRNA levels of any MMP or TIMP in BMDM either alone or in the presence of LPS (Figures 1C,D). No effect of IFN γ was observed in blood derived macrophages either (results not shown). Alternative activation with IL-4 increased mRNA expression of only MMP-19

Table 2 | Primary and secondary antibodies and *Griffonia simplicifolia* Lectin II.

	Catalog number	Type	Supplier
Primary			
GSL II	B1215	Lectin	Vector labs
α -Smooth muscle actin	M0851	M_Mab	Dako
arginase 1	sc-20150	Rb_PAb	Santa Cruz
BrdU	B2531	M_Mab	Sigma
COX2	ab15191	Rb_PAb	Abcam
iNOS	ab15323	Rb_PAb	Abcam
MOMA-2	ab33451	R_Mab	Abcam
MMP-12	ab52897	Rb_MAb	Abcam
MMP-13	ab39012	Rb_PAb	Abcam
MMP-14	ab51074	Rb_MAb	Abcam
STAT1p (phospho Y701)	ab30645	Rb_PAb	Abcam
STAT6p (phospho Tyr641)	06-937	Rb_PAb	Millipore
TIMP-3	Ab39206	Rb_PAb	Abcam
Ym1/2	01404	Rb_PAb	Stemcell
rabbit IgG negative control	I5006		Technologies
mouse IgG2a negative control	M5409		Sigma
Goat serum	G9023		Sigma
Rabbit serum	R9133		Sigma
Secondary antibody			
G α M	E0433	Biotinylated	Dako
G α M	B6649	Biotinylated	Sigma
G α Rb	E0432	Biotinylated	Dako
G α Rb	B6649	Biotinylated	Sigma
R β oR	E0468	Biotinylated	Dako

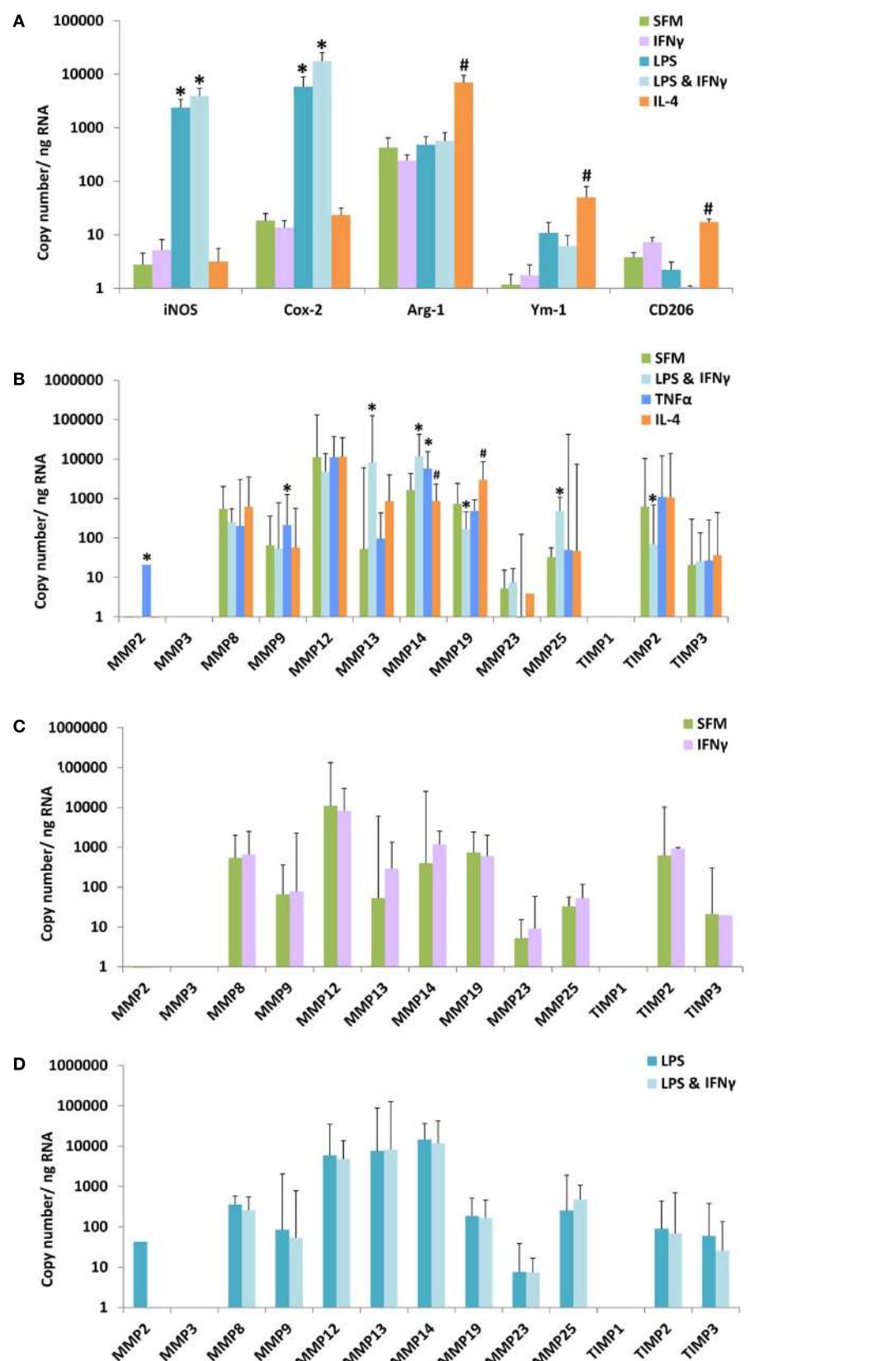


FIGURE 1 | Continued

FIGURE 1 | Continued

Effect of M1/M2 polarization on mRNAs for markers, MMPs, and TIMPs in mouse bone marrow-derived macrophages. Monocytes from mouse bone marrow were differentiated to macrophages in M-CSF and then treated with bacterial lipopolysaccharide (LPS), interferon- γ (IFN γ), tumor necrosis factor- α (TNF α), or interleukin-4 (IL-4) alone or in combination, as indicated. SFM = serum-free medium. **(A)** The effect of

polarization by IFN γ , LPS, LPS + IFN γ , or IL-4 on M1/M2 markers ($n = 4–5$). **(B)** The effect of polarization by IFN γ + LPS, TNF α , or IL-4 on MMPs and TIMPs ($n = 3–8$). **(C)** Effect of IFN γ alone on macrophages incubated in SFM. **(D)** Effect of IFN γ on macrophages incubated in SFM containing LPS. Values are expressed as mean and SEM **(A)** or mean and 95% confidence intervals **(B–D)**. *M1 different from M0, #M2 different from M0, $P < 0.05$.

Table 3 | Concentration of inflammatory and anti-inflammatory cytokines in plasma from ApoE KO or ApoE/Rag1 KO mice.

	Male ApoE KO		Male DKO		P-value (males)	Female ApoE KO		Female DKO		P-value (females)
	Mean	SEM	Mean	SEM		Mean	SEM	Mean	SEM	
IFN γ	11.88	4.353	5.680	2.902	0.2440	11.33	6.345	6.012	2.595	0.6275
TNF α	297.4 ^a	56.00	206.3	23.16	0.1495	167.9	21.46	191.0	19.26	0.4331
GM-CSF	118.0	24.55	106.4	16.66	0.6959	72.85	11.24	113.6	13.22	0.0304
IL-12p70	133.6	38.79	129.9	37.60	0.9457	58.65	12.61	127.7	29.90	0.0302
IL-10	15.23	3.023	23.99	3.852	0.0964	10.32	3.003	25.38	2.486	0.0012

Concentrations (picograms per milliliter) are expressed as mean and SEM, $n = 9–10$. Bold type indicates $P < 0.05$ for ApoE KO vs. DKO.

^aIndicates significant gender difference for that genotype.

Table 4 | Concentration of cholesterol-containing lipoproteins in plasma from ApoE KO or ApoE/Rag1 KO mice.

	Male ApoE KO		Male DKO		P-value (males)	Female ApoE KO		Female DKO		P-value (females)
	Mean	SEM	Mean	SEM		Mean	SEM	Mean	SEM	
Total	1360 ^a	116.5	1215 ^a	47.04	0.3939	802.4	64.65	707.3	59.73	0.3050
HDL	58.64	32.99	108.0	21.20	0.2364	14.39	3.177	85.86	41.76	0.0022
LDL + VLDL	931.3 ^a	71.77	508.4	99.79	0.0063	504.4	55.29	362.3	68.75	0.1384

Concentrations (milligrams per deciliter) are expressed as mean and SEM, $n = 6$. Bold type indicates $P < 0.05$ for ApoE KO vs. DKO.

^aIndicates significant gender difference for that genotype.

in BMDM (**Figure 1B**). These results revealed widely different levels of expression and divergent patterns of regulation of MMPs and TIMPs, which informed our choice of genes to measure in the subsequent *in vivo* experiments (see below).

CIRCULATING INFLAMMATORY CYTOKINE AND CHOLESTEROL LEVELS IN ApoE KO AND ApoE/Rag-1 DOUBLE-KNOCKOUT MICE

To investigate the impact of lymphocytes on M1/M2 polarization and MMP/TIMP expression, we compared ApoE KO and ApoE/Rag-1 DKO mice, which were genotyped using PCR to verify the deletion of ApoE alone or ApoE and Rag-1. The weight of the spleens of ApoE KO mice (115 ± 6 mg, $n = 8$) was reduced to (56 ± 9 mg, $n = 10$) in DKO mice and histological analysis of cut sections confirmed the absence of lymphocytes from DKO spleens (not shown). As expected, the expression of CD3 mRNA in the spleen, as a further marker of the presence of lymphocytes, was measurable in all ApoE KO mice tested (mean value 1.6 ± 0.5 copies/ng RNA, $n = 10$) but undetectable in all DKO mice tested. We used blood levels of cytokines related to Th1 and Th2 lymphocytes to further characterize the inflammatory status of ApoE KO and DKO mice. As in **Table 3**, circulating levels of IFN γ were low and not different between genotypes, whereas IL-4 levels

were below the limits of detection of our assays. Of the more abundant cytokines, TNF α levels were not different, whereas GM-CSF, IL-12, and IL-10 levels were elevated (1.6, 2.2, and 2.5-fold, respectively) in female DKO compared to ApoE KO mice. The same trend was seen for IL-10 levels in male mice (**Table 3**). These results showed, unexpectedly, that cytokines associated with M1 polarization (i.e., GM-CSF and IL-12) and M2 polarization (IL-10) were at least the same or even elevated in DKO mice. We also noted that TNF- α levels were significantly 1.8-fold higher in male than female ApoE KO mice but not different in DKO mice (**Table 3**).

Plasma cholesterol levels play a fundamental role in determining the extent of atherosclerosis in human beings and animal models. As shown in **Table 4**, the plasma concentrations of total cholesterol were not different between ApoE KO and DKO mice. HDL cholesterol was significantly increased sixfold in female DKO vs. ApoE KO mice and there was the same trend (1.9-fold) in the male mice. Conversely, LDL plus VLDL concentrations were significantly 1.8-fold lower in male DKO than ApoE KO mice and same trend (1.4-fold) was seen for the females. We noted that total cholesterol levels were significantly higher in males than females of either genotype. Furthermore, VLDL + LDL levels

were significantly higher in the male than female ApoE KO mice (**Table 4**). Based on increased HDL and decreased VLDL + LDL, DKO mice of either gender might be expected to be protected from atherosclerosis compared with ApoE KO mice.

M1/M2 MARKER AND MMP mRNA LEVELS IN GRANULOMA FCMs FROM SUBCUTANEOUS SPONGES

We sought a ready source of foam cells generated *in vivo* to investigate expression of the M1/M2 markers and MMPs and TIMPs measured in our *in vitro* study of non-foamy macrophages. Atherosclerotic plaques are small and difficult to disrupt but hypercholesterolemia promotes the accumulation of foam cells at several more accessible sites in human beings and mice. For example, foam cells accumulate in the peritoneum (28) or in granulomas that form in sterile sponges implanted subcutaneously into atherosclerosis-prone mice (23). In this study, FCMs were isolated from subcutaneous granulomas. They were purified based on their decreased buoyant density by flotation over a density gradient. The yield of foam cells from subcutaneous sponges implanted for 6 weeks into ApoE KO or DKO mice was $4.07 \pm 0.61 \times 10^6$ and $5.87 \pm 1.21 \times 10^6$ cells, respectively, and did not significantly vary between genders. Foam cells from ApoE KO mice were $95.5 \pm 1.6\%$ macrophages (using MOMA-2 as a marker). FCMs had detectable levels of the same M1 and M2 markers seen in non-foamy BMDM, irrespective of whether they came from ApoE KO or DKO mice

(**Table 5**). We concluded that FCMs acquired M1 marker genes in the absence of T and B lymphocytes. Indeed, there was a trend toward higher levels of mRNA expression of M1 markers, iNOS and COX-2, in DKO animals, although this was not significant (**Table 5**). Given this somewhat surprising conclusion, we measured additional M1 markers, namely IL-12 p35 and p40 and SOCS3, which were also detectable and showed no significant difference between ApoE-KO and DKO mice (**Table 5**). The mRNAs for M2 markers arg-1, and Ym-1 were also expressed at similar levels in granuloma FCMs from ApoE KO or DKO mice (**Table 5**), whereas CD206 was slightly elevated in male DKO compared to ApoE KO mice. The data suggested that M2 polarization also occurred efficiently in the absence of lymphocytes. To confirm this, we added measurements of FIZZ1 and IL-10, which also showed no difference between ApoE KO and DKO FCMs (**Table 5**). We noted a few significant gender differences. The mRNA levels of the M1 marker, IL-12p40, and the M2 markers, CD206 and Ym-1, were approximately 50% lower in granuloma FCMs from male compared to female ApoE KO mice.

The MMPs that were increased by classical activation in blood or BMDM, that is MMP-2, MMP-9, MMP-13, MMP-14, and MMP-25, were all expressed in granuloma FCMs, irrespective of Rag-1 genotype (**Table 5**). TIMP-2 (that was decreased by classical activation) and MMP-19 (that was decreased by classical activation and increased by alternative activation) were also expressed

Table 5 | Characteristics of foam cell macrophages obtained from subcutaneous sponges.

	Male KO		Male DKO		P-value (males)	Female ApoE		Female DKO		P-value (females)
	Mean	SEM	Mean	SEM		Mean	SEM	Mean	SEM	
COX2	915	135	2053	729	0.3692	931	134	1306	243	0.1981
iNOS	56	7	474	305	0.3793	69	13	331	242	0.3357
IL-12p35	1085	88	904	130	0.2451	1563	299	1154	276	0.3316
IL-12p40	6 ^a	1	9	3	0.7304	13	2	11	4	0.6432
SOCS3	1797 ^a	204	4117	1060	0.1191	2679	327	3038	587	0.5896
Arg-1	79646	5095	87511	7825	0.3953	87172	14259	90946	10539	0.8345
CD206	1317 ^a	198	2150	285	0.0224	2420	212	2293	372	0.7707
FIZZ1	377	58	518	82	0.1640	365	65	594	136	0.2810
IL-10	10262	1115	10234 ^a	780	0.9852	12984	2289	19852	4366	0.1853
Ym-1	35 ^a	5	78	31	0.3311	103	20	124	29	0.5530
MMP2	387	77	614	136	0.1462	535	97	681	173	0.4586
MMP9	104 ^a	18	217	63	0.0720	182	22	155	14	0.3537
MMP12	176264	11743	150381	15260	0.1860	215896	28916	160661	26809	0.1831
MMP13	43800	3821	33103	3990	0.0664	48821	8573	34538	4202	0.1770
MMP14	4888	570	5402	462	0.4963	5214	917	5856	942	0.6332
MMP19	8486	1684	7893	930	0.7723	8756	998	7814	1268	0.5699
MMP25	23	3	112	47	0.6049	27	6	84	42	0.9307
TIMP-1	1674 ^a	237	2321	399	0.1625	2543	330	1771	401	0.1571
TIMP-2	6624 ^a	533	6984	730	0.6884	10456	682	6455	606	0.0008
TIMP-3	1203 ^a	160	2728	689	0.0352	2228	356	2452	586	0.7434

FCMs were isolated by density gradient centrifugation and differential adhesion from sponges implanted under the skin of ApoE KO or ApoE/Rag-1 DKO mice, as indicated. Levels of mRNAs of M1/M2 markers and MMPs/TIMPs are expressed as copies/ng total RNA (mean and SEM, n = 7–13). Bold type indicates P < 0.05 for ApoE KO vs DKO.

^aIndicates significant gender difference for that genotype.

at high levels in both genotypes (**Table 5**). Female ApoE KO mice had 1.6-fold more TIMP-2 mRNA than the corresponding males. MMP-12 and TIMP-1 [that showed no relationship with classical/alternative phenotype under the conditions of our *in vitro* studies (**Figure 1B**)] were expressed irrespective of genotype, but TIMP-3 was significantly increased 2.3-fold in male DKO compared to ApoE KO mice. The proportion of granuloma FCMs able to degrade gelatin was determined using *in situ* zymography. A significantly higher percentage of granuloma FCMs had gelatinase activity from female DKO (91%) compared with ApoE KO animals (76%), and the same trend was evident in the males (86 vs. 74%) (**Figure 2A**). Pooling the data, the 88% of the DKO mice had gelatinase activity compared to 75% in the ApoE KO mice ($P = 0.0026$). The proliferative capacity of FCMs was assessed by measuring BrdU incorporation (**Figure 2B**). Proliferation was not different in male mice of either genotype but was increased almost twofold in female ApoE KO mice compared to DKO mice ($P < 0.0001$). Migration through a matrigel layer in a modified Boyden chamber assay ($n = 3–5$) was not significantly different amongst granuloma FCMs from the two genotypes (results not shown).

M1/M2 MARKER PROTEIN EXPRESSION AND PATHWAY ACTIVATION IN GRANULOMA FCMs FROM SUBCUTANEOUS SPONGES

We used immunohistochemistry to confirm the protein expression of M1 and M2 markers *in vivo* by using sections taken from excised subcutaneous sponges. A subpopulation of granuloma cells in sponge sections stained for iNOS (**Figures 3A,B**) or COX-2 protein (**Figures 3C,D**) in either Rag-1 genotype. Control sections stained with isotype matched non-immune immunoglobulins had no staining (**Figures 3E,F**). Furthermore, a fraction of the granuloma cells in sponge sections stained for nuclear localized NF- κ B (**Figures 4A,B**) or phosphorylated STAT-1 (**Figures 4C,D**), indicating that these cells had undergone activation of the signaling pathways that are associated with M1 activation. Sections stained with isotype matched non-immune immunoglobulins had no staining (**Figures 4E,F**). Some FCMs in sections also stained for arg-1 (**Figures 5A,B**), Ym-1 (**Figures 5C,D**), or phosphorylated STAT-6 (**Figures 5E,F**), demonstrating the presence of marker

proteins and active signaling pathways that are associated with M2 macrophages. Sections stained with isotype matched non-immune immunoglobulins again had no staining (**Figures 5G,H**). Similar results to those with sponge sections were found by immunocytochemistry of FCM isolated from sponges (data not shown).

PLAQUE SIZE, COMPOSITION, M1/M2 MARKERS, AND MMP PROTEIN EXPRESSION IN FCMs IN ATHEROSCLEROTIC PLAQUES FROM ApoE KO AND DKO MICE

Plaques in the aortic sinus tended to be smaller in DKO compared with ApoE KO mice but this did not reach statistical significance (**Figure 6A**). However, in agreement with previous reports (18, 19), significant gender differences were observed (**Figures 6A,B**). Aortic sinus plaques were significantly bigger in females compared with males in ApoE KO (twofold: $P = 0.0057$) and DKO (2.5-fold: $P = 0.0043$) mice. Plaques in the BCA were 2.1 times smaller in the DKO compared to ApoE KO males ($P = 0.004$) but there was no significant difference between the two genotypes in the females (**Figure 6B**). Interestingly, ApoE KO males had 2.9 times larger plaques when compared with ApoE KO females ($P < 0.0001$) (**Figure 6B**), which was the opposite of the relationship observed in the aortic sinus. This difference has also been noted before (29). These differences reinforced our decision to stratify our data by gender.

Buried fibrous layers in BCA plaques have been suggested as a marker of plaque complexity or instability (30). However, we observed no linear regression between the number of buried layers and plaque size (P -values range from 0.20 to 0.68, $n = 15–17$) or macrophage content (P -values range from 0.29 to 0.99, $n = 15–17$), which are other measures that have been associated with plaque vulnerability. In any event, there were no significant differences in the number of buried layers in plaques from DKO compared to ApoE KO mice (**Figure 6C**). The area occupied by plaques in the aorta (as demonstrated by *en face* staining with Oil-Red-O) gives another measure of the extent of plaque progression in these mice. This did not change with phenotype or gender (**Figure 6D**).

The comparable plaque size in the aortic root of ApoE KO and DKO mice has been reported previously (20), and was consistent

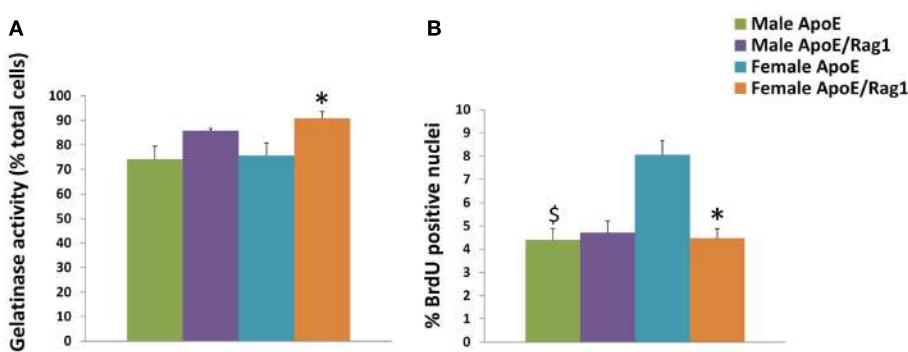


FIGURE 2 | Characteristics of foam cell macrophages (FCMs) obtained from subcutaneous granulomas. Granuloma FCMs were isolated by flotation over a density gradient and differential adhesion from sponges implanted under the skin of ApoE KO or ApoE/Rag-1 DKO mice, as indicated. **(A)** *In situ* zymography (gelatinase activity, mean and SEM, $n = 9–13$). **(B)** Proliferative capacity (8 h BrdU pulse, mean and SEM, $n = 8–14$). * $P < 0.05$ vs. DKO, $^{\$}P < 0.05$ indicates gender differences for that genotype.

indicated. **(A)** *In situ* zymography (gelatinase activity, mean and SEM, $n = 9–13$). **(B)** Proliferative capacity (8 h BrdU pulse, mean and SEM, $n = 8–14$). * $P < 0.05$ vs. DKO, $^{\$}P < 0.05$ indicates gender differences for that genotype.

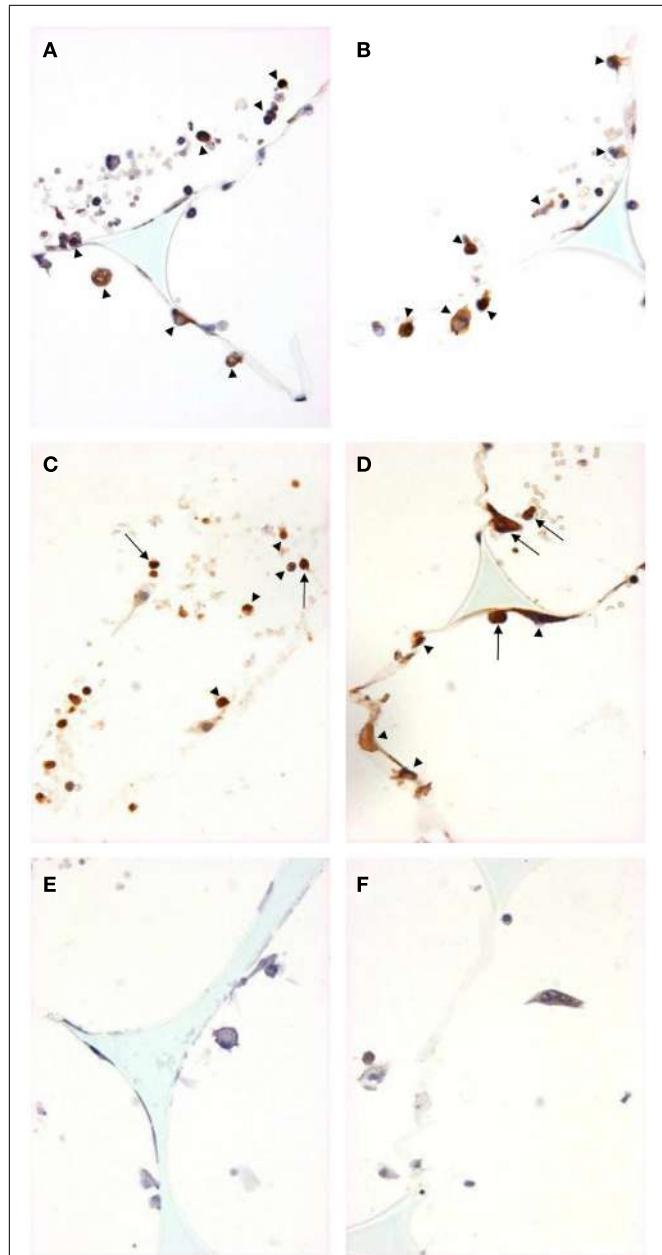


FIGURE 3 | M1 marker staining of sponge sections from ApoE KO or ApoE/Rag-1 DKO mice. Sections from ApoE KO (**A,C,E**) or DKO mice (**B,D,F**) were stained to show immunolocalization of inducible nitric oxide synthase (iNOS) (**A,B**) or cyclooxygenase-2 (COX-2) (**C,D**). Control sections (**E,F**) were exposed to non-immune IgG. Positive staining appears brown (DAB), nuclei blue-purple (hematoxylin), and sponge spicules light blue. Sections from either mouse strain could contain cells with positive cytoplasmic staining (arrowheads). Some cells did not stain, and some appeared to have COX-2 in their nucleus (arrows) (Magnification: $\times 600$).

with the objective of our experimental design. It ensured that any difference in foam cell phenotype in the aortic sinus would be independent of plaque size. However, the larger plaque size in male BCA plaques in ApoE KO than DKO mice could complicate the interpretation of data relating to phenotypes in these mice.

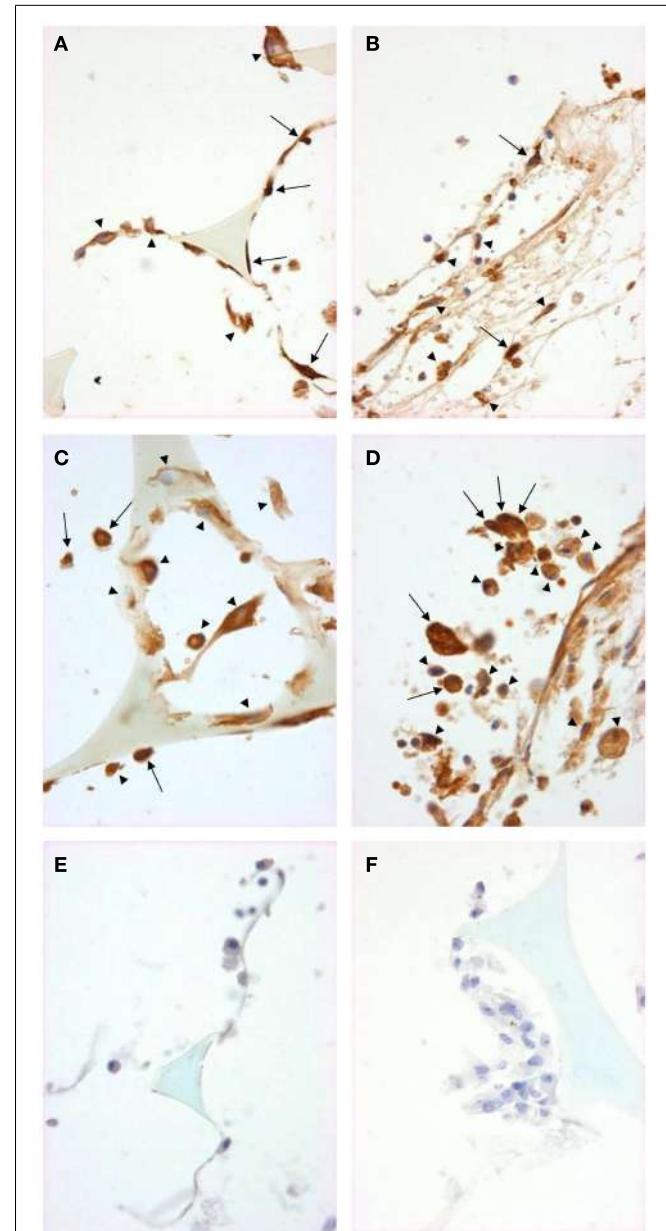
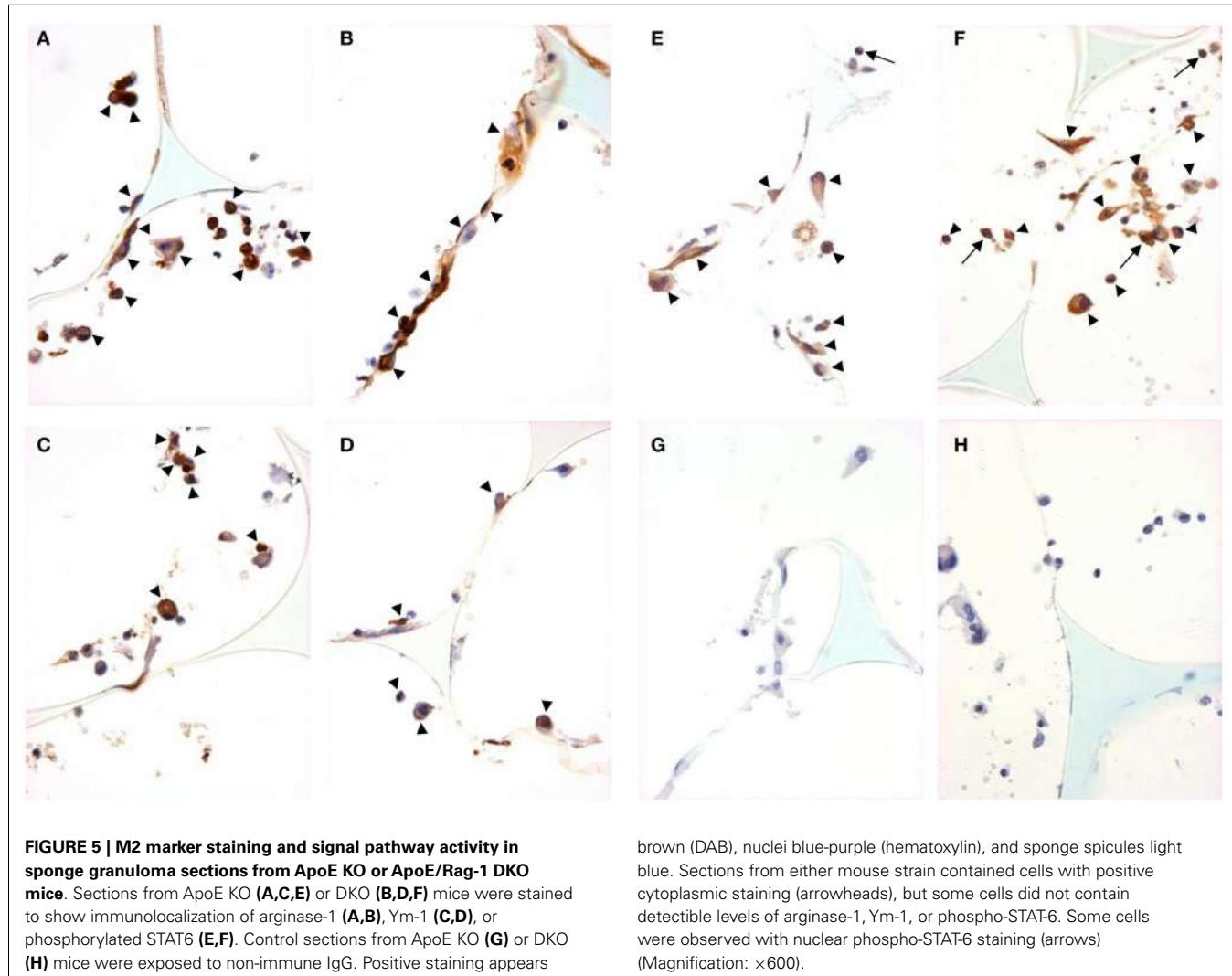


FIGURE 4 | Signal pathway activation in sponge granuloma sections from ApoE KO or ApoE/Rag-1 DKO mice. Sections from ApoE KO (**A,C,E**) or DKO (**B,D,F**) mice were stained to show immunolocalization of NF-κB (**A,B**) or phosphorylated STAT1 (**C,D**). Control sections (**E,F**) were exposed to non-immune IgG. Positive staining appears brown (DAB), nuclei blue-purple (hematoxylin), and sponge spicules light blue. Sections from either mouse strain contained many cells with positive cytoplasmic staining (arrowheads). Some cells did not contain detectable levels of NF-κB or phospho-STAT1, while others appeared to have NF-κB or phospho-STAT1 present in their nucleus (arrows) (Magnification: $\times 600$).

Immunohistochemistry for α -smooth-muscle-cell-actin (α -SM-actin) was used to quantify the presence of vascular SMC and staining for GSL to quantify macrophages. As expected SMC were mainly found in the media and fibrous cap of plaques (Figures 7A–D). Most GSL positive had a foamy



appearance on close examination and were therefore mainly FCMs (**Figures 7A–D**). However, some medial cells, presumably synthetic state SMC or SMC transdifferentiating toward macrophages (31) also stained with GSL (**Figures 7A–D**). Given the small size of atherosclerotic plaques in the AS and BCA, it was impractical to extract mRNA for qRT-PCR, or total protein for Western blotting. We therefore used immunohistological methods to quantify the presence of M1 and M2 markers as well as selected MMPs and TIMP-3. Our *in vitro* studies together with the availability of suitable antibodies (**Table 2**) guided our choice of iNOS as suitable M1 marker and arg-1 and Ym-1 as suitable M2 markers. Based again on our *in vitro* studies and the availability of suitable antibodies (**Table 2**), we chose to study MMP-13 and MMP-14 as potentially related to classical activation. MMP-12 and TIMP-3 were also chosen for comparison because they are abundantly expressed *in vitro*, irrespective of classical and alternative activation. For each of the antibodies and lectin used the staining was specific, both in the aortic sinus (**Figures 7A,B**) and the BCA (**Figures 7C,D**) of either genotype. Interestingly, phenotypic markers, MMPs and TIMP-3

brown (DAB), nuclei blue-purple (hematoxylin), and sponge spicules light blue. Sections from either mouse strain contained cells with positive cytoplasmic staining (arrowheads), but some cells did not contain detectable levels of arginase-1, Ym-1, or phospho-STAT6. Some cells were observed with nuclear phospho-STAT6 staining (arrows) (Magnification: $\times 600$).

were mainly associated with GSL-positive areas rather than α -actin (**Figures 7A–D**). The percentage of the total plaque area stained with each antibody was measured using image analysis. By confining measurements to the plaque, we avoided any influence of staining from the media layer. Furthermore, some of the antibodies stained cardiac myocytes surrounding the aortic root (**Figures 7A,B**) but this did not distort our subsequent measurements because these areas were excluded from the quantification.

The area stained with α -SM actin in the aortic sinus (**Figure 8A**) was less than 20% under all conditions, consistent with the lipid-rich nature of plaques in this model at this time point. Nevertheless, SMC area was 1.5 times higher in the female DKO compared with ApoE KO mice ($P = 0.0051$) and the same trend was seen in the male mice. The pooled data for both genders were also significant ($P = 0.0058$). There were no differences between genotypes in the BCA (**Figure 8B**), although male DKO mice had 3.8-times more SMC staining than females (**Figure 8B**). Plaque areas stained with GSL were 30–40% in AS or BCA, consistent with highly inflamed nature of these plaques. GSL areas in the AS (**Figure 8A**)

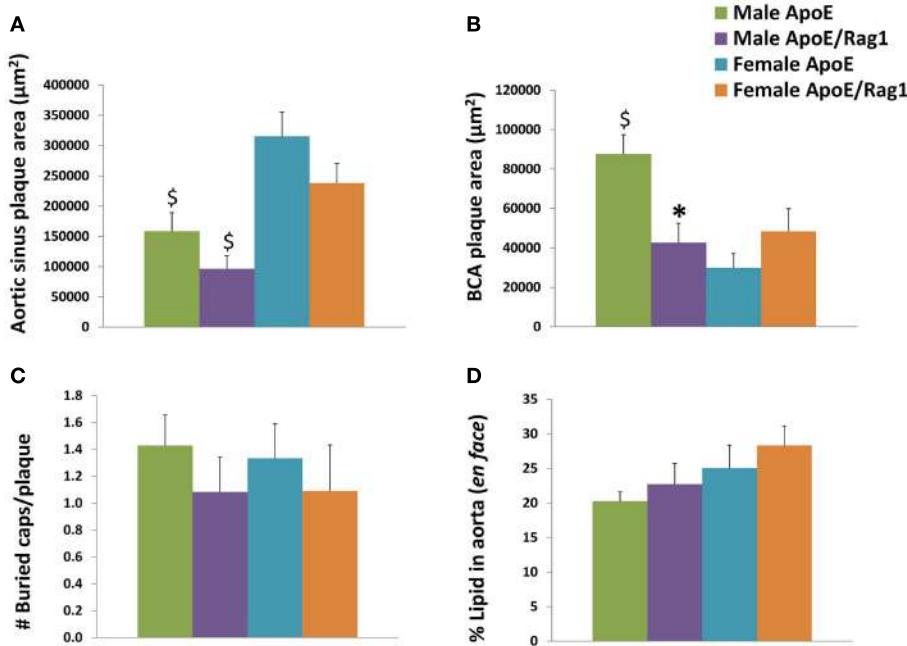


FIGURE 6 | Histological appearances of aortic sinus, brachiocephalic artery and aortic plaques in ApoE KO and ApoE/Rag-1 DKO mice. (A) Area of plaque in the aortic sinus. The first section showing leaflet of the aortic valve was stained using Miller's elastin/van Gieson (EVG) and the area of plaque in each section calculated using computer aided planimetry, $n = 16–22$. **(B)** A section taken 3 μm after the bifurcation of the brachiocephalic artery (BCA) from the aorta was treated and stained and

examined in a similar fashion to A, $n = 13–19$. **(C)** The number of buried layers in plaque from BCA were assessed in EVG and α -SM-actin stained sections, $n = 11–14$. **(D)** Whole aorta were opened longitudinally and stained *en face* for the presence of neutral lipid using Oil-Red-O. The area of lipid-rich plaque in each aorta was calculated using computer aided planimetry, $n = 10–11$. Values are expressed mean and SEM. * $P < 0.05$ vs. DKO, $\$P < 0.05$ indicates gender differences for that genotype.

and BCA (Figure 8B) did not significantly differ in the two mouse genotypes or genders.

Quantitative measurements of staining for iNOS, arg-1, or Ym-1 showed that these areas were similar to or less extensive than GSL (Figures 8A,B), consistent with the concept of their being restricted to subpopulations of the GSL-positive cells (Figures 7A–D). Approximately, the same proportion of cells stained for iNOS and Ym-1, implying that approximately half of the FCMs had M1 or M2 markers, consistent with previous literature (12). Staining for arg-1 was more extensive and appeared to overlap with that for iNOS in some cells (Figures 7A–D and 8A,B). Staining for iNOS tended to be higher in DKO than ApoE KO mice in the aortic sinus (Figure 8A) and BCA (Figure 8B), although this was not significant. Based on this evidence, M1 activation of plaque FCMs occurs and may even be increased in the absence of T and B lymphocytes, irrespective of gender, and consistent with the PCR data on granuloma FCMs described above. Female DKO mice had 1.4-fold more arg-1 staining than ApoE KO mice in the aortic sinus (Figure 8A) and the same trend was seen in the BCA (Figure 8B). There were no differences in arg-1 staining for male DKO and ApoE KO mice at either site (Figures 8A,B). Staining for Ym-1 was also not different between genotypes in the aortic sinus (Figure 8A) or BCA (Figure 8B). Based on this evidence, M2 activation of FCM was present in the absence of T and B lymphocytes in mice of both sexes, and may be increased in the DKO females.

Additional gender differences were also noted. Staining for iNOS in the aortic sinus was 2.4 times greater in the male than female mice of both ApoE KO and DKO mice (Figure 8A). Staining for arg-1 was 2.1 and 1.4-fold higher in male compared with female ApoE and DKO mice, respectively in the aortic sinus (Figure 8A) although not the BCA (Figure 8B). Staining for Ym-1 was similar between males and females of either genotype in the aortic sinus, and in ApoE KO in the BCA (Figures 8A,B).

The areas stained for MMPs-12, -13, -14, and TIMP-3 were extensive in the aortic root and BCA plaques, which shows that these proteins are widely expressed in plaques (Figures 8A,B). The area of staining for MMP-13 and MMP-14 was similar in ApoE KO and DKO mice of either gender in the AS (Figure 8A) or BCA (Figure 8B). Clearly, the absence of T and B lymphocytes had little impact on extent of MMP-13 or MMP-14 staining, consistent with the PCR data obtained from granuloma FCMs. MMP-12, however, showed fourfold increased staining in male DKO compared to ApoE KO mice ($P = 0.0261$), although this was not replicated in the BCA (Figure 8B) or in female mice. The areas of TIMP-3 staining in the BCA were in all cases similar irrespective of genotype or gender (Figures 8A,B). Male mice of either genotype had approximately twice as much staining for MMP-13, MMP-14, or TIMP-3 than females in the AS (Figure 8A), although there was no difference in the BCA (Figure 8B).

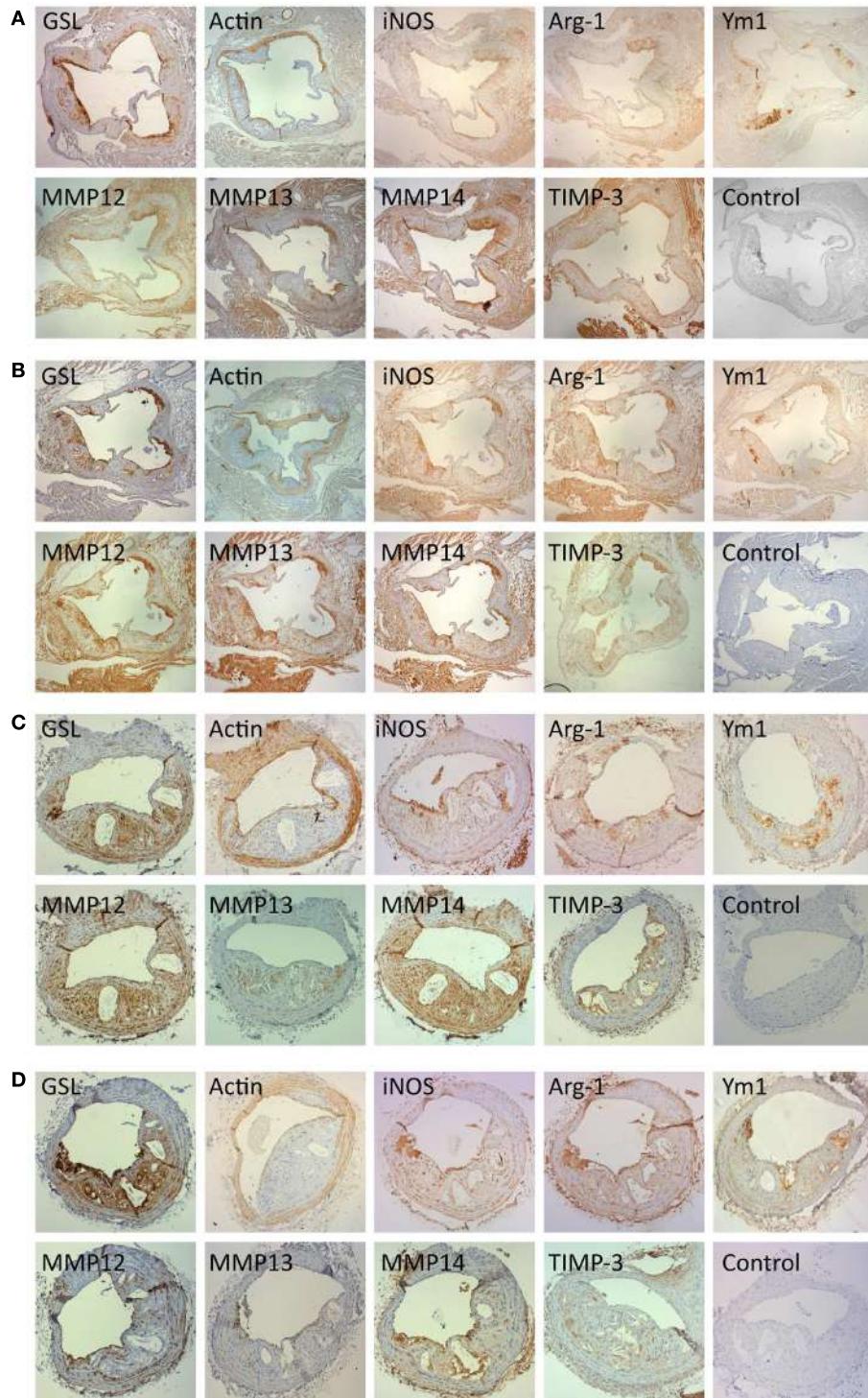


FIGURE 7 | Immunohistochemical staining for cell type, M1 and M2 markers and for MMPs and TIMPs. Near consecutive sections to the section stained with elastin/van Gieson (EVG) were subjected to immunohistochemistry for macrophages [*Griffonia simplicifolia* lectin II (GSL)], smooth muscle cells (α -smooth muscle actin; actin), iNOS, arg-1, Ym-1, MMP-12, MMP-13, MMP-14, and TIMP-3, using the antibodies detailed in

Table 2. Controls were performed with non-immune IgG or normal serum replacing the primary antibody. **(A)** Aortic sinus plaques from ApoE KO mice (Magnification: $\times 4$). **(B)** Aortic sinus plaques from ApoE/Rag1 DKO mice (Magnification: $\times 4$). **(C)** Brachiocephalic artery plaques from ApoE KO mice (Magnification: $\times 10$). **(D)** Brachiocephalic artery plaques from ApoE/Rag1 DKO mice (Magnification: $\times 10$).

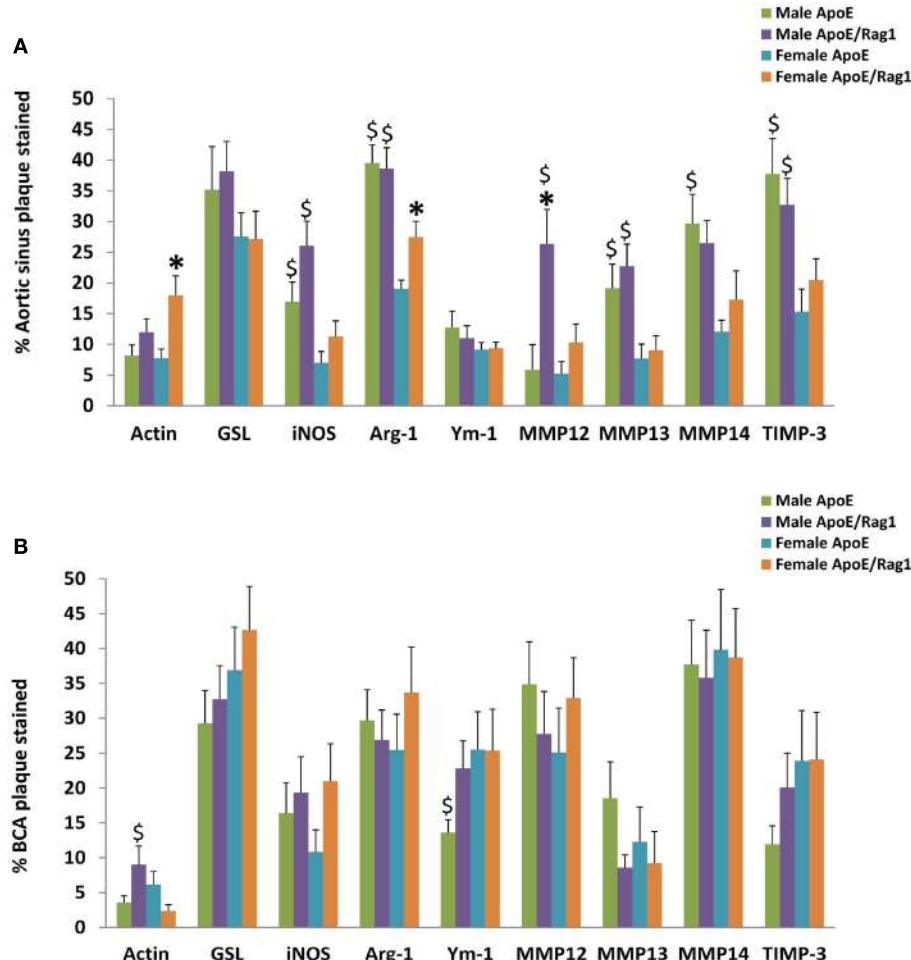


FIGURE 8 | The proportion of staining for cell type, M1/M2 markers, MMPs, and TIMPs in plaques from ApoE KO or ApoE/Rag1 DKO mice. (A) Aortic sinus plaques. **(B)** Brachiocephalic artery plaques. Values are expressed as mean and SEM. * $P < 0.05$ vs. DKO, \$ $P < 0.05$ indicates gender differences for that genotype, $n = 11–19$.

DISCUSSION

The clear conclusion from our experiments is that FCMs in subcutaneous granulomas and atherosclerotic plaques acquired markers of classical (M1) and alternative (M2) activation even in the absence of lymphocytes. This conclusion was valid in male and female mice, despite differences in the size and composition of plaques between the two genders. Furthermore, since nuclear localized NF- κ B, phospho-STAT-1 and phospho-STAT-6 were detected in granuloma FCMs, activators of the pathways leading to M1 and M2 polarization also existed in adequate quantities in the presence or absence of T or B lymphocytes. We also observed that expression of several MMPs and TIMPs occurred in granuloma and plaque FCMs *in vivo* independently of T and B lymphocytes.

Recent work demonstrated that foam cell formation did not, in itself, lead to M1 or M2 activation (28). By contrast, peritoneal FCM generated *in vivo* were resistant to M1 activation, owing to stimulation of the LXR pathway. Nevertheless, several studies showed that there are FCMs in mouse atherosclerotic plaques that

express both M1 and M2 markers either *in situ* (12, 13) or after collagenase isolation (32). M1 and M2 markers were associated with distinct cell populations even though they had overlapping distributions in the intima of advanced mouse plaques (32). Our histological observations confirmed these findings but showed, in addition, that FCMs produced in the context of the foreign body reaction caused by a polyurethane sponge implantation also prominently expressed M1 and M2 markers. Granuloma FCMs mimicked the gene expression pattern of plaque FCMs and may therefore be useful as a more-easily isolated surrogate. Functionally, iNOS and arg-1 appear to compete for substrate. Indeed, in a previous study, we demonstrated this directly in rabbit FCMs where down-regulation of arg-1 compared to non-foamy macrophages led to decreased urea and increased nitrate release (24).

In previous papers dealing with atherosclerosis, it has been acknowledged that Th1- and Th2-lymphocyte related cytokines can polarize macrophages toward M1 and M2 phenotypes, respectively (11–13). This suggests that lymphocytes and hence,

by implication, adaptive immunity plays an essential role in macrophage polarization, albeit in concert with other factors (11). On the contrary, our new data show clearly that M1/M2 polarization of FCMs can take place efficiently in the absence of T and B cells. To do this, we recreated a previously-characterized cross (20) between the well-established ApoE KO mouse and the equally well-characterized Rag1 KO mouse. We obtained similar development of plaques in these mice as previously observed (20) and then went on to make novel observations of macrophage phenotypes in subcutaneous granulomas and plaques.

It is worth noting that the absolute copy numbers of the M1 and M2 marker mRNAs measured in granuloma FCMs were of the same order of magnitude as those in measured in classical and alternatively activated macrophages *in vitro* (compare **Table 5** to **Figure 1A**). Despite the obvious limitations of comparing data across such different experimental conditions, it is hard to escape the conclusion that the FCMs produced *in vivo* express M1 and M2 marker genes to a substantial degree, irrespective of the presence of lymphocytes. Moreover, the canonical pathways of classical activation, NF- κ B and STAT-1, and alternative activation, STAT-6, are also triggered in T and B cell deficient mice. One possible explanation is that TLR-4 mediated induction of so-called interferon response factors (IRFs, specifically IRF-3 and IRF-7) can lead to the secretion of IFN α and IFN β , thereby bypassing the requirement for IFN γ (9). Plaques contain several potential activators of TLRs (33); and these could well be the sources of classical activation. In addition to TLR agonists, other stimulators of the NF- κ B pathway, including TNF α and IL-1, that are known to occur in mouse atherosclerotic plaques (3) could also act as classical activators independently of IFN γ , as shown in many previous studies (3, 14). The presence of these alternative mediators therefore provides a rationale for M1 activation in T and B cell depleted mice, although additional experiments beyond the present scope would be needed to identify the specific mediators. M2 polarization can also occur in response to a variety of mediators, although activation of STAT-6 appears to indicate the mediation of IL-4 and/or IL-13 in our mice even in the absence of lymphocytes. An additional, non-exclusive explanation for our findings is that there are sources other than lymphocytes for the cytokines associated with M1 and M2 activation in mice. For example, natural killer cells were shown to be an active source of IFN γ in Rag-1 KO mice (34) and could therefore account for the residual levels of IFN γ we observed in the blood of ApoE/Rag-1 DKO mice (**Table 3**). Likewise, mast cells (35) and neutrophils (36) are plausible sources of IL-4 and IL-13 in lymphocyte-depleted mice.

MMPs have been strongly implicated in the progression of atherosclerosis, and more particularly in ECM degradation as well migration, proliferation and apoptosis of vascular cells (5, 37). The results in **Figure 1B** showing that MMP-2, MMP-9, MMP-13, MMP-14, and MMP-25 were up-regulated and TIMP-2 down-regulated in mouse macrophages during classical activation are consistent with previously reviewed data for MMP-9 and MMP-13 (38). However, IFN γ had no effect on MMP or TIMP mRNA expression in mouse macrophages at the 18-h time point we used (**Figures 1C,D**), which was chosen to allow time for priming effects to be observed. Up-regulation of MMP-19 and down-regulation of MMP-14 were the only changes that we observed in response

to IL-4 (**Figure 1B**). Furthermore, there was no difference in the expression level of any of the MMPs or TIMPs in FCMs isolated from sponge granulomas in ApoE KO and DKO mice. Despite this, there was a small increase in the proportion of cells able to degrade gelatin in DKO mice, which might be explained by the trend toward increases in MMP-2 and MMP-9 mRNA expressions. Further experiments would be needed to verify this. Recent work has placed increased emphasis on macrophage proliferation in mouse atherosclerosis (39). Specifically, it has been suggested that proliferation, rather than recruitment, is the major factor leading to accumulation of FCMs into atherosclerotic plaques of ApoE null mice at early time points (40). We found a relative decrease in FCMs from female DKO mice, but this did not appear to be associated with differences in M1/M2 polarization or MMP expression. Turning to our immunohistochemical studies of atherosclerotic plaques, few changes were noted in the extent of MMP-12, -13, -14, or TIMP-3 staining. Only MMP-12 staining appeared to be increased in male DKO mice in the AS but not BCA. Since, we found no effect of classical or alternative activation on MMP-12 expression *in vitro*, this isolated observation might be explained by another mediator such as GM-CSF, which has been shown to up-regulate MMP-12 in several, previously reviewed studies (38). However, the plasma cytokine levels (**Table 3**) provide no corroboration for this contention.

During the course of our studies, we noted significant differences in cytokine and lipid levels, plaque sizes, and content of SMC between male and female mice that obliged us to consider these data separately. We found smaller AS plaques in male than female ApoE KO mice, similar to what has been previously noted and attributed to the effects of estrogens (19) and prostaglandins (41). On the other hand, we found that males develop larger lesions in the BCA, confirming what we previously published in thesis form (29) and consonant with findings in the aorta at longer time points (18). This is most likely related, at least in part, to the higher total cholesterol and VLDL + LDL levels, we observed in male mice (**Table 4**). The important fact to stress, however, is that acquisition of M1 and M2 markers and expression of MMPs and TIMP was independent of T and B lymphocytes, irrespective of the gender of mice we analyzed.

Human atherosclerotic plaques also have prominent populations of FCMs that show M1 markers (42, 43) and have been known for many years to have nuclear localized NF- κ B (44). Consistent with our present results, work on cells isolated from human atherosclerotic plaques, placed emphasis on innate immune mechanisms, by showing that TLR-2 activation plays an important role in M1 polarization and MMP secretion (45). There are also foci of FCMs in the intima that express M2 markers, which are distinct from FCMs carrying M1 markers (11). Non-foamy macrophages carrying M2 markers are even more prevalent in the adventitia (43). Hence, the distribution of cells carrying M2 markers in human plaques appears to be more restricted than in the mouse plaques in our study (**Figures 7A–D**) and in those previously published (12, 13).

With respect to MMPs and TIMPs, comparison of our *in vitro* results with published data from human macrophages isolated and incubated under very similar conditions (38, 42, 46) demonstrates many differences. For example, MMP-1 is absent but MMP-13

is abundant in mouse macrophages, whereas MMP-13 is absent and MMP-1 is abundant in human macrophages (42, 47), consistent with the limited distribution of MMP-13 in human tissues (48). Furthermore, MMP-12 is apparently much more abundant in mouse macrophages (**Figure 1B**) than human macrophages (42). Conversely, mRNAs for MMP-2, MMP-3, MMP-7, MMP-9, MMP-10, MMP-11, MMP-17, TIMP-1, and TIMP-3 appear much less abundant in mouse macrophages (**Figure 1B**) than human macrophages (42). Only MMP-8, MMP-14, MMP-19, MMP-25, and TIMP-2 show similar (within 10-fold) abundance in both species at the mRNA level. Responses of MMPs and TIMPs to classical and alternative also present stark contrasts between mice and men, under the conditions of our experiments. For example, expression of MMP-1, MMP-3, MMP-7, MMP-10, MMP-12, and TIMP-1 was increased by classical activation of human macrophages, whereas MMP-9 was constitutive (42), in contrast to what we observed here (**Figure 1B**). Furthermore, IFN γ increased MMP-1, MMP-10, MMP-12, and MMP-14 expression and decreased TIMP-3 expression in human macrophages (42), none of which we observed in mouse macrophages at the same time point (**Figure 1C**). Finally, IL-4 treatment increased MMP-11, MMP-12, and TIMP-3 expression in human macrophages (42) but MMP-19 expression in mice (**Figure 1B**). Similar disparity has been previously noted with respect to the expression levels of M1 and M2 markers in human and mouse macrophages (15). Moreover, the overall transcriptomic response to several *in vivo* models of inflammation appears highly divergent in mice and men (49). These limitations therefore caution against over-extrapolating our present results from a mouse model to human atherosclerosis.

In conclusion, our results definitively counter the hypothesis that lymphocytes are necessary for M1 or M2 polarization in mouse atherosclerosis, although more work will be needed to define the mediators responsible. Lymphocytes are also not needed for MMP and TIMP expression in FCMs *in vivo*. However, our studies do not rule out a modulatory role for T or B lymphocytes on either macrophage polarization or MMP production. It is conceivable that deletion of different lymphocyte populations has opposing effects of macrophage and foam cell activation, leading to a neutral effect overall. Subsequent studies using more selective interventions will be needed to investigate the role of specific lymphocyte subsets in mice.

ACKNOWLEDGMENTS

This work was supported by funding from the British Heart Foundation. The authors would like to thank Mrs. Kristina Baker for her help with some tissue harvests and KWS BioTest Ltd. for measuring the cytokine levels.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 29 July 2014; **accepted:** 10 October 2014; **published online:** 28 October 2014. **Citation:** Hayes EM, Tsaousi A, Di Gregoli K, Jenkinson SR, Bond AR, Johnson JL, Bevan L, Thomas AC and Newby AC (2014) Classical and alternative activation and metalloproteinase expression occurs in foam cell macrophages in male and female ApoE null mice in the absence of T and B lymphocytes. *Front. Immunol.* **5**:537. doi:10.3389/fimmu.2014.00537

This article was submitted to Inflammation, a section of the journal *Frontiers in Immunology*.

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Cholesterol efflux pathways regulate myelopoiesis: a potential link to altered macrophage function in atherosclerosis

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Atherosclerotic cardiovascular disease is a chronic inflammatory disease of the blood vessels that can lead to myocardial infarction or stroke. The major cell in the atherosclerotic lesion, the macrophage, is thought to be an important contributor to the production of inflammatory mediators that exacerbate this disease. Macrophages are generally derived from circulating monocytes, which are in turn produced by hematopoietic stem and multipotential progenitor cells (HSPCs) in the bone marrow and other medullary organs. Recent studies suggest that disruption in cholesterol homeostasis or prolonged exposure to a hypercholesterolemic environment can influence HSPCs to over-produce monocytes, resulting in monocytosis. These monocytes may carry a pre-programmed ability to become M1-like macrophages once they enter the atherosclerotic lesion. Future studies may help to differentiate the role of such pre-programming versus responses to local environmental cues in determining M1, M2, or other macrophage phenotypes in atherosclerotic lesions.

Keywords: atherosclerosis, hematopoiesis, cholesterol efflux, monocytes, macrophages, HDL

Innate immunity has long been considered a driving force in the initiation and progression of atherosclerotic cardiovascular disease (CVD) (1). Indeed, inflammation is a process that has attracted considerable attention as a potential therapeutic target in atherosclerosis. It has also become well accepted that cholesterol metabolism is intimately linked to inflammation and innate immune processes. This close relationship is not only important in the effector cells of atherosclerotic disease such as monocytes (2) and macrophages (3) but cholesterol metabolism has also been shown to play a central role in their hematopoietic precursors (4, 5). This is important as changes in cholesterol homeostasis in the hematopoietic stem and multipotential progenitor cells (HSPCs) control the rate of production of monocytes/macrophages, and possibly have an influence on their function (4–6). Increased numbers of circulating monocytes are a predictor of cardiovascular risk (7–14) and studies in mice have shown a causal role (4–6, 15, 16). These key studies in mice have also revealed that HSPCs can mobilize from the bone marrow (BM) to extramedullary sites such as the spleen (4–6, 17), where they can also produce monocytes that contribute to atherogenesis (6). In this article, we will review these topics and also explore the hypothesis that the mechanisms contributing to monocyte production from HSPCs could also influence the type and function of lesional macrophages.

INNATE IMMUNE CELL PRODUCTION AND CARDIOVASCULAR RISK

Monocytosis is associated with CVD and atherosclerotic plaque severity in prospective and cross-sectional human studies (7, 11–13). Monocytosis is also closely linked to plasma lipids, where

a positive correlation is observed with total cholesterol levels (15, 18–20), and an inverse correlation with plasma high-density lipoprotein (HDL) levels (11, 14, 20, 21). Gerrity et al. first suggested that excessive monocyte production contributed to atherosgenesis in rabbit and pig 1 models of hypercholesterolemia and atherosclerosis (18, 19). These studies also made the link between hypercholesterolemia and enhanced monocyte production from the BM using colony-forming assays and suggested that this could be driving the atherogenic phenotype (18). Through the use of mouse models, a causal relationship between monocyte levels and severity of atherosclerotic lesions has been shown (4, 5, 15). Studies employing the *op/op* mouse that carries a mutation in the gene encoding macrophage colony-stimulating factor (CSF-1; M-CSF) have a gene dose-dependent decrease in monocyte levels that is reflected by smaller atherosclerotic lesions (22). Conversely, western diet (WTD)-fed *Apoe*^{-/-} mice display monocytosis that is proportionate to the length of feeding and reflects the size of the atherosclerotic lesion (15). We have also shown that monocytosis, largely independent of activation, accelerates atherosclerosis in mouse models (4). In addition to the abundance of monocytes that circulate, the site of production may play an important role, as monocytes produced in the spleen appear to have an atherogenic phenotype (6). While monocytes may directly contribute to atherosclerosis by secreting inflammatory cytokines, ROS, and proteases, their most important role is probably to act as precursors to lesional macrophages. Macrophages are a heterogeneous population of cells and have been categorized into two main groups known as M1 and M2. This classification is based on function and the expression of a number of genes. M1 macrophages

are thought to be inflammatory cells, expressing a gene signature including *iNos*, *IL-6*, *Tnf- α* , and *IL-1 β* , while M2 cells are thought to play a resolving role and expression genes such as *IL-10*, *Tgf- β* , and *Arg1*. However, these cells due retain plasticity and can sit at various points along the scale [see recent reviews on suggested nomenclature (23, 24)]. In *Apoe*^{-/-} mice, CCR2⁺ Ly6-Chi monocytes preferentially enter the atherosclerotic lesion (15, 16), and this monocyte subset has been suggested to differentiate into a macrophage with an inflammatory phenotype. Interestingly, lesional macrophages can also undergo local proliferation to sustain their population within the advanced atherosclerotic plaque (25). The phenotype of proliferating macrophage or its product cells has not yet been studied in detail; however, as the proliferation of these cells is dependent on SR-A (25), it could perhaps be of the M2 variety (26). Below we will discuss how defects in cholesterol metabolism pathways influence the HSPCs, monocytes, and macrophages to promote atherosclerosis, and will make the speculative suggestion that events in the hematopoietic stem and progenitor populations may influence the ultimate functions of the macrophage.

CHOLESTEROL EFFLUX PATHWAYS LINK HSPC PROLIFERATION, MONOCYTE PRODUCTION, AND ATHEROSCLEROSIS

Impaired cholesterol efflux has long been associated with atherosclerosis, and more recently, the ability of HDL to promote efflux from cholesterol loaded cells was shown to be a stronger predictor or atherosclerotic burden than HDL cholesterol or apoA-I levels (27). In line with this is the experimental evidence in animal models of atherosclerosis where increasing HDL levels either therapeutically (rHDL infusions) (28) or genetically (ApoA-I transgene) (5, 29) is protective. This is thought to be due to the ability of HDL or ApoA-I to prevent foam cell formation, inhibit leukocyte adhesion, and protect the endothelium from activation (30, 31). However, recent studies have shown that HDL via cholesterol removal from the cell membrane can regulate the production of innate immune cells (4, 5, 32), particularly monocytes, by acting on HSPCs (4, 5). In respect to the anti-atherogenic properties of HDL, this may be an important function that could affect the types and/or functions of the downstream cells that eventually mature into lesional macrophages.

The removal of cholesterol from HSPCs can be facilitated by a number of pathways. We discovered that HSPCs express *Abca1*, *Abcg1*, and *Apoe* at high levels and these key efflux genes could further be induced *in vivo* by the administration of Liver-X-Receptor (LXR) agonists (4). Co-deletion of two key cholesterol efflux genes ATP bind cassette transporter (ABC) A1 and *Abcg1*, in the hematopoietic compartment and transplantation into *Ldlr*^{+/-} mice resulted in prominent monocytosis and neutrophilia, which was accompanied by a dramatic acceleration in atherosclerotic lesion formation (5). A myeloproliferative phenotype was suggested, as myeloid cells infiltrated many major organs, including the spleen, liver, and intestine. Mice with *Abca1/g1* KO BM had a dramatic expansion of the HSPCs, which were proliferating at higher rates compared to mice that received WT BM. The enhanced proliferation in the *Abca1/g1* KO HSPCs was found to be due to an increase in the expression of the common

β subunit of the IL-3/GM-CSF receptor (IL-3R β ; aka CD131), making these cells more sensitive to these cytokines. Promoting cholesterol efflux with an apoA-I transgene reversed the proliferative defects and reduced the severity of the atherosclerosis. *Abca1*^{-/-}, *Abcg1*^{-/-}, and *Apoe*^{-/-} HSPCs also mobilized into the circulation in increased amounts and established extramedullary hematopoiesis in the spleen and other organs (17). These sites of extramedullary hematopoiesis provide an important reservoir for monocytes in acute coronary disease (33), highlighting the multiple links between hypercholesterolemia, defective cholesterol efflux pathways, and the over-production of monocytes and neutrophils that contribute to atherosclerosis.

As mentioned above, we and others have also reported that WTD-fed *Apoe*^{-/-} mice display prominent monocytosis (4, 15, 16). We found that this was also due to expansion and proliferation of the HSPCs as a result of increased expression of the IL-3R β . Treating *Apoe*^{-/-} mice with reconstituted HDL (rHDL; CSL-111) to promote cholesterol efflux normalized this proliferative defect (4). The role for the IL-3R β in promoting HSPC proliferation and monocytosis in *Apoe*^{-/-} mice was confirmed in mice with deficiency of both genes (34). Through the use of competitive BM transplant (cBMT) studies, we found that these efflux pathways at least partly functioned in a cell intrinsic manner (4). For example, deletion of *Apoe* in cells marked by CD45.2 produced more monocytes and lesional macrophages compared to WT cells marked by CD45.1 that were transplanted into the same recipients. We also found that the *Ldlr*^{-/-} mice that received the mix of *Apoe*^{-/-}(CD45.2)/WT(CD45.1) had larger lesions compared to those that received WT(CD45.2)/WT(CD45.1). The increase in lesion size was independent of monocyte activation and supports the idea that increased production of monocytes directly impacts lesion monocyte/macrophage content, size, and severity. However, we speculate that other explanations may be involved, including that increased entry of *Apoe*^{-/-} monocytes results in macrophages that have an altered phenotype/function, or that alterations in cholesterol metabolism in HSPCs pre-program their daughter cells (i.e., monocytes and macrophages) into an inflammatory phenotype (**Figure 1**).

To further explore the contribution of cholesterol efflux in macrophages versus HSPCs, cell specific knockouts of *Abca1*/*Abcg1* have been employed to examine the role of these transporters in cells down stream of HSPCs (35). Using the Lysozyme M Cre mouse crossed with *Abca1*^{fl/fl} *Abcg1*^{fl/fl} mice (Mac-DKO), Westerterp et al. (35) were able to reduce the expression of *Abca1*/*Abcg1* by approximately half in the granulocyte-macrophage progenitors (GMPs) and monocytes, and by ~90% in macrophages. Transplantation of the *Mac*^{ABCDKO} BM into *Ldlr*^{-/-} mice resulted in a ~1.7-fold increase in atherosclerosis plaque area compared to the mice that received the control BM. However, the lesions in the mice that received *Abca1/g1* KO BM has significantly larger lesions (~3-fold) compared to control and *Mac*^{ABCDKO} mice (~1.7-fold). This implies that there is a major effect of cholesterol efflux in cells upstream of macrophages in controlling atherosclerosis, likely HSPCs.

The studies of Westerterp et al., in the *Mac*^{ABCDKO} mice also provided a novel insight on effects of altered cholesterol homeostasis in regulating the production of monocytes from the BM.

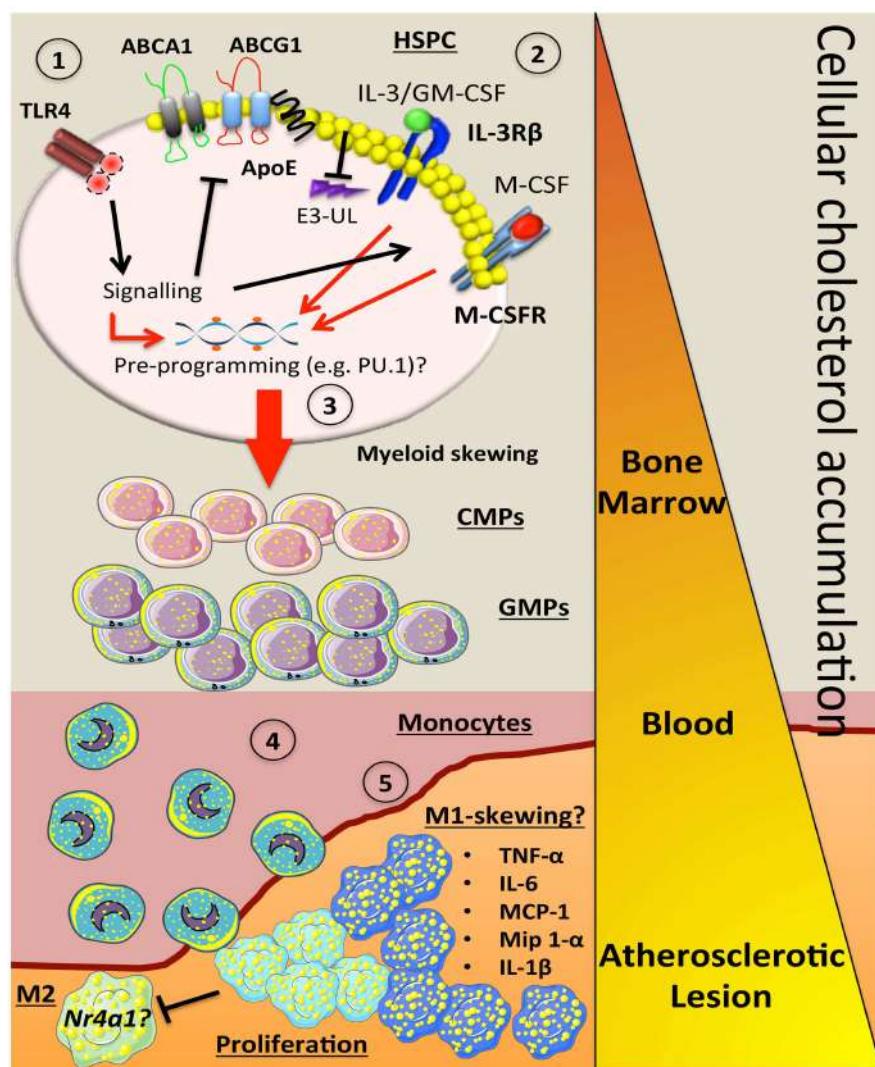


FIGURE 1 | How alterations in cholesterol metabolism and myeloid skewing contribute to atherosclerosis. In the setting of hypercholesterolemia, inflammatory signals could be sensed by receptors such as TLR4 on HSCPs to trigger a number of downstream signaling events. This could (1) inhibit key cholesterol efflux pathways (ABCA1, ABCG1, ApoE), which would result in cellular accumulation. The increase in membrane cholesterol could lead to (2) increased cell surface expression of cytokine receptors such as IL-3R β and the M-CSFR due to a failure to activate E3-ubiquitin ligases (E3-UL). (3) Sustained signaling from myeloid cytokines (IL-3, GM-CSF, M-CSF) along with the hypercholesterolemic environment could pre-program the HSPC via PU.1

to produce more myeloid cells. As these cells mature into CMPs and GMPs, they have the potential to carry more cellular cholesterol if their cholesterol efflux pathways are suppressed. (4) Once the blood monocyte is circulating, more lipid is acquired and it can carry this into the atherosclerotic plaque. (5) These lipid-laden monocytes could then differentiate into an M1-like macrophage that can also undergo local proliferation, which enhance inflammation by producing a number of cytokines and chemokines. These M-1 cells may also have a defect in *Nr4a1* and lack the ability to convert into M-2 resolving macrophages. Triangle indicates cellular cholesterol accumulation as the myeloid cells mature (orange to yellow).

Macrophages deficient in *Abca1/Abcg1* produced more M-CSF, G-CSF, and MCP-1, key cytokines involved in myelopoiesis and monocyte emigration from the BM (36). Interestingly, there was also a significant number of lipid-laden foam cells in the BM and spleen of the *Mac^{ABCDKO}* mice, probably representing BM monocytes and macrophages. Consistent with this idea, there was a significant portion of the blood monocytes from the *Mac^{ABCDKO}* mice that were loaded with cholesterol. Thus, deletion of *Abca1/Abcg1* in hematopoietic progenitors (i.e., GMPs)

could predispose these and their daughter cells (monocytes) to accumulate cholesterol. This could result in monocytes carrying lipid into the atheroma and perhaps facilitating their maturation into M1 macrophages as the macrophages from the *Mac^{ABCDKO}* mice also displayed enhanced inflammatory gene expression (Figure 1). Additionally, *in vitro* migration studies revealed a severe migratory defect of cultured macrophages deficient in *Abca1/Abcg1* (37), how this translates into the *in vivo* setting is unknown.

MONOCYTE TO MACROPHAGE DIFFERENTIATION

The origin of the macrophage itself is not always from a blood monocyte (38). Yolk-sac derived tissue macrophages can sustain their population under steady-state conditions, without recruitment of blood monocytes (39–41). After an inflammatory insult, blood monocytes can be recruited to increase the macrophage pool and to enhance the inflammatory response (39–41). In the heart, an organ with abundant resident macrophages that are established during embryonic development, it was found that CCR2⁺ Ly6-C^{hi} monocyte-derived macrophages coordinate the inflammatory response after cardiac injury by AngII infusion (39) or myocardial infarction (MI) (39). These Ly6-C^{hi} monocytes express *Nr4a1*, a transcription factor critical in the development of Ly6-C^{lo} monocytes (42), at low levels (43). However, in the healing phase after a MI, *Nr4a1* (Nur77) levels are increased permitting the maturation and differentiation of Ly6-C^{hi} monocytes into Ly6-C^{lo} monocyte/macrophages (43). These macrophages contribute to healing and tissue remodeling by producing factors such as TGF- β , IL-10, and VEGF- α . In essence, these studies revealed that the Ly6-C^{hi} monocyte orchestrate the initial inflammatory event, likely by forming M1 macrophages and then also develop into the reparative, M2-like macrophage (43).

Extending these key findings to the atherosclerotic lesion, as M1 macrophages can develop into M2 macrophages after *Nr4a1* induction (43), and deletion of *Nr4a1* results in M1 polarized macrophages and increased atherosclerosis (44), it is possible that the environment of the atherosclerotic lesion could affect the M1 macrophages resulting in a failure to upregulate *Nr4a1* and prevents the differentiation into M2 cells (Figure 1). It should also be noted that Ly6-C^{lo} monocytes do enter the lesion (45), and while these cells could become M2-like macrophages, they may not frequent the lesion in large enough numbers to make an impact.

Another newly discovered macrophage subset is the Mox macrophage. These macrophages are distinct to the classical M1 or M2 macrophage, as these cells display a unique gene expression profile with induction of redox-related genes including heme oxygenase-1 under the control of the transcription factor *Nrf2* (46). Mox macrophages also display a decrease in phagocytic and chemotactic capacity. Interestingly, both M1 and M2 macrophages can differentiate into the Mox macrophage when incubated with oxidized phospholipids. The *in vivo* relevance of these cells is noted as approximately 30% of all lesional macrophages are of the Mox phenotype.

HYPERCHOLESTEROLEMIA INFLUENCES HSPCs TO PRODUCE ATHEROGENIC MACROPHAGES

It is clear from animal studies that a hypercholesterolemic environment enhances the production of myeloid cells, namely monocytes, which contribute to atherosclerosis. However, a hypercholesterolemic environment could also induce a “memory” effect in the HSPCs, which could also alter the function of their daughter cells. This hypothesis was recently explored by Seijkens and co-workers (47). Similar to our studies (4), they found that hypercholesterolemic *Ldlr*^{-/-} mice had an expanded pool of HSPCs in the BM. Interestingly, when they harvested the BM from hypercholesterolemic *Ldlr*^{-/-} mice and transplanted it competition with BM from normocholesterolemic mice, they found that the BM

from the hypercholesterolemic mice had an enhanced propensity to produce myeloid cells (47). This was even observed in a normocholesterolemic environment. Evidence was provided to support the hypothesis that the hypercholesterolemic-primed HSPCs produced atherogenic (i.e., M1) macrophages as the macrophages from these HSPCs produced higher amounts of TNF- α , IL-6, and MCP-1. It was also found in the subsequent atherosclerosis studies that hypercholesterolemic-primed HSPCs produced leukocytes that more readily entered the atherosclerotic lesion. This resulted in larger more macrophage-rich lesions.

The cbMT studies into hypercholesterolemic and normocholesterolemic mice suggest that there is a memory effect in the HSPCs (47). This idea was recently brought to light by Kampen et al., who discovered that BM harvested from WTD-fed mice has a loss of epigenetic control of key myeloid genes such as PU.1 and IRF8 (48). Transplantation of the BM from the WTD-fed mice into *Ldlr*^{-/-} recipients, like the studies of Seijkens et al., also resulted in larger lesion compared to recipient mice that received BM from chow fed donors. Consistent with the changes in PU.1 and IRF8, the WTD-conditioned BM produced more leukocytes, particularly of the myeloid variety. There were also signs of extramedullary hematopoiesis as the WTD-conditioned BMT mice had splenomegaly. However, one caveat of this study was the mice that received the WTD-conditioned BM-developed hyperglycemia, which has been shown to have independent effects on BM progenitors to induce monocyte production and contribute to atherosclerosis (49).

Another important point to note is that these studies either performed BMTs using total BM or the total pool of HSPCs and not just the long-term repopulating cells. Thus, as we have also noted, a predominant expansion of the multipotential progenitor 2 (MMP2) HSPCs that is thought to give rise to myeloid cells in *Apoe*^{-/-} mice (4), it is possible that hypercholesterolemia-priming promotes the expansion of a subset of HSPCs that preferentially produces atherogenic myeloid cells.

EARLY MYELOID LINEAGE SKEWING IN ATHEROSCLEROSIS: EMERGING CONCEPTS

The idea is emerging that signaling events in hematopoietic stem cells (HSCs) are able to influence lineage selection in these cells. Recently, it was discovered that HSCs express the M-CSF receptor and the engagement with M-CSF activated the myeloid master regulator, PU.1 (50). Injection of mice with LPS increased M-CSF levels and PU.1 expression in HSCs, which is likely to be the initiating step of myeloid lineage skewing in response to an infection. However, the LPS receptor TLR4 is also expressed on BM stem and progenitor cells (51) and could have been an additional contributor to the early lineage selection in these studies. Linking these findings with cholesterol metabolism, macrophages deficient in either *Abca1* and/or *Abcg1* express more TLR4 on their surface and like *Apoe*^{-/-} macrophages are more responsive to TLR4 ligands (3, 52, 53). Thus, it is also conceivable that defective cholesterol efflux pathways in HSCs could lead to enhanced expression of TLR4 that could sense endogenous ligands, priming these cells to sense myeloid promoting cytokines. Whether ligands of pattern recognition receptors (PRRs) such as damage associated molecular pattern (DAMPs) (including S100A8/A9 and

HMGB1), heat shock proteins, and modified LDL particles (54), some of which are increased in people with CVD, are present in the BM and bind to TLR4 on HSPCs is unknown. Assuming TLR4 ligands are present within the stem cell niche, it is possible that their interaction with TLR4 on HSPCs could downregulate *Abca1*, *Abcg1* (55), and *Apoe* (56) by the activation of IRF3, preventing LXR activating these target genes (57). This lead to increased cholesterol in the cell membrane and increased levels of cytokine receptors (4, 5). This could occur through the prevention of key feedback loops, such as activation of the E3-ubiquitin ligase c-CBL, which we recently reported was perturbed in progenitor cells lacking ABCG4 (32), and is also reported to downregulate the M-CSFR (58). Taken together, it is conceivable that defective cholesterol efflux and a hypercholesterolemic environment could influence the HSCs to respond to myeloid promoting cytokines to produce more monocytes that may have an altered function, which could ultimately contribute to the pool of inflammatory lesional macrophages in the atherosclerotic plaque.

Dissecting out the contribution of changes in the HSPCs to the function of the macrophage will be critical in further understanding the mechanisms contributing to not only atherogenesis but also lesion regression. The lesion milieu is also critically important, and is a dynamic environment with the newly recruited cells also contributing to and being influenced by the environment. However, taken together, the emerging theme from recent literature suggests that therapeutic interventions aimed at targeting HSPCs (i.e., cholesterol efflux pathways) may be an effective strategy to treat atherosclerosis by not only inhibiting monocyte production and entry into lesions but also to change the function/phenotype of the mature macrophage.

ACKNOWLEDGMENTS

Andrew James Murphy was supported by a Viertel award from Diabetes Australia Research Trust Australia, and a National Health and Medical Research Council program grant (APP10363652). Dragana Dragoljevic was supported by an Australian Postgraduate Award. Alan Richard Tall was supported by a grant from the NIH (HL107653).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 04 August 2014; **paper pending published:** 19 August 2014; **accepted:** 22 September 2014; **published online:** 13 October 2014.

Citation: Murphy AJ, Dragoljevic D and Tall AR (2014) Cholesterol efflux pathways regulate myelopoiesis: a potential link to altered macrophage function in atherosclerosis. *Front. Immunol.* **5**:490. doi:10.3389/fimmu.2014.00490

This article was submitted to Molecular Innate Immunity, a section of the journal *Frontiers in Immunology*.

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Dynamic aspects of macrophage polarization during atherosclerosis progression and regression

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INTRODUCTION

Atherosclerotic cardiovascular diseases, which include myocardial infarction and stroke, are the most common causes of morbidity and mortality in western society and will soon be the same world-wide. Atherosclerosis represents a failure to resolve the inflammatory response in the arterial wall initiated by the retention of apolipoprotein B (apoB)-containing lipoproteins (1). These lipoproteins are taken up by tissue macrophages, which ultimately become engorged with cholesterol (foam cells) and activated. The continuing stimulus of the entry and retention of apoB-lipoproteins fuels not only the accumulation of foam cells to form a plaque, but also the chronicity and amplification of the inflammatory response, which contribute to the vulnerability of some plaques to rupture and cause acute tissue ischemia. The central role of macrophages in atherosclerosis pathophysiology has, therefore, focused attention on their properties in plaque initiation and progression, and more recently, in regression (2–5).

The study of macrophages over the past decade is characterized by a remarkable expansion of knowledge concerning their origin, functional properties, and potential to both protect from and contribute to disease [e.g., see in Ref. (6, 7)]. Admitting the complexity of macrophage biology, for the purposes of this review, we have restricted ourselves to considering how aspects of macrophage polarization in the M1/M2 classification system (8) relate to atherosclerosis progression and regression. In this system, which is influenced by the Th1 and Th2 classification of lymphocytes, macrophages can be grossly divided into pro-inflammatory, M1 cells and anti-inflammatory, M2 cells based mainly on *in vitro* criteria (9). It is important to note, however, that while the classification of lymphocytes into Th1 and Th2 preceded the classification

It is well recognized that macrophages in many contexts *in vitro* and *in vivo* display a spectrum of inflammatory features and functional properties. A convenient system to group together different subsets of macrophages has been the M1 (inflammatory)/M2 (anti-inflammatory) classification. In addition to other sites of inflammation, it is now established that atherosclerotic plaques contain both M1 and M2 macrophages. We review results made possible by a number of recent mouse models of atherosclerotic regression that, taken with other literature, have shown the M1/M2 balance in plaques to be dynamic, with M1 predominating in disease progression and M2 in regression. The regulation of the macrophage phenotype in plaques and the functional consequences of the M1 and M2 states in atherosclerosis will also be discussed.

Keywords: macrophages, atherosclerosis, regression, cholesterol, polarization

scheme of macrophages as M1/M2, the Th1 and Th2-like responses result from polarization of macrophages to M1 and M2 states, respectively. Furthermore, M1/M2 polarization is not dependent on T cells, as has been demonstrated in Rag KO and other immune deficient mice (8).

Polarization toward the M1 state is induced by several stimuli *in vitro*, including Toll-like receptor (TLR) ligands (such as lipopolysaccharide, LPS) and interferon γ (potential endogenous stimuli in atherosclerosis will be discussed below). M1 macrophages express several pro-inflammatory mediators, such as inducible nitric oxide synthase, tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), IL-6, IL-12, and proteolytic enzymes. M1 macrophages have been found in both human and mouse atherosclerosis, and their secretion of the pro-inflammatory mediators is thought to maintain local inflammation and the degradation of extracellular matrix components, resulting in disease progression [e.g., Ref. (10)], and ultimately, unstable plaques in humans. As alluded to earlier, unstable plaques are at increased risk of rupture and causing thrombosis, resulting in myocardial infarction and stroke (11–14).

On the other side of the polarization spectrum, M2 macrophages are induced *in vitro* by Th2-type cytokines, such as IL-4 and IL-13. T regulatory cells (Tregs) have also been implicated in the induction of M2 polarization (15), possibly through IL-10. M2 polarized macrophages have been characterized by their expression of CD163, mannose receptor 1 (also known as CD206), FIZZ1, and high levels of arginase 1. In addition, they secrete anti-inflammatory cytokines, such as transforming growth factor- β , IL-1 receptor antagonist, IL-10, and have increased secretion of collagen. Based particularly on the role of M2 macrophages in wound

healing, the combination of the factors they express are thought to be particularly suited for tissue repair, which is consistent with their increased presence in regressing plaques in mouse models of atherosclerosis (16, 17).

In addition to the M1 and M2 macrophages, oxidized phospholipids present in oxidized LDL induce a macrophage phenotype that is distinct from M1 or M2 phenotypes and that has been termed Mox; these macrophages are characterized by the increases in the expression of nuclear factor erythroid 2-related factor 2 (NRF2)-dependent genes and in reactive oxygen species, and are found in the progressing plaques (18). Their role in atherosclerosis regression, however, has not been established.

With this background, we will now turn to a more complete consideration of the inflammatory states of macrophages in atherosclerosis progression and regression.

ATHEROSCLEROSIS PROGRESSION

DRIVERS OF MACROPHAGE INFLAMMATION IN PROGRESSIVE PLAQUES

As mentioned above, M1 macrophages are thought to have significant roles in progressing and vulnerable plaques (19). As noted above, a potent inducer of the M1 state *in vitro* is LPS, which is part of the outer membrane of Gram-negative bacteria, and which binds and activates TLR4 (20). Dozens of studies have demonstrated associations among high-fat diet, the metabolic syndrome, and endotoxemia (i.e., increased levels of LPS in the plasma) (21–23). It appears that a high-fat diet, the consumption of which leads to metabolic syndrome in mouse models, also induces a change in the gut bacterial flora, which, in turn, causes an increase in the LPS plasma levels. Circumstantial considerations that support a link between the metabolic syndrome and LPS signaling include the increased cardiovascular risk in patients with the metabolic syndrome and the positive association of TLR4 activity with atherosclerosis progression in mice and human beings (24–28). Despite the evidence for association between LPS, atherosclerosis progression, and M1 polarization, a study in germ-free apoE-deficient mice on low-fat chow diet showed increased atherosclerosis progression (29), suggesting that some bacteria in the gut flora have an anti-inflammatory effect (30).

Cholesterol and macrophage activation

The most accepted and robust risk factor for atherosclerosis is low-density lipoprotein-cholesterol (LDL-C). Thus, several studies have tried to understand how cholesterol can induce inflammation in general, and specifically to an activated state. The different mechanisms by which cholesterol can drive macrophage activation could be divided into those direct – how cholesterol affects macrophages, and indirect – how cholesterol affects other cell types through which activation could be induced, for example by the secretion of pro-inflammatory cytokines from T cells (**Figure 1**).

Direct mechanisms that link cholesterol to macrophage inflammation. Accumulation of cholesterol leads to the formation of crystals that are both intra- and extracellular. The presence of cholesterol crystals in early lesions in Apoe^{-/-} mice was recently demonstrated (31, 32); in addition, it was shown that both macrophage engulfment of cholesterol crystals and *de novo* formation of intracellular cholesterol crystals activate the NLRP3

(NOD-, LRR- and pyrin domain-containing 3) inflammasome (33). Activation of NLRP3, in turn, results in the secretion of the pro-inflammatory cytokine IL-1 β . This pathway appears to be necessary for atherosclerosis progression, as LDL receptor (Ldlr)^{-/-} mice, transplanted with bone marrow cells deficient in IL-1 β or in components of the NLRP3 inflammasome, had reduced plaque progression (31, 34).

A second direct mechanism that can explain macrophage activation by cholesterol is mediated through oxidized LDL. Oxidized LDL is present in both human and mouse atheromas. LDL oxidation is thought to be mediated by enzymes (such as 12/15-lipoxygenase and myeloperoxidase) and by free radicals that are abundant in the atherosclerotic plaque (35). Several studies have demonstrated that oxidized LDL can act as a ligand for both the scavenger receptors and TLRs on macrophages. Depending on the extent of oxidation, species of oxidized LDL were found to be agonists of CD14–TLR4–MD2 or CD36–TLR4–TLR6 complexes *in vitro*; these complexes can induce a pro-inflammatory signaling cascade involving IL-1 receptor-associated kinase 4 (IRAK4) (36, 37), myeloid differentiation primary-response protein 88 (MYD88) (28, 38) and other signaling proteins, culminating in activation of Nf- κ B targets. Whole-body knock out mice of TLR2, TLR4 and some of the signaling proteins mentioned here were demonstrated to have reduced atherosclerosis progression (28, 39).

A third direct mechanism points toward the increase in plasma membrane cholesterol. The change in the microenvironment of TLRs (40, 41) renders them more sensitive to ligands, thereby heightening the inflammatory responses.

Despite the predominance of data that show a direct link between cholesterol and macrophage inflammation, there are a number of studies that show the opposite – an anti-inflammatory phenotype induced by intracellular cholesterol. The basis for this is rooted in the LXRs and PPARs, which are important nuclear receptors. The ligands for these receptors include lipids, and in addition to regulating many steps of their metabolism, LXRs and PPARs can also suppress inflammatory signaling in macrophages (42). For example, a recent study demonstrated that cholesterol-loading induced LXR-dependent downregulation of inflammatory gene expression in macrophages as a consequence of the accumulation of the cholesterol precursor desmosterol (43).

Indirect mechanisms that link cholesterol to macrophage inflammation. It has been long known that T cells participate in plaque inflammation (4). For example, using flow cytometry of whole aortic digests, Galkina et al. could demonstrate the presence of diverse cell-types of the adaptive immune system in the atherosclerotic plaque and the surrounding adventitia (44). Moreover, the same group showed that antigen presentation to CD4+ T cells in the arterial wall causes local T cell activation and the secretion of pro-inflammatory cytokines, which promote atherosclerosis by maintaining chronic inflammation and inducing foam cell formation (45). Other studies have shown a direct pro-inflammatory role of intracellular cholesterol in T cells, mediated by cholesterol-induced nano-clustering of T cell receptors (46). In addition, mice lacking T cells can have a significant reduction in atherosclerosis progression (47). Some studies, however, have demonstrated only a minor role for T cells in atherosclerosis progression (48).

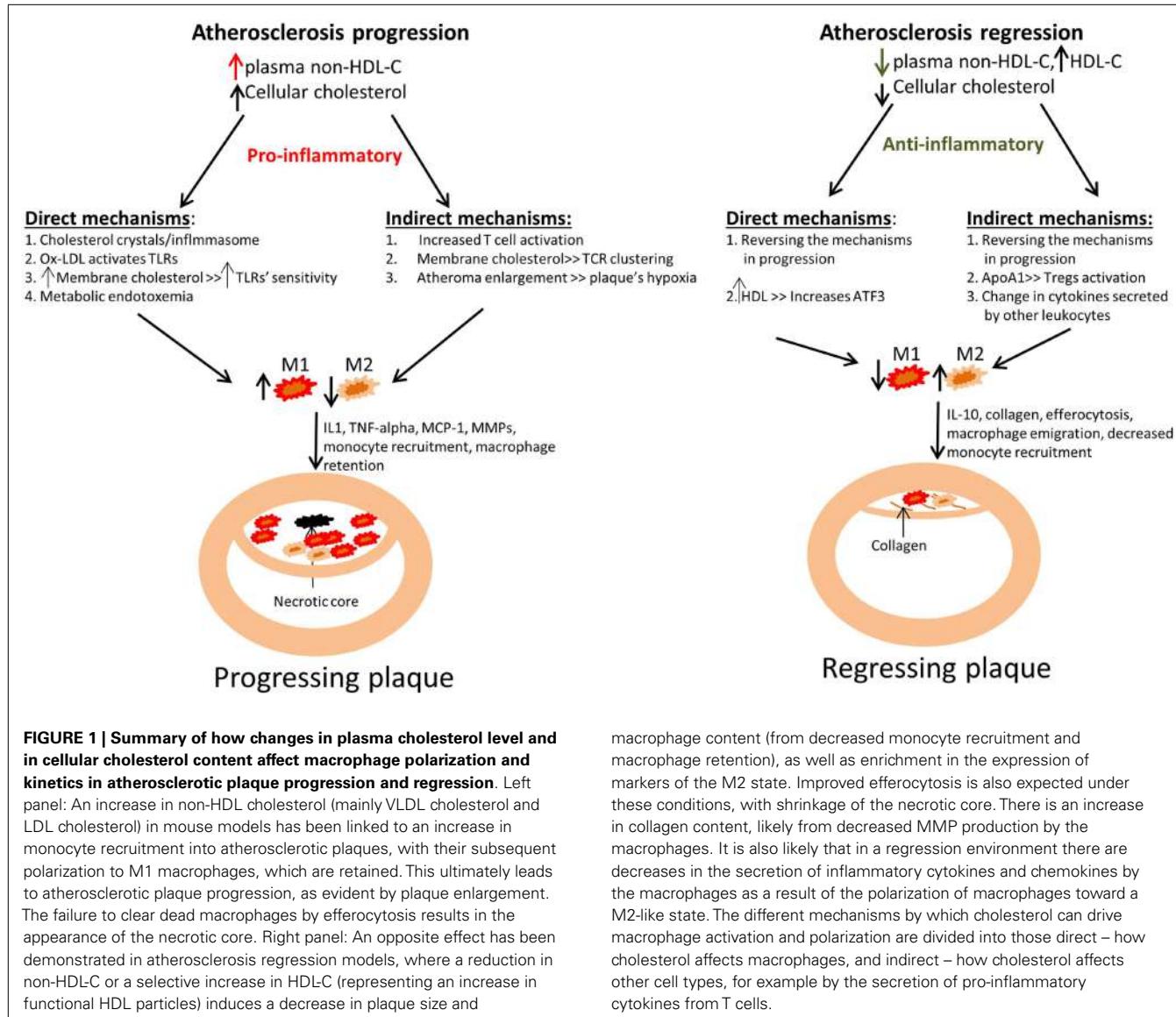


FIGURE 1 | Summary of how changes in plasma cholesterol level and in cellular cholesterol content affect macrophage polarization and kinetics in atherosclerotic plaque progression and regression. Left panel: An increase in non-HDL cholesterol (mainly VLDL cholesterol and LDL cholesterol) in mouse models has been linked to an increase in monocyte recruitment into atherosclerotic plaques, with their subsequent polarization to M1 macrophages, which are retained. This ultimately leads to atherosclerotic plaque progression, as evident by plaque enlargement. The failure to clear dead macrophages by efferocytosis results in the appearance of the necrotic core. Right panel: An opposite effect has been demonstrated in atherosclerosis regression models, where a reduction in non-HDLC or a selective increase in HDLC (representing an increase in functional HDL particles) induces a decrease in plaque size and

macrophage content (from decreased monocyte recruitment and macrophage retention), as well as enrichment in the expression of markers of the M2 state. Improved efferocytosis is also expected under these conditions, with shrinkage of the necrotic core. There is an increase in collagen content, likely from decreased MMP production by the macrophages. It is also likely that in a regression environment there are decreases in the secretion of inflammatory cytokines and chemokines by the macrophages as a result of the polarization of macrophages toward a M2-like state. The different mechanisms by which cholesterol can drive macrophage activation and polarization are divided into those direct – how cholesterol affects macrophages, and indirect – how cholesterol affects other cell types, for example by the secretion of pro-inflammatory cytokines from T cells.

Another indirect effect on macrophage polarization/activation by cholesterol is related to its being a major structural component of progressing plaques, so its ongoing accumulation will contribute to enlargement of the atheroma. This, in turn, is likely to contribute to a hypoxic environment because as the atheroma grows, the distance between the intimal cells from an oxygen supply will increase, particularly in mice, which have little capacity to form vasa vasorum. Hypoxia triggers a heavy reliance on glycolysis for energy production and it has been recently recognized that M1 cells are more glycolytic and M2 cells are more fatty acid oxidizing, and that factors that promote one pathway of energy generation over the other will promote the polarization state corresponding to the favored pathway (49). In addition, hypoxia can contribute to the formation of the necrotic core, a characteristic feature of advanced plaques that in humans increases the possibility of rupture (50). In response to the hypoxic conditions in the arterial wall, the development of vasa vasorum is enhanced,

and this has also been related to atherosclerosis progression, possibly by the recruitment of monocytes to the plaque through this vascular route (51).

MACROPHAGE POLARIZATION AND LOCATION IN PROGRESSING PLAQUES

Recent studies have found that although both M1 and M2 macrophage numbers are increased during human plaque progression, M1 macrophages were the predominant phenotype in rupture-prone shoulder regions, whereas M2 markers were predominant in the adventitia and in stable cell-rich areas of the plaque (19). In another study of human plaques, using the mannose receptor as a marker, M2 macrophages were found located far from the lipid core of the plaque (52).

In mouse plaques, M1 macrophages tend to be diffusely distributed in, and characteristic of, progressing plaques at the usual age that atherosclerotic mice are examined in detail (typically after

12–16 of the consumption of a high-fat, high-cholesterol diet, or ~16–20 weeks of age). Khalou-Laschet et al. have found in apoE-deficient mice of a similar age, but with less advanced atherosclerosis because they were maintained on a low-fat, chow diet that these early plaques were infiltrated by M2 macrophages, with M1 macrophages appearing later. Disease progression correlated with the dominance of M1 over the M2 phenotype (53). Based on serial histologic examination, they further propose that the M2 → M1 shift in balance was due to a phenotypic switch of the infiltrated cells, but the data to support this were indirect and did not exclude the possibility of replacement of macrophages by new ones with a different phenotype, or the local proliferation of M2-like tissue-resident cells, which has been reported in other contexts as well (54, 55). In regard to the location of the different macrophage phenotypes in the plaque, they show that M2 macrophages accumulated in the luminal side of the plaques in young mice, while at 55 weeks of age, both M1 and M2 labeling was evenly distributed across the plaque.

FUNCTIONAL CONSEQUENCES OF M1 POLARIZATION IN PROGRESSING PLAQUES

The secretion of a wide range of cytokines and chemokines (e.g., IL-1, TNFalpha, MCP-1) by M1 macrophages serves to further activate macrophages, as well as other cell types in the atheroma, such as endothelial and smooth muscle cells. There are also effects on cellular lipid metabolism. In one study of human plaques, macrophages with a marker of M2 macrophages had small lipid droplets and in studies *in vitro*, this was associated with a decreased ability to efflux cholesterol (52). In a study of murine macrophages, when the cells were polarized to the M1 state, there was downregulation of ABCA1 and reduced cholesterol efflux (56). If this were to happen *in vivo*, a vicious cycle would be formed – cholesterol accumulation would contribute to macrophage activation and M1 polarization, which would further induces cholesterol accumulation. Before concluding that this happens, given the divergent results on the effects of the M1 and M2 phenotypes on cholesterol efflux, more research in this area is clearly needed.

M1 macrophages also secrete chemokines (such as MCP-1) and cytokines (such as IL-12) that induce chemotaxis of other white blood cells (57, 58). In addition, M1 macrophages secrete several matrix metallo-proteinases (MMPs), such as MMP2 and MMP9, that can degrade the extracellular matrix in the plaque, which is thought to lead to destabilization and rupture. Indeed, MMPs were shown to co-localize with M1 macrophages in atherosclerotic plaques (59).

ATHEROSCLEROSIS REGRESSION

Fatty streaks, the initial phase in the development of plaques, were found in children as young as 3 years of age (60), with well-established plaques developing by adolescence (61). Thus, while most studies are focused on the progression of atherosclerosis and finding means to delay it, the more frequent clinical scenario is that by the time the patient comes for treatment of cardiovascular risk factors, as a frequently middle-aged adult, he or she may already have a significant burden of atherosclerosis, making the optimal goal of therapy the induction of plaque regression.

Plaque regression can be defined in various ways, such as a reduction in plaque size, plaque cholesterol content, plaque macrophage number/percentage, or a decreased inflammatory state. Of course, multiple changes can occur simultaneously, but not in every case. For example, if the plaque macrophage content decreases, while collagen content increases, as we have observed experimentally in some models of regression [e.g., Ref. (16)], the size may not change, but there will be less inflammation and more stabilizing material. Nevertheless, though size changes may vary, one consistent finding in various mouse models of atherosclerosis regression in which the issue of macrophage polarization was examined, as will be summarized below, is that the plaque content of M1 markers decreased, while those of M2 markers increased [e.g., Ref. (16, 17, 62)].

ATHEROSCLEROSIS REGRESSION MODELS AND CHANGES IN PLAQUE MACROPHAGES

Some of the currently available regression models include aortic arch transplantation model (17, 63), Reversa mouse model (16, 64), adenoviral gene transfer of the LDL receptor, apoA1 or apoE, a “hypomorphic apoE” model, administration of an inhibitor of MTP, and infusion of apoA-I (the major protein in HDL) or apoA-I mimetics (5, 65–69). By necessity, all models begin with a progression phase, in which the total plasma cholesterol, and in particular, LDL-C and VLDL-C, are very high. After a certain period of time, preferably at least 12–16 weeks of western diet (rich in saturated fat and cholesterol) in order to accelerate in apoE-deficient-based or enable in Ldl receptor-deficient-based models the development of a complex atherosclerotic plaque, regression is typically induced by a major change in the plasma lipid profile. This change is either a reduction of LDL/VLDL-C or an increase in apoA1/HDL, both of which would decrease the plaque content of cholesterol.

In the transplantation model, the plaque-containing aortic arch from a donor apoE-deficient (*apoE*^{-/-}) or *Ldl* receptor-deficient (*ldlr*^{-/-}) mouse fed a high-fat diet for 16 weeks is transferred into the abdominal aorta of a normo-lipidemic wild-type recipient mouse or an *apoE*^{-/-} mouse made transgenic in human *apoA1* (“*hA1/EKO*”) (17, 63, 70, 71). In either case, the regression of the plaque in the transplanted arch occurs within a few days. The advantage of the transplant model is that it can be used to test the effects of specific genes on regression by using knock out or transgenic strains, either for the donor or recipient mice, as well as to conveniently study leukocyte trafficking in and out of the plaques if the donors and recipients are mismatched in isoforms of the pan-leukocyte marker for CD45. The disadvantage lies in the technical difficulty in performing such a surgery in the mouse, thereby limiting the throughput. In addition, there is inherent inflammation induced by the surgery itself, which theoretically can affect the process of regression, though control transplants into *apoE*^{-/-} mice are used as a standard control for these and other effects related to the surgical procedures.

As noted above, HDL and its major protein, apoA1, can be increased by injections of apoA1, apoA1 mimetics, or an adenoviral vector expressing apoA1 (69, 72). In advanced atherosclerotic plaques of *apoE*^{-/-} mice, every other day injections of native human apoA-I over only 1 week led to atherosclerosis regression, as demonstrated by significant decreases in plaque lipid content,

macrophage number, and an increase in collagen content; moreover, apoA1 injections led to a significant reduction in the plaques of inflammatory M1 and an increase in anti-inflammatory M2 macrophage markers, mannose receptor 1 and arginase 1 (73). Treating mice with a recombinant adenovirus encoding human apoA1 with relatively early atherosclerotic plaques resulted in a 70% reduction in aortic lesion area characterized by a significant decrease in the fraction of lesions occupied by macrophages and macrophage-derived foam cells. The inflammatory status of this population of cells was not reported (69).

Another example for plaque regression induced by an increase in HDL was shown with our collaborators using an inhibitor of microRNA-33 (miR-33). miR-33 suppresses HDL formation in the liver and its ability to efflux cholesterol from macrophages by suppressing the expression of cholesterol transporter ATP-binding cassette transporter 1 (*ABCA1*) (74). It was hypothesized that inhibiting it by an antagonim (anti-miR-33) would promote atherosclerosis regression. *Ldlr*^{-/-} mice with established plaques were treated with anti-miR-33 over 4 weeks. As expected, anti-miR-33 treatment led to increased reverse cholesterol transport through an increase in HDL levels and expression of *ABCA1* in the liver and macrophages. Consistent with that, and consistent with the apoA1 injection study, atherosclerotic lesions regressed by anti-miR-33 treatment, as shown by reduced plaque size, lipid and macrophage content, increased collagen content and a diminished inflammatory state of the macrophages in the plaque (75).

The Reversa (*Ldlr*^{-/-}*ApoB100/100Mtpfl/fLMx1Cre*^{+/+}) mouse is a non-surgical regression model, based on the *Ldlr*^{-/-} mouse, in which the hyperlipidemia can be reversed by inducing the conditional knock out of the microsomal triglyceride transfer protein (MTTP) gene (64). MTTP is required for the proper assembly of VLDL, the precursor of LDL (76). The reversal of hyperlipidemia by inactivation of MTTP leads to regression of atherosclerosis over a few weeks accompanied by favorable changes in the composition of the atherosclerotic plaque. Again, plaque lipid content decreases, collagen content increases, and M1 markers are decreased while M2 markers are increased in the plaque macrophages (16). The advantage of the Reversa model is that it does not require any surgery in order to get extreme reduction in LDL/VLDL-C. In addition, it is important to note that unlike the transplant models, there is no increase in HDL after inducing the conditional knock out of the MTTP gene. This might be part of the reason for the reduced regression rate that can be seen in the Reversa model compared with the transplant model. The disadvantage of the model lies in the complicated genetic manipulations that were performed to create it – there are four different gene insertions/deletions in the Reversa mouse, and thus breeding it with another transgenic/knock out mouse to test the importance of a specific gene for regression is extremely time-intensive, making bone marrow transfer for myeloid-specific factors a more convenient manipulation.

The hepatic overexpression of *apoE* in *apoE*^{-/-} or *ldlr* in *ldlr*^{-/-} mice is two gene transfer strategies to induce regression, again by normalization of the lipid profile (5). The drawback of this method is a potential immune response of the host after the adenoviral gene transfer (77), which might complicate the interpretation of the inflammatory state of cells in the plaque.

Also, especially with early versions of viral vectors, there can be limitations related to the duration or amplitude of expression.

Inducing regression just by a diet change, from western diet to chow, in the *ldlr*^{-/-} mouse model has also been tried. Many times, no significant changes have been observed, presumably because the plasma cholesterol levels remain elevated, and perhaps, the experiments were not continued long enough. In one recent report, we and our collaborators have observed a reduction in plaque macrophage content and inflammatory state over 4 weeks (78). Notably, these favorable changes were impaired by hyperglycemia, consistent with our previous report using Reversa mice (79).

Mechanisms for M2 macrophage enrichment in atherosclerosis regression

There are two major questions we will consider in this section – the origin of the M2 macrophages in regressing plaques and the mechanisms for their increase.

One possibility is that the re-balancing from enrichment in M1 to M2 markers in regressing plaques represents either a change in an individual cell, as can be accomplished *in vitro* by changing the cytokine environment, or as proposed to happen in the “early to advanced” plaque transition in *apoE*^{-/-} mice. Indeed, it is generally accepted that the phenotype of a macrophage is quite “plastic” and responsive to microenvironmental changes (9). It is also possible that M1-like macrophages leave and are replaced by M2-like cells, as occurs in wound healing. Support for this scenario is our demonstration, particularly in the aortic transplantation model, of emigration of macrophages from, and the ongoing recruitment of monocytes to, plaques in the regression environment (80). A third possibility is the induction of the proliferation of a resident population of yolk-sac-derived M2 macrophages, as observed in other settings (54). Support for this possibility is the recent demonstration in progressing plaques for macrophage proliferation (55).

It is tempting to speculate that the pro-activating direct and indirect effects of cholesterol described above are reversed under conditions of regression, under which the plaque content of cellular and extracellular cholesterol is typically reduced. Little experimental evidence, however, is available *in vivo* to prove this. Even for factors with known cholesterol-removing function, such as HDL, there could be lipid-independent reasons for their effects. For example, we have recently reported that murine bone marrow derived-macrophages (BMDM) not loaded with cholesterol will increase their M2 marker expression when incubated with HDL (81) [though in human monocytes this was not found to be the case (82)], and Latz and colleagues have found that HDL will induce in macrophages the transcriptional regulator ATF3, a repressor of a number of inflammatory factors (83). There is also a study in which injection of apoA1 reduced skin inflammation in *ldlr*^{-/-}, *apoA1*^{-/-} DKO mice (which show signs of autoimmunity) by augmenting the effectiveness of the lymph node Treg cells (84); Tregs were shown to induce an M2 polarization in yet another study (15). In this example, there may have been a lipid-removal aspect in that *apoA1*^{-/-} mice are deficient in cholesterol efflux because of reduced numbers of HDL particles. Although unrelated directly to macrophage polarization, it has been shown that defects in cholesterol efflux can enrich the plasma membranes of

monocyte precursors in the bone marrow with cholesterol, which results in their greater proliferation, circulating moncytosis, and increased entry into plaques of monocytes, which subsequently promotes atherogenesis (85). Importantly, we have recently found that this mechanism might also be related to the impairment of atherosclerosis regression in diabetes (7).

Turning to the issue of what regulates the enrichment in M2 macrophages in regressing plaques, there are no results to discuss at this time, but there are a number of possibilities. As mentioned earlier, potent cytokines that polarize macrophages are IL-4 and IL-13. These can be derived from a variety of leukocytes, namely Th2 lymphocytes, eosinophils, and basophils. Even if one or more of these types of leukocytes were the source of polarizing signals, there is still the mystery of how the change in the lipoprotein/lipid environment causes either the recruitment of the cells to the plaque or the stimulation of secretion from either pre-existing or newly recruited cells.

Functional contribution of M2 polarized cells to atherosclerosis regression

Another major and incompletely understood area is the requirement for, and the function of, the enrichment in M2 macrophages in atherosclerosis regression. Because their properties include tissue remodeling and inflammation resolution, it is tempting to attribute such changes in regressing plaques to M2 macrophages. This would be consistent with studies in which treatment of *ldlr*^{-/-} mice with IL-13 resulted in pre-existing plaques the following: an increase in collagen, a reduction in vascular cell adhesion molecule-1 (VCAM-1)-dependent monocyte recruitment, decreased macrophage content, and the induction of M2 macrophages, despite ongoing hyperlipidemia (86). Another reflection of the importance of the M2 macrophages for regression comes from our studies in diabetic Reversa mice, in which hyperglycemia impaired their enrichment in plaques despite lipid lowering and limited the favorable changes in macrophage content and inflammatory state (79).

The promotion of the resolution of inflammation by M2 macrophages in regressing plaques is likely a consequence of their secretion of IL-10. The plaque remodeling may represent at least two other properties of M2 macrophages, namely their secretion of collagen (87) and their enhanced capacity for efferocytosis (88). Efferocytosis is the clearance of apoptotic cells by macrophages. During atherosclerosis progression, the ability to store cholesterol in plaque macrophages in the form of cholesteryl ester lipid droplets wanes and free cholesterol accumulates, causing ER stress and apoptosis (89). If efferocytosis of the dying cells does not keep up with their formation, the cellular debris that are not cleared would be expected to accumulate and become a necrotic core. Indeed, in both mouse and human plaques, as plaques advance, the efferocytotic activity of their macrophages decreases (50). Thus, an enrichment in M2 macrophages with enhanced efferocytosis would be expected to clear apoptotic cells and thereby stop, and even reverse, necrotic core expansion.

CONCLUDING REMARKS

During both the progression and the regression of atherosclerosis, macrophages have central roles. While macrophage phenotypes

are diverse and form a continuum (90), we chose a simple and dichotomous approach in order to emphasize the differences between macrophage properties in atherosclerosis progression and regression. M1 macrophages predominate in progression and contribute to the inflammatory state, whereas M2 macrophages are enriched in many models of regression and appear to participate in inflammation resolution and plaque remodeling. Interestingly, M2 macrophages seem to have a beneficial role even when plasma cholesterol levels remain high [e.g., Ref. (17, 86)], and the opposite is also true – M1 macrophages, though activated by direct and indirect effects of cholesterol, have also been linked to an increase in atherosclerosis progression despite similar levels of plasma cholesterol, for example, in diabetes (91). Thus, there are complex interactions between macrophage phenotypes and plasma cholesterol levels, a situation likely to also exist with other known (and yet to be discovered) risk factors.

As noted throughout this review, there are many areas in which our knowledge of macrophage biology in plaques is inadequate. Yet, it is already clear that the inflammatory state of these cells is dynamically influenced by multiple metabolic, genetic, and pharmacologic factors. Deeper understanding of how these factors effect changes in plaque macrophages will likely advance the development of new strategies to reduce the huge burden of cardiovascular morbidity and mortality that persists with existing therapies.

ACKNOWLEDGMENTS

We thank all of our lab members, past and present, as well as our collaborators, who have contributed to our studies cited in this review. Support for these studies comes from the following NIH grants: HL098055, HL084312, DK95684, and HL117226.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 25 September 2014; accepted: 29 October 2014; published online: 12 November 2014.

Citation: Peled M and Fisher EA (2014) Dynamic aspects of macrophage polarization during atherosclerosis progression and regression. *Front. Immunol.* 5:579. doi:10.3389/fimmu.2014.00579

This article was submitted to Molecular Innate Immunity, a section of the journal *Frontiers in Immunology*.

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Functions of arginase isoforms in macrophage inflammatory responses: impact on cardiovascular diseases and metabolic disorders

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Macrophages play a paramount role in immunity and inflammation-associated diseases, including infections, cardiovascular diseases, obesity-associated metabolic imbalances, and cancer. Compelling evidence from studies of recent years demonstrates that macrophages are heterogeneous and undergo heterogeneous phenotypic changes in response to microenvironmental stimuli. The M1 killer type response and the M2 repair type response are best known, and are two extreme examples. Among other markers, inducible nitric oxide synthase and type-I arginase (Arg-I), the enzymes that are involved in L-arginine/nitric oxide (NO) metabolism, are associated with the M1 and M2 phenotype, respectively, and therefore widely used as the markers for characterization of the two macrophage phenotypes. There is also a type-II arginase (Arg-II), which is expressed in macrophages and prevalently viewed as having the same function as Arg-I in the cells. In contrast to Arg-I, little information on the role of Arg-II in macrophage inflammatory responses is available. Emerging evidence, however, suggests that differential roles of Arg-I and Arg-II in regulating macrophage functions. In this article, we will review recent developments on the functional roles of the two arginase isoforms in regulation of macrophage inflammatory responses by focusing on their impact on the pathogenesis of cardiovascular diseases and metabolic disorders.

Keywords: arginase, arginine, macrophages, nitric oxide synthase, cardiovascular diseases

INTRODUCTION

Macrophages are important sentinel cells in our body and are involved in maintenance of tissue homeostasis, immune responses, and inflammation-associated diseases. Recent findings have revised our traditional view on the origin and biological functions of macrophages. We now know that tissue macrophages are not only recruited from bone marrow-derived monocytes but also differentiated from yolk sac-derived embryonic stem cells (1–3). Moreover, tissue macrophages are not terminally differentiated and are maintained throughout life by local proliferative self-renewal (4, 5). Importantly, macrophages are highly heterogeneous and undergo phenotypic changes, i.e., macrophage plasticity, in response to specific signals as a consequence of adaptation to local tissue environmental cues (6, 7). The original and the best known types of macrophage responses are the pro-inflammatory M1 type (killer cells) and the anti-inflammatory M2 type (repair type cells) (3, 7). There are convincing evidences from research of recent years showing that different phenotypic macrophages are indeed importantly participating in the process of immune and inflammatory responses, which have been reviewed by many comprehensive articles (7, 8).

MACROPHAGE POLARIZATION

Macrophage polarization describes acquisition of distinctive phenotypic and functional characteristics of fully differentiated macrophages in response to microenvironmental stimuli. Functional polarization of macrophages and the underlying

mechanisms that control the cell phenotypes are complex and have been extensively investigated in recent years. As mentioned, the M1 and M2 classifications of macrophages described the two major and opposing activities committed to killing and repairing functions of the cells. It is emerging that macrophage polarization is regulated by a broad spectrum of recognition receptors, cytokines, specific signaling pathways, and genetic programs. Some of them are used as markers or functional repertoire of the macrophage phenotypes. There are, however, no standard guidelines for classification of macrophage phenotypes. Most importantly, information about functions of these markers in regulation of macrophage inflammatory responses or phenotypes is either lacking or controversial. The conclusions are usually based on association studies. It is generally the view that M1 macrophages express enhanced genes, which are pro-inflammatory and cytotoxic, typically inducible nitric oxide synthase (iNOS)/NO, IL-12, class II MHC, and the chemokines IL-8 and CCL2, participating in killing intracellular parasites and tumor development. In contrast, M2 macrophages produce more anti-inflammatory cytokines and substances involved in repairing function, typically, arginase/ornithine, EGF, VEGF, and TGF- β , and mannose receptor (9). This phenotypic cell is mainly participating in resolution of inflammation, tissue repairing, angiogenesis, allergy, and tumor progression (10). It is, however, to notice that M1 and M2 activation programs display differences, but they may not form clear-cut activation subsets and reveal overlapping effects. A discussion about the complexity of macrophage phenotype markers,

differentiation mechanisms, and the roles in human diseases is beyond the scope of this review article. For these aspects, readers are kindly asked to refer to several comprehensive review articles (11, 12). In the following section of this article, we will focus on discussing the role of the enzymes arginase and nitric oxide synthase (NOS) that are involved in L-arginine metabolism in various cell types including vascular endothelial cells and macrophages and widely used as markers to distinguish M1 and M2 macrophage phenotypes.

L-ARGININE METABOLISM, iNOS, AND ARGINASE IN MACROPHAGE FUNCTIONAL POLARIZATION

The suggestion that L-arginine metabolism could be involved in regulation of macrophage phenotypes was from early studies with macrophages isolated from the Th1 strain mouse C57B1/6 and Th2 strain BALB/c mouse (13, 14). These studies demonstrate that isolated macrophages from Th1 strain C57B1/6 mouse are more readily activated to produce nitric oxide (NO) upon stimulation with IFN- γ or lipopolysaccharide (LPS) than the macrophages from Th2 strain BALB/c mouse. Later on, it was characterized that M1 macrophages are more easily activated by LPS to produce cytotoxic NO via iNOS, whereas M2 macrophages generate little NO but more ornithine from the same substrate L-arginine via arginase (15). The iNOS and arginase are thought to affect inflammatory responses in the opposite way. NO production from iNOS in M1 macrophages inhibits cell proliferation and kills pathogens, a M1 killing type response (16, 17), while ornithine production promotes cell proliferation and repairs tissue damage through generation of polyamines and collagen in M2 macrophages, a M2 repairing type response (11, 18). Both NO and ornithine are generated from the same substrate L-arginine via iNOS and arginase, respectively (11, 18) (Figure 1). From these studies, one can consider dominant NO production as M1 activity, whereas dominant ornithine production as M2 activity of macrophages.

ARGINASE ISOENZYMES AND L-ARGININE METABOLIZING FUNCTIONS

In human beings and mammals, there are two isoforms of arginases, arginase-1 (Arg-I) and arginase-II (Arg-II). Both isoenzymes are

encoded by two separate genes. In human beings, Arg-I gene maps to chromosome 6q23 and encodes a 322 amino acid protein (19–21), while Arg-II gene maps to chromosome 14q24.1 and encodes a 354 amino acid protein (22–24). At the subcellular level, Arg-I is mainly localized in cytoplasm and Arg-II in mitochondrion (25). The physiological role of the different subcellular compartmentation of the two isoenzymes is not known. The two isoenzymes, however, share similar structure, reveal more than 50% of homology of their amino acid residues with 100% homology in the areas, which are critical for their L-arginine metabolizing function (22, 23, 26). Although both Arg-I and Arg-II are to hydrolyze L-arginine to produce urea and L-ornithine (25), the functional impact of the two isoenzymes is either similar or different depending on specific organs/cells. For example, increased activity and/or expression of either Arg-I or Arg-II in endothelial cells impair the vasoprotective endothelial NO production via eNOS (27). However, in macrophages, Arg-I and Arg-II seem to play an opposite function, which we will discuss later in this article. The primary function of Arg-I is to remove excessive nitrogen produced from amino acid metabolism through hepatic urea cycle, which is otherwise toxic for our body (28, 29), because Arg-I is constitutively and abundantly expressed as a cytosolic enzyme in the liver (30). No Arg-II could be detected in hepatocytes. The vital effect of hepatic Arg-I is evidenced by the studies showing that Arg-I knockout mice reveal severe symptoms of hyperammonemia and die between postnatal days 10 and 14 (31). Patients with Arg-I deficiency due to gene mutation reveal urea cycle disorder, hyperargininemia, and exhibit progressive neurologic impairment, development retardation, and hepatic dysfunction associated with cirrhosis and carcinoma in early childhood (28, 29). Arg-I has been reported to be expressed also in many extrahepatic tissues such as stomach, pancreas, and lung (32). The functions of Arg-I in these organs are far from clear. Unlike Arg-I, Arg-II is confined mainly to kidney, brain, prostate, intestine, and also pancreas (22, 23, 32). The functions of Arg-II in these organs are not known. The best characterization of Arg-II function is done in vascular endothelial cells in which the isoenzyme, similar to Arg-I, metabolizes L-arginine to urea and L-ornithine, which limits L-arginine bioavailability for generation of the vasoprotective NO via eNOS, resulting in vascular endothelial dysfunction (33, 34). This effect of arginases on endothelial cells is attributable to eNOS-uncoupling, a situation that eNOS enzyme produces increased superoxide anion, but decreased NO (34–39), which is thought to be attributed to L-arginine deficiency, leading to oxidative stress, and enhanced expression of endothelial inflammatory adhesion molecules such as VCAM-1 and ICAM-1 (39), since endothelial NO reveals important anti-oxidative and anti-inflammatory functions and suppresses expression of the adhesion molecules (40). These effects of Arg-II are dependent on the enzymatic activity, since loss-of-function point mutation of histidine to phenylalanine at position 160 in Arg-II abolishes its L-arginine-urea hydrolase activity and is unable to cause eNOS-uncoupling and the inflammatory responses in endothelial cells (39). We have recently reviewed the aspect of arginase in eNOS-uncoupling (41). Arginase also exerts pleiotropic effects, i.e., L-arginine-urea hydrolase activity-independent effect, which we will discuss later in this article.

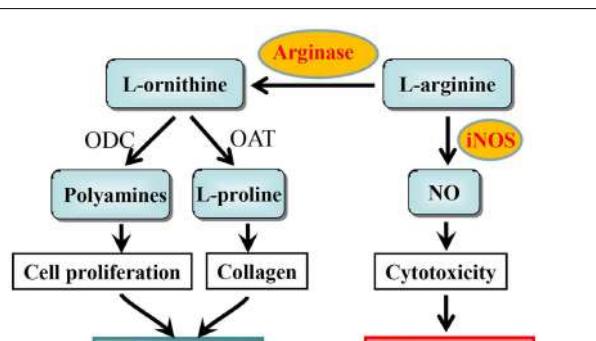


FIGURE 1 | L-arginine metabolism by iNOS and arginase and the functional consequences in macrophages. ODC, ornithine decarboxylase; OAT, ornithine aminotransferase.

ARGINASE ISOENZYMES AND MACROPHAGE FUNCTIONS

As mentioned above, macrophages are heterogeneous and undergo phenotypic changes, depending on microenvironmental stimuli. The expression of Arg-I and Arg-II is inducible in macrophages depending on external stimuli (42, 43). As discussed, NO from iNOS in macrophages is linked to M1, whereas ornithine generated from arginase is associated with M2 phenotype (11). Substantial number of studies demonstrates that Arg-I is dominantly expressed in M2 cells and reduces NO production from iNOS through limiting bioavailability of intracellular L-arginine, resulting in dampening of inflammatory tissue damage and suppression of clearance of intracellular pathogens (44–48). In contrast to Arg-I, only very little and even contradictory information is available about the expression and role of Arg-II in macrophage phenotype regulation and inflammatory responses. Until we have systematically investigated this specific aspect in macrophage inflammatory responses (43), the function of Arg-II in macrophages is believed to be anti-inflammatory, which is extrapolated from its similar function as Arg-I on L-arginine/NO metabolism. An early study showed that Arg-II gene is a direct target of liver X receptor that has been shown to exert inhibitory effects on expression of inflammatory genes in macrophages (49). Based on this association, the authors suggest that Arg-II is anti-inflammatory. The functional analysis is, however, not done. It is of particular importance to note that LPS stimulation exclusively enhances iNOS in macrophages associated with M1 phenotype (43, 50). We could demonstrate that iNOS induction in macrophages is paralleled with enhanced expression of Arg-II, but not Arg-I (43), which suggests that Arg-I and Arg-II shall have different functions in macrophage inflammatory responses or phenotype regulation. In line with this observation, accumulation of Arg-II-expressing macrophages is associated with advanced atherosclerotic lesions in which pro-inflammatory cells are dominant (42), suggesting that Arg-II is associated with pro-inflammatory responses. Because of this contradictory concept about the role of Arg-II and lack of functional analysis of Arg-II in macrophage inflammatory responses, we recently systematically characterized the role of Arg-II in regulation of macrophage inflammations at the cellular and whole body levels in mouse models of chronic inflammatory diseases such as obesity-linked insulin resistance, type-II diabetes mellitus, and atherosclerosis (43).

In this study, we demonstrate that M1 activation of macrophages by LPS exclusively up-regulates iNOS and Arg-II, but not Arg-I expression in murine and human macrophages (43). Silencing Arg-II gene in human monocyte/macrophage cell lines decreases the cell adhesion to endothelial cells with reduced production of pro-inflammatory cytokines in response to LPS or ox-LDL at both the mRNA and protein levels. Moreover, LPS-induced up-regulation of numerous pro-inflammatory mediators, including MCP-1, TNF- α , IL-6, MMP14, and iNOS, is significantly suppressed in macrophages isolated from Arg-II^{-/-} mice as compared with those from wild-type control animals. Convincingly, introducing Arg-II gene back to the Arg-II^{-/-} macrophages restores or enhances the LPS-stimulated expression of the pro-inflammatory genes to much higher levels compared to the Arg-II^{+/+} cells from wild-type mice. Importantly, Arg-II^{-/-} mice are protected from systemic pro-inflammatory macrophage

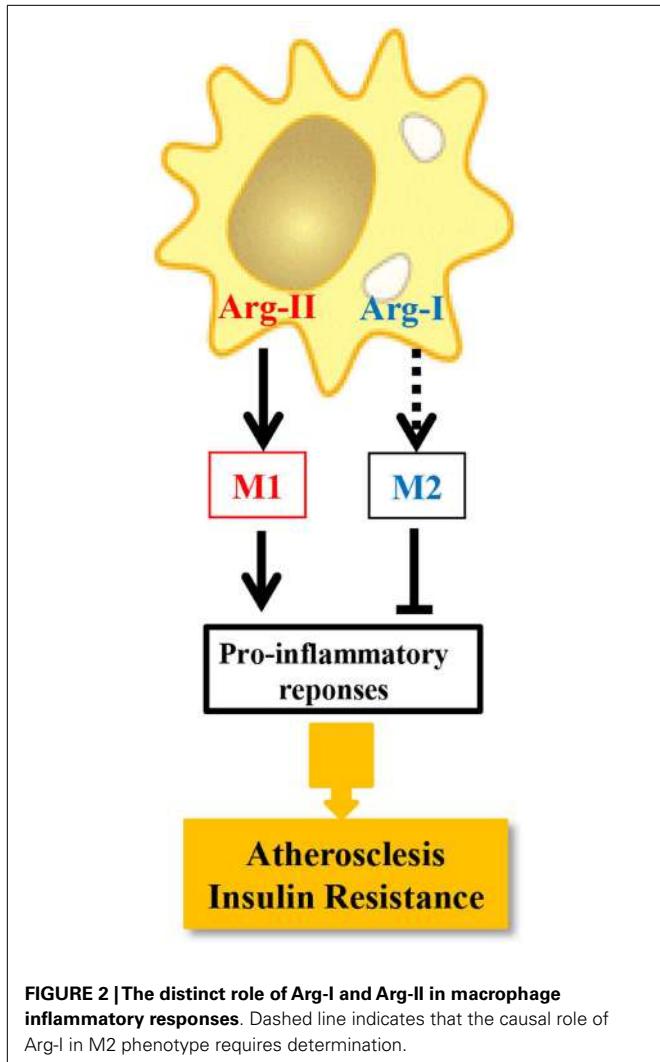
infiltration in various organs and expression of pro-inflammatory mediators in high-fat diet (HFD)-induced obesity. Arg-II^{-/-} mice, when fed a HFD, although have similar body weight as WT controls, reveal lower fasting plasma glucose concentration, are more glucose tolerant and insulin sensitive (43) as compared to WT mice on HFD. Interestingly, Arg-II levels in macrophages are significantly increased in WT mice fed HFD, which is associated with pro-inflammatory responses. The pro-inflammatory function of Arg-II in macrophages is further demonstrated in another chronic inflammatory disease model, i.e., atherosclerosis mouse model (43). Knocking-out Arg-II gene in the atherosclerosis-prone ApoE^{-/-} mice (ApoE^{-/-}/Arg-II^{-/-}) decreases inflammatory cytokine levels and macrophage content in the aortas, reduces atherosclerotic plaque formation, and reveals more stable plaque features as compared to ApoE^{-/-} Arg-II^{+/+} control mice. Since M1 pro-inflammatory macrophages play crucial role in development of insulin resistance and type-II diabetes and atherogenesis (51–55), our results demonstrate that Arg-II promotes pro-inflammatory or M1 phenotype of macrophages and favors chronic inflammatory disease development such as obesity-associated insulin resistance, type-II diabetes, and atherosclerosis. It is to mention that the pro-inflammatory effect of Arg-II in macrophages does not seem to be relying on iNOS, since inhibition of iNOS does not significantly affect expression of several pro-inflammatory genes in macrophages. The dissociation of arginase activity from iNOS has been reported by several studies, showing that alteration of arginase activity in macrophages is not necessarily associated with functional changes in iNOS (56–59). These iNOS-independent pro-inflammatory responses mediated by Arg-II in macrophages are due to enhanced mitochondrial ROS, since reintroduction of the Arg-II gene into Arg-II^{-/-} macrophages enhances mitochondrial O₂⁻ and H₂O₂ generation and inhibition of mitochondrial ROS significantly reduces Arg-II-mediated inflammatory responses. The function of Arg-II in comparison with Arg-I in macrophage inflammatory responses and chronic inflammatory diseases, i.e., atherosclerosis and insulin resistance is summarized in Figure 2. It is not very surprising, since Arg-II is a mitochondrial enzyme (60). The question remains elusive how Arg-II affects mitochondrial function leading to mitochondrial ROS production in macrophages.

Although we have characterized the function of Arg-II in macrophages, many important questions remain unanswered. In the following section, we will briefly discuss several important remaining questions regarding the role of arginase isoenzymes in macrophage functions.

FUTURE RESEARCH QUESTIONS AND PERSPECTIVES

DOES Arg-I PLAY A CAUSAL ROLE IN M2 MACROPHAGE PHENOTYPE?

As discussed, Arg-I is constitutively expressed in hepatocytes and is inducible in macrophages, e.g., by Th2 cytokines IL-4 and IL-13 (44, 61, 62). It is highly upregulated in M2 macrophages and widely used as a M2 macrophage marker (11). It has been suggested that Arg-I in macrophages promotes Th2 cytokine production, contributing to resolution of inflammation and tissue repairing (63). A study in human smooth muscle cells showing that overexpression of Arg-I gene is able to decrease LPS-induced pro-inflammatory cytokine production (64), seems to support the



anti-inflammatory function of Arg-I. However, mice with specific Arg-I gene deficiency in macrophages show exacerbated Th2 response and fibrosis in the liver of *Schistosoma mansoni* infected mice (65), which does not seem to support previous suggestions in promotion of Th2 responses (63). Most studies demonstrate only the positive correlation of Arg-I with M2 macrophage phenotype, the causal relationship of Arg-I with macrophage phenotype is, however, not fully clear. Importantly, a recent study demonstrates that Arg-I deficient mouse macrophages has even higher polyamine production and does not impair gene expression in response to IL-4 (66), which raises the question about the role of Arg-I in M2 macrophage regulation. Future research shall elucidate the causal role of Arg-I in regulation of macrophage functional polarization.

DOES L-ARGININE DEFICIENCY EXPLAIN THE FUNCTIONS OF ARGINASE ISOENZYMES IN MACROPHAGES?

There is continuing debate about the role of L-arginine deficiency in arginase-induced alterations of cellular functions. It is generally believed that arginase including Arg-I and Arg-II

causes L-arginine deficiency, resulting in decreased NO production from eNOS in endothelial cells (endothelial dysfunction) and from iNOS in macrophages (M2 type function) (41). It has been demonstrated that the concentration of L-arginine in adult human and mouse plasma (0.1 mmol/L), as well as intracellular arginine concentration (0.05–0.2 mmol/L) far exceed the K_m of eNOS (2–20 μ mol/L) (67). Even though, acute L-arginine supplementation in cells, isolated blood vessels, or in animals or in patients is able to enhance NO production and improve endothelium-dependent relaxations, a situation called “arginine paradox” (68, 69). This phenomenon led to doubt whether L-arginine deficiency caused by arginase is true. Several hypotheses have been proposed to explain the “arginine paradox.” First, a “relative” intracellular L-arginine deficiency, resulting from an increased level of endogenous competitors for eNOS substrate L-arginine such as ADMA that binds to eNOS but could not be metabolized by the enzyme on top of increased arginase activity either Arg-I or Arg-II in endothelial cells has been suggested (70). Experiments showed that inhibition of arginase improves eNOS function and overexpression of Arg-I or Arg-II causes eNOS-uncoupling, leading to oxidative stress, and decreased bioavailability of endothelial NO production, which is associated with only 11–25% reduction in intracellular L-arginine concentration in the presence of high-extracellular concentration of L-arginine (0.4 mmol/L) (71). These results seem to support the “relative L-arginine deficiency” hypothesis. It is worthy of noting that NO production, particularly, iNOS/NO can be inhibited by TGF- β , which is a strong Arg-I up-regulator and present in very high amount during wound healing (72). It is presumable that NO production is inhibited even under the condition of high-plasma L-arginine concentration because of high concentration of TGF- β . Whether this could explain the “arginine paradox” is not known. Another alternative hypothesis is that a specific intracellular pool of L-arginine for NO production may exist in endothelial cells and could be depleted by enhanced arginase (73), yet, it is highly speculative. It is not known whether enhanced arginase activity, particularly Arg-II, could also cause iNOS-uncoupling, affecting macrophage functions. Another puzzling is that why Arg-I and Arg-II share the same L-arginine metabolizing function but seem to exert distinct effects on macrophages.

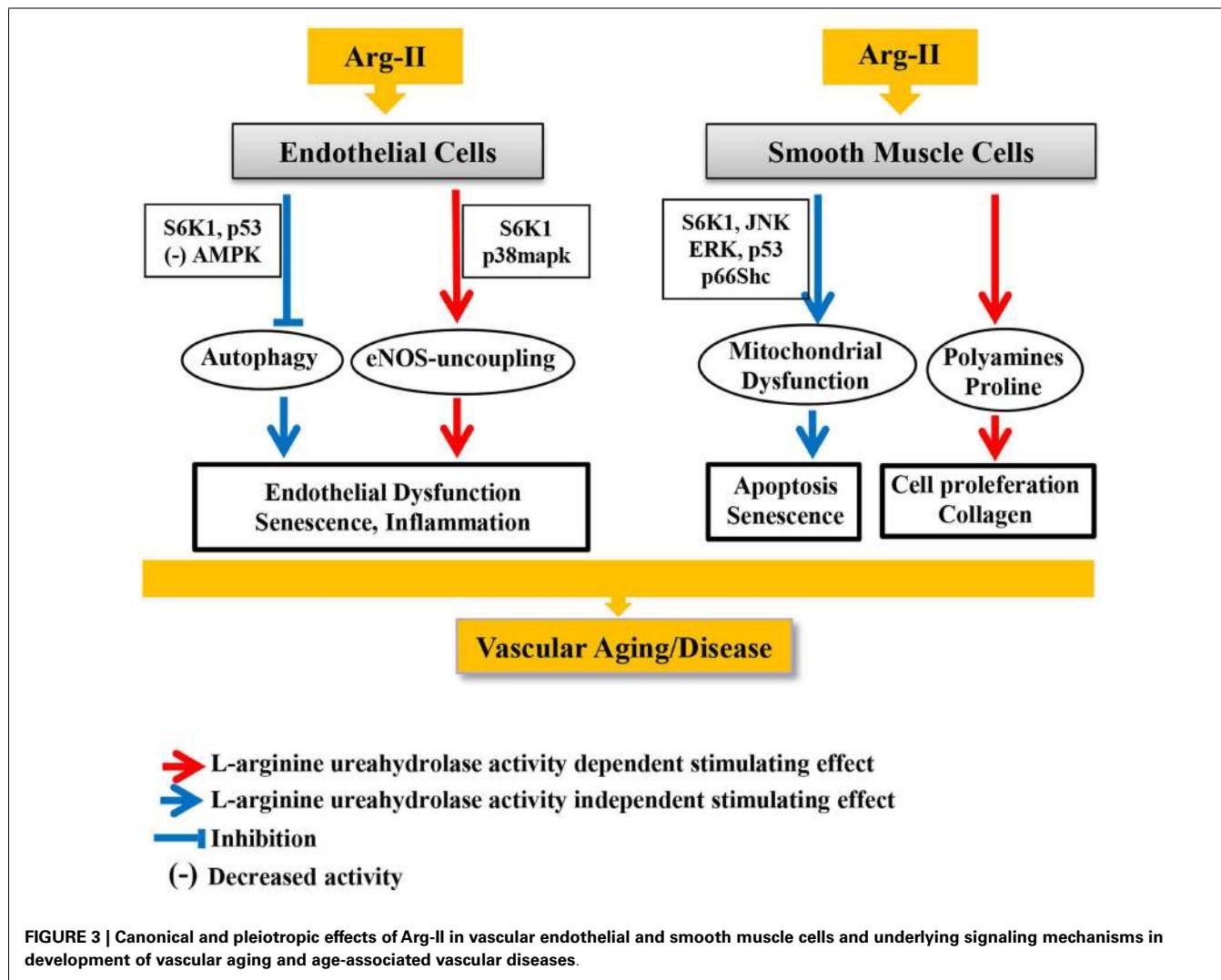
If there is no real L-arginine deficiency caused by arginase either Arg-I or Arg-II, L-arginine supplementation therapy aiming to enhance endothelial NO production and to treat vascular disease shall not work. In accordance, clinical studies in patients with acute myocardial infarction or with peripheral arterial disease demonstrate that 6-month oral L-arginine supplementation (3 g three times a day on top of standard medications) increase mortality and shorten walking distance accompanied with decreased NO production as compared to the placebo group (74). The underlying mechanisms are not known and may be related to the induction of arginase, particularly Arg-II in vascular endothelial cells by chronic L-arginine exposure as demonstrated by recent studies including our own (37, 69). These studies show that acute supplementation of L-arginine to endothelial cells increases NO bioavailability, while chronic L-arginine supplementation causes eNOS-uncoupling mediated by up-regulation of Arg-II levels, leading to endothelial senescence (69). Similar to this finding, L-arginine has also been reported to cause iNOS-uncoupling in

macrophages (75). These studies do not support a role of absolute L-arginine deficiency caused by arginase, but strongly implicate that too much L-arginine is even harmful, which is probably due to production of other undesired metabolites from L-arginine as speculated (76). Alternatively, a pleiotropic effect may also provide explanation for the detrimental effects of arginase at least for Arg-II under the condition of sufficient L-arginine supply. This point will be discussed below.

DOES ARGINASE EXERT PLEIOTROPIC EFFECTS: L-ARGININE-UREA HYDROLASE ACTIVITY-INDEPENDENT EFFECTS?

Any proteins or enzymes may have pleiotropic or off-target effects that are not necessarily related to their canonical functions. We have recently discovered that Arg-II exhibits its biological functions in vascular cells through both mechanisms, which are either dependent or independent on L-arginine metabolizing function (L-arginine–urea hydrolase activity) (77). We show that the catalytically inactive mouse Arg-II mutant with a point mutation of histidine to phenylalanine at position 160 (referred to as H160F), which lost its L-arginine–urea hydrolase activity, although does

not cause eNOS-dysfunction in endothelial cells, promotes cell apoptosis and senescence to the same extent as the WT Arg-II in vascular smooth muscle cells (VSMC). In contrast, only the WT Arg-II (not the H160F inactive mutant) exerts function to promote VSMC proliferation (Figure 3), which can be attributed to the production of polyamine from L-arginine/ornithine pathway. This intriguing result provides evidence that Arg-II on one hand promotes VSMC proliferation and on the other hand causes VSMC apoptosis and senescence. While the cell proliferation-stimulating effect of Arg-II is dependent on its L-arginine–urea hydrolase activity via synthesis of ornithine and polyamines (71, 78), the cell apoptosis/senescence-promoting effect is independent of its enzymatic activity (Figure 3). Further experiments show that this L-arginine–urea hydrolase activity-independent effect is mediated through signaling pathways including mTORC1/S6K1, JNK, and ERK1/2, converging on p66^{Shc} leading to H₂O₂ production and mitochondrial dysfunction leading to cellular apoptosis and senescence (77) (Figure 3). In parallel to these signaling pathways, p53 is also activated by Arg-II independently of its L-arginine–urea hydrolase activity, contributing to the cell senescence of the



apoptosis process. Importantly, expression of Arg-II and activities of S6K1, ERK1/2, p66Shc, and p53 are all augmented in senescent VSMC, and genetic inhibition or ablation of Arg-II not only reduces these signaling pathways and VSMC senescence/apoptosis *in vitro* but also in atherosclerosis-prone ApoE^{-/-} mice *in vivo*, which at least in part accounts for the reduced plaque lesion formation and a more stable plaque characteristics in aortic roots in Arg-II-deficient ApoE^{-/-} mice (43, 77) (**Figure 3**). Moreover, we also show that Arg-II negatively regulates autophagy function – a cell protective mechanism of lysosomal proteolysis aiming to remove harmful proteins (79) – in endothelial cells, which is also independent on its L-arginine–urea hydrolase activity [Ref. (69), **Figure 3**]. Decreased autophagy function in vascular cells and macrophages are implicated in vascular aging and atherosclerotic vascular disease. Indeed, recent studies provide evidence suggesting that adequate induction of autophagy protects against cellular injury in endothelial and smooth muscle cells and formation of foam cells, resulting in anti-atherosclerotic effects (80–82). In line with this finding, genetic ablation of Arg-II in atherosclerotic ApoE^{-/-} mice preserves endothelial autophagy in aortas, which associates with reduced atherosclerosis lesion formation (69). In this study, we also demonstrate that Arg-II impairs endothelial autophagy independently of the L-arginine–urea hydrolase activity through activation of mTORC1/S6K1 and p53, resulting in inhibition of AMPK in endothelial cells, which contributes to development of atherosclerosis (**Figure 3**). How Arg-II, independently of its L-arginine–urea hydrolase activity, impacts vascular cell functions, remains to be investigated. Further, whether these enzymatic dependent and independent effects also exist for Arg-I and whether the pleiotropic effects of arginase account for functional regulations in macrophages are unknown.

WHAT ARE THE MECHANISMS THAT REGULATE Arg-I AND Arg-II IN MACROPHAGES

Arginase-I gene expression is inducible in macrophages by a variety of stimuli, for example, by elevated cAMP, IL-4, and TGF- β (50). The regulation of Arg-I gene expression is mainly investigated at the transcriptional levels in murine macrophages, while it remains to be investigated whether the findings are also true in human cells. A number of transcription factors and nuclear receptors such as RXR, PPAR γ , PPAR δ , STAT6, C/EBP β , KLF4, PU.1, IRF8, and AP-1 have been shown to bind directly to specific sites in the promotor region of Arg-1 gene and in turn to enhance Arg-1 expression. The complexity of Arg-I gene regulation mechanisms are further complicated by the fact that these transcription factors can be regulated by post-translational modification mechanisms such as SUMOylation and ubiquitination that are participating in the regulation of Arg-1 gene [for detailed description of these mechanisms please refer to the review articles (83, 84)]. There is, however, little information available regarding the upstream regulatory mechanisms involved in gene expression and enzymatic activity of Arg-I in macrophages. Also, very limited information is provided on regulation of Arg-II in macrophages. The stress sensor p38mapk has been demonstrated to participate in up-regulation of activity and expression of Arg-I and Arg-II in macrophages (85, 86). This seems to be also the case in bovine and rat aortic endothelial cells for Arg-I expression

(87) and in human endothelial cells and mouse penile tissues for Arg-II (88, 89). In accordance, *in vivo* treatment of hypertensive mouse induced by angiotensin-II infusion with a p38mapk inhibitor prevents elevation of Arg-II expression and activity and enhances endothelium-dependent relaxation (88). These studies demonstrate that p38mapk is the upstream regulator of Arg-II in endothelial cells and macrophages. Our most recent study provides evidence showing that p38mapk also functions as downstream effector of Arg-II in endothelial cells, causing oxidative stress through eNOS-uncoupling, since overexpression of Arg-II in human endothelial cells causes eNOS-uncoupling and augments p38mapk activation (90), and inhibition of p38mapk either pharmacologically by SB203580 or genetically by silencing the major isoform p38mapk α in endothelial cells prevents eNOS-uncoupling effect by Arg-II gene overexpression (90). Furthermore, mice fed HFD, an obesity mouse model, exhibit enhanced Arg-II expression/activity and p38mapk activity and eNOS-uncoupling in the aortas and inhibition of p38mapk recouples eNOS activity in the obese mice. Moreover, mice deficient in Arg-II (Arg-II^{-/-}) on the same obesogenic diet reveal decreased p38mapk activity and eNOS function is fully preserved. These results demonstrate that Arg-II causes eNOS-uncoupling through activation of p38mapk in HFD-induced obesity (90). Together with the experiments discussed above, there might be a positive regulatory circuit between p38mapk and Arg-II at least in vascular endothelial cells. Whether this mechanism is also involved in Arg-I and/or Arg-II gene expression in macrophages is not known. A similar positive regulatory circuit between S6K1 and Arg-II has also been demonstrated by our recent studies in vascular endothelial cells (39).

In this study, we show that a persistent hyperactive S6K1 activity is found to play a causal role in eNOS-uncoupling, leading to vascular endothelial aging and senescence (39, 91). Overexpression of a constitutively active S6K1 mutant up-regulates Arg-II (not Arg-I) gene expression and arginase activity in non-senescent cells by stabilizing Arg-II mRNA (39). Conversely, silencing S6K1 in senescent cells reduces Arg-II gene expression and activity and genetic or pharmacological inhibition of S6K1 in senescent cells or in old rat aortas decreases Arg-II gene expression and activity, demonstrating a critical role of hyperactive S6K1 in up-regulating Arg-II gene expression, resulting in enhanced arginase activity in endothelial aging. Interestingly, silencing Arg-II gene in senescent endothelial cells or deficiency in Arg-II gene in mice reduces S6K1 activity, recouples eNOS function in aging, and inhibits endothelial expression of adhesion molecules such as ICAM-1 and VCAM-1, resulting in inhibition of monocyte-endothelial cell interaction, demonstrating a positive vicious cycle between S6K1 and Arg-II in vascular endothelial aging. These studies provide evidence showing that a mutual positive regulation between S6K1 and Arg-II gene expression accelerates endothelial aging through eNOS-uncoupling, leading to oxidative stress and inflammation (39). Further studies will analyze whether S6K1 is also involved in regulation of Arg-I and/or Arg-II in macrophages, participating in macrophages phenotype determination. Also, the relationship between p38mapk, S6K1, and arginase remain to be analyzed.

Other signaling pathways such as GTPase RhoA and its downstream kinase ROCK have been reported to mediate Arg-I gene

expression in porcine coronary arterioles in response to hydrogen peroxide (H_2O_2) and peroxy nitrite (92) and Arg-II (but not Arg-I) expression and/or activity in women with preeclampsia (93) and in human endothelial cells in response to thrombin (35), oxidized LDL (94), and hyperglycemia (36). In macrophages, however, ROCK kinase inhibitor enhances Arg-I expression and shift M1 to M2 phenotype (95), suggesting that ROCK pathway may inhibit Arg-I expression. No information is available so far whether Rho/ROCK pathway is involved in Arg-II regulation in macrophages. For the detailed regulatory signaling mechanisms of Arg-I and Arg-II expression/activity in vascular cells, please refer to the review article (41).

CONCLUSION

The two isoforms of arginase, i.e., Arg-I and Arg-II, although located in different subcellular compartments, share the same function on L-arginine metabolism. Both isoenzymes hydrolyze L-arginine to urea and L-ornithine, resulting in eNOS-uncoupling in endothelial cells. In macrophages, Arg-I and Arg-II can be differentially induced by external stimuli. Evidence has been provided that Arg-II plays a causal role in M1 functions, whereas Arg-I is associated with M2 function in macrophages and widely used as M2 marker for macrophages. However, the causal relationship between Arg-I and M2 phenotype warrants further investigation. It remains to be characterized how Arg-I and Arg-II share the same L-arginine metabolizing effect, but exhibit distinct or opposite effects in macrophage inflammatory responses. Arg-II as therapeutic target in chronic inflammatory disorders such as age-associated vascular dysfunctions, atherosclerosis, and type-II diabetes and complications has shown promising beneficial effects in genetic modified mouse models (39, 43, 96). Some studies implicate that targeting Arg-I is also beneficial for cardiovascular functions, these studies are solely dependent on the pharmacological inhibitors, which inhibit both isoforms of arginases (97–99), since systemic Arg-I deficient mouse exhibits severe symptoms of hyperammonemia, and die between postnatal days 10 and 14 (31), one should consider that these inhibitors could inhibit liver Arg-I, resulting in hyperammonemia. Taking into account that Arg-I in macrophages may exhibit opposite effects as Arg-II, this is another important reason to develop specific Arg-II inhibitors. Moreover, whether Arg-I and Arg-II exert pleiotropic effects on macrophage functions as demonstrated in vascular cells shall be investigated. If this proves to be true, development of therapeutic drugs that target L-arginine–urea hydrolase activity may have limitation on treatment of inflammatory diseases. Additionally, signaling pathways that are involved in regulation of gene expression and enzymatic activity of both Arg-I and Arg-II shall be further elucidated in macrophages. Characterization of these signaling mechanisms will also provide possibilities or rationales to target arginase isoforms specifically in an indirect way to treat inflammatory diseases. Finally, we have focused on cardiovascular and metabolic diseases here. But, functional analysis of arginase isoenzymes and their roles in macrophage polarization should also help understanding other diseases, notably cancer. In particular, monocytes and macrophages are recruited into tumors and regulate tumor growth by changing their functional phenotypes, which is originally demonstrated by Mills and colleagues (100).

M1 macrophage has been shown to have antitumor immunity, whereas the M2 macrophage exerts protumorigenic properties (101). Regardless of the inflammatory circumstance, it appears that macrophage arginases are key players in influencing disease outcomes.

ACKNOWLEDGMENTS

The original studies from own research were supported by the Swiss National Science Foundation (310000-120435/1 and 310030-141070/1), Swiss Heart Foundation, and National Centre of Competence in Research Program (NCCR-Kidney.ch).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 02 September 2014; accepted: 09 October 2014; published online: 27 October 2014.

Citation: Yang Z and Ming X-F (2014) Functions of arginase isoforms in macrophage inflammatory responses: impact on cardiovascular diseases and metabolic disorders. *Front. Immunol.* **5**:533. doi: 10.3389/fimmu.2014.00533

This article was submitted to Molecular Innate Immunity, a section of the journal *Frontiers in Immunology*.

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Role of alveolar macrophages in chronic obstructive pulmonary disease

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Alveolar macrophages (AMs) represent a unique leukocyte population that responds to airborne irritants and microbes. This distinct microenvironment coordinates the maturation of long-lived AMs, which originate from fetal blood monocytes and self-renew through mechanisms dependent on GM-CSF and CSF-1 signaling. Peripheral blood monocytes can also replenish lung macrophages; however, this appears to occur in a stimuli specific manner. In addition to mounting an appropriate immune response during infection and injury, AMs actively coordinate the resolution of inflammation through efferocytosis of apoptotic cells. Any perturbation of this process can lead to deleterious responses. In chronic obstructive pulmonary disease (COPD), there is an accumulation of airway macrophages that do not conform to the classic M1/M2 dichotomy. There is also a skewed transcriptome profile that favors expression of wound-healing M2 markers, which is reflective of a deficiency to resolve inflammation. Endogenous mediators that can promote an imbalance in inhibitory M1 vs. healing M2 macrophages are discussed, as they are the plausible mechanisms underlying why AMs fail to effectively resolve inflammation and restore normal lung homeostasis in COPD.

Keywords: alveolar macrophage, chronic obstructive pulmonary disease, efferocytosis, lung inflammation, oxidative stress, resolution

INTRODUCTION

Macrophages are essential for pulmonary host defense through their capacity to survey the exposed airways and regulate innate and adaptive immunity. The pulmonary macrophage system consists of several different populations that are found in anatomically distinct compartments, including the airways, alveolar spaces [alveolar macrophages (AMs)], and resident lung tissue. AMs constitute over 90% of the pulmonary macrophage population (1) and have traditionally thought to originate from the bone marrow (2). More recently, it has been shown that although AMs originate from fetal blood monocytes within the first week of life via GM-CSF dependent mechanisms, maintenance of this population during homeostasis is dependent on their self-renewing capacity (3). Once, developed, this lung macrophage pool is long-lived in humans (2) in the absence of an inflammatory insult. This finding has been replicated in mice, where there was approximately 40% turnover of AMs over 1 year in the absence of an insult (4). Hence, the macrophage lung population, particularly during the steady state, is primarily sustained by the self-renewal of pulmonary AMs through local proliferation (5). The local proliferation of AMs during homeostatic repletion is dependent on both GM-CSF and CSF-1, with no dependence on IL-4 signaling (6). Both GM-CSF and M-CSF also control the proliferation and survival of AMs (7). The differentiation of AMs is particularly dependent on GM-CSF, where the leukocyte growth factor regulates essential functions including phagocytosis and surfactant catabolism through the PU.1 transcription factor (8). This is consistent with a unique airway environment that is associated with high oxygen tension and high levels of GM-CSF.

Resident AMs are constantly encountering inhaled substances due to their exposed position in the alveolar lumen. AMs are considered to be major effector cells in innate host defense against inhaled irritants by virtue of their phagocytic ability (9). Therefore, it is vital that resident AMs are kept in a relatively quiescent state with active suppression of inflammation in response to harmless antigens to prevent collateral damage to lung tissue (10). Although AM exhibit microbial, tumoricidal, and parasiticidal activities (9, 11), they are functionally less responsive than tissue-resident macrophages. Relative to tissue macrophages, they display reduced phagocytic capacity, reduced respiratory burst, and a diminished capacity to present antigen to T cells (12–14). Under homeostatic conditions, AMs are closely associated with alveolar epithelial cells (AECs), and this in turn induces the expression of epithelial restricted $\alpha v \beta 6$ integrin that binds and activates latent TGF β (15). TGF β can inhibit macrophage activation that is implicated in alveolar wall destruction (15). Upon recognition of antigens by TLRs, the rapid induction of actin polymerization promotes AMs detachment from AECs (16). The subsequent production of proteases by activated AMs then activates latent TGF β , thereby reinstating AMs to their resting state (16). This illustrates an intricate mechanism of microenvironmental macrophage specialization to keep the macrophage response in check. AMs can also produce TGF β , which suppresses T cell activation and has been shown to promote the emergence of T regulatory cells (17).

ROLE OF AMs IN INNATE HOST DEFENSE

In contrast to the self-renewing capacity of AMs during the steady state, inflammatory and infective insults dramatically change the

dynamics of local lung macrophages. Using chimeric mice, parabiosis, and adoptive cellular transfer models, it has been established that bone marrow-derived blood monocytes replenish the AM pool during lethal irradiation (3). This is context specific, as inoculation with influenza in mice, which dramatically depletes resident AMs in a strain specific manner, led to restoration of this population through self-renewal proliferative mechanisms (6). This infectious model also promotes the substantial recruitment of blood monocytes; however, the fate of monocyte-derived macrophages has yet to be established in this setting. Using a similar approach, LPS has been shown to restore lung macrophage numbers through both local proliferation of resident macrophages and maturation of recruited blood monocytes (18). Mouse blood monocytes can be subdivided according to differential expression of chemokine receptors and adhesion molecules that are involved in cell recruitment. $CCR^{2+}Gr1^{hi}CX_3CR1^{lo}$ is actively recruited to inflamed tissues by virtue of their recognition of CCL2 (also known as MCP-1) (19). Mouse monocytes that are $CCR^{2+}Gr1^{hi}CX_3CR1^{lo}$ are classified as pro-inflammatory but share morphological characteristic and chemokine receptor patterns with the classical human monocytes ($CD14^{hi}CD16^{-}CX_3CR1^{lo}$) (19). Monocyte-derived macrophages can acquire distinct morphological and functional properties as directed by the immunological microenvironment.

Alveolar macrophages coordinate antimicrobial defenses through expression of receptors for immunoglobulin (F_cR), complement, β -glucan, mannose, and several types of scavenger receptors that together facilitates phagocytosis (20). AMs generate reactive nitrogen and oxygen intermediates involved in macrophage-mediated defense against microbial infection (21, 22). Surfactant protein A augments pathogen killing by AMs by stimulating phagocytosis and production of reactive oxygen–nitrogen intermediates (23). In addition, AMs can initiate recruitment of inflammatory cells from pulmonary vasculature into the alveolar space. There are a number of studies that implicate AMs as central effector cells in the production of pro-inflammatory cytokines, which initiate the early phase of neutrophil influx in response to acute lung injury caused by bacterial products (24, 25). More recently, selective targeting strategies that ablate different monocyte/macrophage populations have identified an important role for peripheral blood monocytes. Ablation of $CCR2^{hi}$ monocytes significantly reduced indices of acute lung injury (26). It is plausible that AMs may actually play a role in limiting neutrophil influx by controlling MCP-1 production through AECs (27). Furthermore, in a murine model of pneumococcal pneumonia, AMs depletion resulted in a failure to modulate the inflammatory response with increased levels of pro-inflammatory cytokines (28). AMs are also central regulators of the resolution of inflammation through their ability to engulf apoptotic neutrophils during the resolution phase (28, 29). The active phagocytosis of dying cells by macrophages may also lead to the induction of anti-inflammatory or suppressive properties in macrophages as shown by the inhibition of IL-1 β , IL-8, TNF α , and GM-CSF production (30).

Alveolar macrophages are also indispensable for the clearance of influenza infection in a viral strain-dependent manner in mice. Here, it was established that the depletion of airway macrophages was associated with more severe lung injury following inoculation

with BJx109, which is a viral strain that infects macrophages with high efficiency (31). This is in contrast to the highly virulent PR8 strain that poorly replicated in airway macrophages, suggesting that avoidance of AM engagement may contribute to the virulence of influenza strains (31). There is also the important clinical complication of secondary bacterial infections following a significant viral event, where AM function is likely to be compromised. In murine models of secondary bacterial infection, the initial depletion of AMs as a consequence of influenza infection rendered the host susceptible to *Streptococcus pneumoniae* (Spn) colonization and systemic invasion (32). The repletion of resident AMs occurred 2 weeks after influenza, which resulted in the re-establishment of early innate host protection to Spn (32). This AM replenishment phase may represent a window of opportunity for opportunistic respiratory pathogens such as Spn that take advantage of this immunocompromised state. Interferon- γ production during the recovery phase of a viral infection can also inhibit lung anti-bacterial defenses. Mechanistically, it was shown that viral-induced production of interferon- γ caused downregulation of the scavenger receptor MARCO, and neutralization of interferon- γ prevented secondary pneumococcal infection (33). Using MARCO-deficient mice, it has been established that expression of this scavenger receptor on AMs is critical for efficient clearance of Spn from the lungs (34).

ROLE OF AMs IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE

There is a large body of evidence implicating AMs in the pathogenesis of Chronic Obstructive Pulmonary Disease (COPD). COPD is a major global health problem and has been predicted to become the third largest cause of death in the world by 2020 (35). Cigarette smoking is the major cause of COPD and accounts for more than 95% of cases in industrialized countries (36), but other environmental pollutants are important causes in developing countries (37). COPD is “a disease state characterized by airflow limitation that is not fully reversible.” The airflow limitation is usually progressive and associated with an abnormal inflammatory response of lungs to noxious particles and gases (38). COPD encompasses chronic obstructive bronchiolitis with fibrosis and obstruction of small airways and emphysema with enlargement of airspaces and destruction of lung parenchyma, loss of lung elasticity, and closure of small airways. Most patients with COPD have all three pathologic conditions (chronic obstructive bronchiolitis, emphysema, and mucus plugging), but the relative extent of emphysema and obstructive bronchiolitis within individual patients can vary.

Studies have highlighted that macrophages play a pivotal role in the pathophysiology of COPD (39). There is a marked increase (5- to 10-fold) in the numbers of macrophages in airways, lung parenchyma, bronchoalveolar lavage fluid (BALF), and sputum in patients with COPD (40, 41). A morphometric analysis of macrophage numbers in the parenchyma of patients with emphysema showed a 25-fold increase in the numbers of macrophages in the tissue and alveolar space compared to smokers with normal lung function (42). There is a positive correlation between macrophage numbers in the airways and the severity of COPD (43). In addition, a pathological role for macrophages has been demonstrated, as the depletion of lung macrophages conferred

protection against the development of emphysema in an experimental model of COPD (44). Macrophages are activated by cigarette smoke and other irritants to release inflammatory mediators. AMs also secrete elastolytic enzymes (proteases), including matrix metalloprotease (MMP)-2, MMP-9, MMP-12, cathepsin K, L, and S in response to irritants and infection, which together are responsible for destruction of lung parenchyma (36). In patients with emphysema, there is an increase in BALF concentrations and macrophage expression of MMP-1 and MMP-9 (45). There is an increase in activity of MMP-9 in the lung parenchyma of patients with emphysema (46). AMs from smokers with normal lung function express more MMP-9 than those from non-smoking healthy subjects (47), and there is an even greater increase in cells from patients with COPD, which have enhanced elastolytic activity (48).

Chronic obstructive pulmonary disease subjects can also be very susceptible to bacterial colonization (49, 50) and exacerbations that are commonly caused by respiratory infections of viral and/or bacterial etiology (51). The frequent exacerbator phenotype has been reported, which is associated with a poorer quality of life and increased systemic inflammation (52). Impaired AM function is central to high colonization rates and increased susceptibility to exacerbations observed in COPD. Chronic cigarette smoke exposure is a major cause of COPD, which markedly depletes intracellular GSH stores (53, 54). Oxidative stress leads to disruption of GSH metabolism, which is considered as a key susceptibility feature of lung diseases (55). Excessive oxidative stress is particularly deleterious to AM function, leading to a deficiency in phagocytosis of bacteria (56) and efferocytosis of apoptotic cells (57). Treatment with anti-oxidants such as procysteine can significantly improve efferocytic function of AMs isolated from experimental models of COPD (58). Impaired AM-mediated efferocytosis in COPD can be particularly damaging in COPD as neutrophils are persistently recruited into the airways. Cigarette smoke impairs clearance of apoptotic cells through oxidant-dependent activation of RhoA (59) and inhibition of Rac1 (60), leading to defective actin polymerization normally required for efficient efferocytosis. The inability to efficiently remove exhausted neutrophils has damaging implications in COPD as accumulation of necrotic neutrophils can lead to the indiscriminate release of granule protease pools including neutrophil elastase. Neutrophil elastase localizes to lung elastic fibers in emphysematic patients and degrades extracellular matrix components (61) and can promote the release of mucins through epidermal growth factor receptor (EGFR)-dependent mechanisms (62). EGFR transactivation can also augment inflammatory responses initiated by rhinovirus infection (63). Reactive free radicals also impair clearance mechanisms by directly causing cytoskeletal instability and carbonyl modification of pseudopodia (64–66). Macrophages also interact with carbonyl-adduct modified extracellular matrix proteins, which impair their ability to clear apoptotic neutrophils (67).

In addition to oxidative post translational modification of the host phagocytic machinery, the complex milieu within COPD airways can alter the phenotype of highly plastic airway macrophages. It is becoming increasing clear that different macrophage subpopulations exist in the inflamed lung. Although the existence of such

populations is implicated in COPD, the importance of these subpopulations is unknown (68). The ongoing characterization of disease-associated macrophages clearly demonstrates that they do not conform into the classic M1/M2 dichotomy and it is likely that the inflammatory environment of the COPD airways drives development of both M1 and M2 macrophages (58, 69). Indeed, it has been shown that iNOS and arginase activity are concurrently elevated in COPD airways. Specifically, elevated iNOS expression has been observed in AMs of COPD patients, which increased with severity of disease and during exacerbations (70–72). Of interest, levels of exhaled nitric oxide (NO) are not elevated in stable COPD, which may be consequential to increased production of superoxide that reacts with NO to generate the highly reactive nitrogen species, peroxynitrite (73). In addition, arginase-1 is increased in cigarette smoke exposure models (74), which will reduce L-arginine availability to iNOS. Reduced L-arginine bioavailability also stimulates iNOS to simultaneously produce NO and superoxide, which facilitates rapid formation of peroxynitrite (73). Consistent with the concurrent existence of iNOS and arginase expressing macrophages in COPD, nitrotyrosine (product of peroxynitrite) levels have been shown to be increased in sputum macrophages of COPD patients, which are negatively correlated with their lung function (75). Hence, the relative ratio of iNOS expressing M1 macrophages and arginase expressing M2 macrophages will be particularly important to the oxidative/nitrosative state of the lung.

The relative balance between these polarization states can in turn, have a profound impact on disease progression [reviewed in Ref. (76)]. There is evidence for the reprogramming of AMs as a consequence of chronic smoke exposure that is associated with the induction of a unique set of genes including MMP12 (77). There is also evidence for the transcriptional skewing of AMs toward an M2 gene profile in smokers with normal lung function that was more evident in smokers who had progressed to develop COPD (78). In this study, they also demonstrate the progressive down-regulation of M1 genes (78), which would appear paradoxical to the observation of increased expression of pro-inflammatory mediators in COPD. Hence, there is a need to better define the relative contribution of M1 vs. M2 macrophages as there is emerging evidence that both populations do concurrently exist in COPD airways. Another important consideration in COPD is the interaction between macrophages and T cell subsets. Although it has been broadly stated that T cells control the polarization state of macrophages, macrophages can also potently regulate T cell biology (79, 80). Since T cell subsets including CD8⁺ T cells, T_H17, and iBALT formation are implicated in COPD pathology, the role of macrophages in regulating T cell biology remains to be elucidated.

The accumulation of M2 skewed airway macrophages may be reflective of deficient resolution processes that normally switch off inflammation and restore lung homeostasis. Since the stimulation of non-phlogistic phagocytosis is essential to resolution of inflammation, the oxidant-dependent impairment of efferocytic clearance of damaged tissue may maintain M2 macrophages in COPD. The induction of CD163 is commonly recognized as a marker for M2-alternatively activated macrophages involved in wound-healing (81, 82) and CD163 positive macrophages are

highly prominent in the BAL compartment of current and ex-smokers with COPD (83). CD163 may constitute a major defense mechanism to protect the lung as it functions as a scavenger receptor, which promotes degradation of HbHp complexes and signaling that induces expression of heme-oxygenase-1 (HO-1) (82). The persistence of HO-1 in COPD airways (84) is consistent with an environment where there is excessive oxidative stress and a deficiency in the resolution of inflammation. The mediators that induce expression of M2 markers in COPD have not been comprehensively characterized. Interleukin-10 (IL-10) is a potent inducer of CD163 expression in human monocytes (85); however, there is some data to suggest that the level of IL-10 positive macrophages is reduced in COPD (86). An alternate mediator that has been shown to potently induce expression of CD163 in human monocyte-derived macrophages is serum amyloid A (SAA) (87). SAA is a major acute phase reactant that has now been shown to be expressed in COPD lungs, where its levels correlated with neutrophilic inflammation (88). SAA is known to target the ALX/FPR2 receptor and oppose the actions of pro-resolving ligands such as LipoxinA₄ (88–90), which normally stimulate non-phlogistic clearance pathways. In addition to CD163, SAA can also stimulate the expression of the T_H17 polarizing cytokines, IL-6, and IL-1 β in monocyte-derived macrophages, and neutralization of IL-17A expression suppressed neutrophil airway inflammation stimulated by SAA (91). Hence, the persistence of host defense mediators such as SAA may maintain alternative macrophage populations in COPD airways that not only express M2 markers of wound repair, but also markers of acute inflammation.

CONCLUSION

In this review, the origin and maintenance of AMs and their essential role in innate immunity to respiratory pathogens are discussed. There is emerging evidence for the self-renewal of AMs and lung tissue macrophages through mechanisms dependent on GM-CSF and CSF-1 signaling. The role of peripheral monocytes in replenishing lung macrophages appear to be context specific, as radiation-induced lung injury stimulates monocytic replenishment, whereas influenza infection replenishes AMs through local proliferation of resident macrophages. In diseases such as COPD where there is an accumulation of airway macrophages, the relative contribution of monocyte versus local proliferation of mature AM populations remains to be determined. Lineage tracing of monocytes/macrophages in experimental models of COPD will inform on the origin of macrophages associated with disease pathology.

Alveolar macrophages coordinate the efficient clearance of inhaled irritants and microbes to resolve inflammation. In addition, AMs display efferocytic activity to clear damaged tissue and cells following injury and infection. In COPD, the persistence of inflammation and the inability to efficiently clear damaged tissue and exhausted immune cells such as neutrophils may be due to excessive oxidative stress that impairs the phagocytic capacity of AMs. Airway macrophages in COPD also display a unique phenotype that is associated with the induction of M2-related genes, which are likely to be upregulated in response to local tissue damage (Figure 1). The maintenance of this subpopulation may also contribute to deleterious remodeling in COPD. Thus, there is a

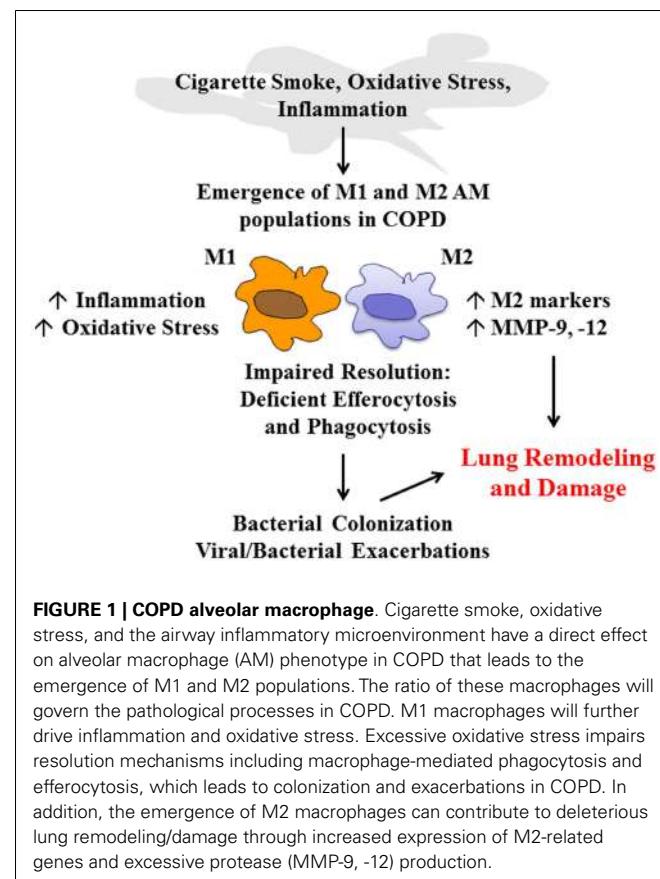


FIGURE 1 | COPD alveolar macrophage. Cigarette smoke, oxidative stress, and the airway inflammatory microenvironment have a direct effect on alveolar macrophage (AM) phenotype in COPD that leads to the emergence of M1 and M2 populations. The ratio of these macrophages will govern the pathological processes in COPD. M1 macrophages will further drive inflammation and oxidative stress. Excessive oxidative stress impairs resolution mechanisms including macrophage-mediated phagocytosis and efferocytosis, which leads to colonization and exacerbations in COPD. In addition, the emergence of M2 macrophages can contribute to deleterious lung remodeling/damage through increased expression of M2-related genes and excessive protease (MMP-9, -12) production.

need to better characterize distinct AM populations present in COPD and their relative contribution to disease pathology, as their highly plastic nature offer a therapeutic opportunity to reprogram macrophages to facilitate restoration of lung homeostasis.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 22 July 2014; paper pending published: 18 August 2014; accepted: 26 August 2014; published online: 10 September 2014.

Citation: Vlahos R and Bozinovski S (2014) Role of alveolar macrophages in chronic obstructive pulmonary disease. *Front. Immunol.* **5**:435. doi:10.3389/fimmu.2014.00435

This article was submitted to Molecular Innate Immunity, a section of the journal *Frontiers in Immunology*.

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Macrophage polarization in obesity and type 2 diabetes: weighing down our understanding of macrophage function?

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Obesity and type 2 diabetes are now recognized as chronic pro-inflammatory diseases. In the last decade, the role of the macrophage in particular has become increasingly implicated in their pathogenesis. Abundant literature now establishes that monocytes get recruited to peripheral tissues (i.e., pancreas, liver, and adipose tissue) to become resident macrophages and contribute to local inflammation, development of insulin resistance, or even pancreatic dysfunction. Furthermore, an accumulation of evidence has established an important role for macrophage polarization in the development of metabolic diseases. The general view in obesity is that there is an imbalance in the ratio of M1/M2 macrophages, with M1 "pro-inflammatory" macrophages being enhanced compared with M2 "anti-inflammatory" macrophages being down-regulated, leading to chronic inflammation and the propagation of metabolic dysfunction. However, there is emerging evidence revealing a more complex scenario with the spectrum of macrophage states exceeding well beyond the M1/M2 binary classification and confused further by human and animal models exhibiting different macrophage profiles. In this review, we will discuss the recent findings regarding macrophage polarization in obesity and type 2 diabetes.

Keywords: macrophage, inflammation, obesity, type 2 diabetes, M1/M2, polarization

Inflammation is a fundamental biological process whose role is not only to enable host protection against pathogens, but also to stimulate and modulate repair and healing when cellular damage occurs. During an inflammatory event, once the initial insult is contained, a primary objective is the restoration of tissue homeostasis. Failure to appropriately resolve an inflammatory stimulus can result in persistent immune system activation, which can actually cause tissue damage and disease. Significant literature over the last decades has established obesity to induce a state of chronic low-grade systemic inflammation (1). Importantly, the inflammation accompanying obesity is distinctly different to that of acute inflammation, as the inflammatory stimulus fails to be resolved. This is of particular significance as chronic low-grade inflammation is implicated in the etiology of atherosclerosis, hypertension, type 2 diabetes (T2D), and even certain cancers, which can all be associated with obesity (2, 3). Considering the economic burden from the increasing prevalence of these chronic metabolic diseases, it is not surprising that considerable scientific attention has focused on how and why obesity promotes chronic low-grade inflammation.

Immune cells are the primary effectors of most inflammatory reactions and are categorized into the innate and acquired immune systems. With respect to obesity, leukocytes from both immune

systems have been implicated in the development of chronic low-grade inflammation and metabolic dysfunction (1). However, adipose tissue macrophages have received the lion's share of attention of the immune cells involved. Macrophages can display remarkable phenotypic heterogeneity with the ability to perform vastly different roles depending on the biological situation (4). Thus, a spectrum of many different macrophage populations has been characterized by combinations of membrane markers and gene expression profiles. This led to the establishment of a complex nomenclature, which over time has become simplified into two main macrophage phenotypes, M1 pro-inflammatory and M2 anti-inflammatory macrophages. This classification is obviously a simplistic view of the situation as it is now clear that macrophages dynamically evolve from one phenotype to the other according to their environment and can occupy various points of the spectrum with mixed characteristics [for updated nomenclature see Ref. (5, 6)]. For clarity purposes, we will mostly mention macrophages as either M1 or M2.

MACROPHAGE ACCUMULATION IN THE OBESE ADIPOSE TISSUE

Phagocytosis is a main function of macrophages that allows them to contribute to tissue homeostasis through surveillance,

maintenance, and repair. Though long-lived macrophage populations reside within almost all bodily tissues, in 2003, two separate laboratories reported macrophage accumulation in the white adipose tissue (WAT) of both obese patients and rodents (7, 8). Importantly, this was completely in line with Hotamisligil's landmark article 10 years earlier observing TNF α secretion from the adipose tissue in obese rodents (9). Indeed, it appeared these macrophages were the source of the elevated inflammatory cytokines reported in obesity and their accumulation was associated with insulin resistance (7, 8). These landmark studies were the first to link obesity and insulin resistance with adipose tissue macrophage content and inflammation. Later it appeared that the accumulation of macrophages is not limited to the WAT in obesity with macrophages found to accumulate in many other organs critical for glucose homeostasis such as liver, pancreas, gut, and even the brain (3). Regardless, the most significant immunological changes occurring during obesity originate within the adipose tissue. Of interest, despite the adipose tissue representing a small portion of whole body glucose disposal, immune dysfunction within this tissue is sufficient to impair systemic glucose metabolism (1).

MACROPHAGE POLARIZATION IN OBESITY AND T2D

Macrophages constitute an important fraction of non-adipocyte cells within the WAT. Indeed, within a "normal" lean WAT, they can account for almost 10% of total cell number. Profiling of lean adipose tissue revealed these macrophages to possess an M2 phenotype. These macrophages perform tissue surveillance and remodeling functions and are associated with maintaining WAT insulin sensitivity. Indeed, the manipulation of peroxisome proliferator activator receptor (PPAR) transcription factors required for macrophage M2 polarization was associated with metabolic dysfunction (10–12). The current theory supports that weight gain induces local inflammation and chemokine production to promote recruitment of circulating pro-inflammatory (Ly6C hi) monocytes. Recruited monocytes differentiate into an M1 macrophage phenotype and their accumulation leads to an imbalance between M1 and M2 macrophages. Increased cytokine production from M1 macrophages and/or reduced anti-inflammatory signals from the M2 macrophages promote adipose tissue dysfunction and impairs glucose tolerance.

Evidence for the detrimental role of M1 macrophages in promoting adipose tissue insulin resistance has been reported in several studies. Lumeng and colleagues first reported that the macrophages accumulating in obese WAT possessed an inflammatory CD11c $+$ M1 phenotype and gathered around necrotic adipocytes in Crown like structures (CLS) (13, 14). Furthermore, M1 macrophage numbers were shown to correlate with insulin resistance in high-fat fed rodents (15). Whether WAT M1 macrophages could be targeted therapeutically has proven more challenging. Clodronate is a toxic compound administered in liposomes that gets taken up by macrophages thereby inducing their apoptosis. Clodronate liposome injections succeed in decreasing visceral WAT macrophage accumulation and improve glucose metabolism and insulin sensitivity in HFD-fed mice (16). However, this approach has limitations as clodronate liposomes targeted all WAT macrophage phenotypes as well as

liver Kuppfer cells. One could envisage a deleterious effect from depleting M2 macrophages on a long term basis. A more specific removal of M1 macrophages was achieved in an elegant study published by Olefsky's group. They demonstrated that ablation of M1 macrophages, achieved by targeting diphtheria-sensitive CD11c $+$ cells with diphtheria toxin, was associated with improved glucose tolerance (17). While CD11c expression allows discrimination between M1 and M2 macrophages, its expression is not exclusive for M1 macrophages. Dendritic cells and neutrophils also express CD11c and the elevation of these cells in obese WAT may contribute to the increased CD11c $+$ cell population and insulin resistance (18, 19). From these studies, it is clear that targeting established M1 populations within obese WAT will prove complex. For these reasons, studies investigating mechanisms leading to macrophage accumulation may yield more promising therapeutic targets.

POTENTIAL MECHANISMS FOR MACROPHAGE ACCUMULATION IN OBESITY

White adipose tissue macrophage accumulation is thought to occur through two main processes. First, increased chemokine secretion from adipocytes and resident macrophages promotes the recruitment of Ly6C $+$ blood monocytes to obese WAT. Most of these monocytes subsequently differentiate into M1 macrophages in response to inflammatory signals within the adipose tissue. Of the chemokines produced from obese WAT, monocyte chemoattractant protein-1(MCP-1) and its receptor C-C motif receptor-2 (CCR2) appear particularly important. For example, mice lacking either CCR2 or MCP-1 have reduced ATMs, whereas adipocyte specific over-expression of MCP-1 leads to enhanced ATMs (20, 21). However, these knockout models did not normalize macrophage numbers suggesting additional mechanisms are likely to be involved in obesity-induced macrophage accumulation (20). Indeed, obese WAT secretes many chemokines including LTB4, MIP, MIF, and MCP-3 implicated in macrophage accumulation and glucose intolerance (22). Interestingly, in addition to chemokine secretion, signals from obese WAT also influence bone marrow progenitor cells to increase myelopoiesis. Indeed, we have recently demonstrated that in obese mice, IL1 β production from CD11c $+$ ATMs promoted bone marrow myelopoiesis further perpetuating adipose tissue inflammation (23). Second, resident ATMs have a strong proliferation capacity in both human beings and rodents. Indeed, Jenkins et al. showed that IL4 is a strong promoter of macrophage proliferation (24). Hence, in the lean state where eosinophils secrete high levels of IL4 in the WAT, proliferation is considered the major mechanism to maintain resident M2 macrophage populations (24–26). Interestingly, Amano et al. revealed that MCP-1 could enhance macrophage proliferation in the visceral WAT independent of its chemokine function (27). It is clear that further studies are required to determine the contribution of macrophage proliferation and recruitment to adipose tissue macrophage accumulation in obesity.

MACROPHAGE FATE

While the recruitment and source of macrophages present in obese adipose tissue are well documented, the fate of these recruited

macrophages remains less studied. Unlike resolving inflammation in which levels of inflammatory leukocytes subside following the restoration of tissue homeostasis, adipose tissue inflammation induced by excessive adiposity fails to resolve naturally. During resolving inflammation, it is well appreciated that the initially recruited polymorphonuclear neutrophils undergo swift apoptosis prompting their phagocytosis by macrophages. Despite early evidence for macrophage emigration and drainage to lymph nodes as a significant contributor to macrophage disappearance following acute inflammation, recent work from the Randolph laboratory has revealed that macrophage apoptosis is largely responsible for their removal in acute inflammation (28). Whether these mechanisms contribute to adipose tissue macrophage accumulation in non-resolving inflammation is not well studied. For example, whether M1 macrophages accumulate within obese adipose tissue due to pro-survival/anti-apoptotic signals remains unknown. However, it is also possible that macrophages are actively retained within the adipose tissue in response to various cues. Netrin-1, best described for its role in neural development, has recently been implicated in macrophage retention within obese adipose tissue (29). However, these findings are somewhat at odds with the plethora of studies showing the importance of monocyte/macrophage recruitment, repopulation (e.g., after bone marrow transplantation), and proliferation to the macrophage burden in the obese adipose tissue and perhaps plays only a minor role.

A MORE COMPLEX PICTURE THAN M1 OR M2

Macrophages are able to modify their phenotype according to their environment. However, whether macrophage phenotype switching occurs in obesity remains unresolved (30, 31). Shaul et al. demonstrated that in HFD-fed animals' classical M1 macrophage accumulation is observed after 8 weeks of diet, however, after 12 weeks of diet, these CD11c⁺ macrophages exhibited an increased expression of M2 associated transcripts (32). While the M1 macrophages maintained their pro-inflammatory phenotype, they also adopted some remodeling features in a context of increased adipogenesis reminiscent of M2. Conversely, M2 macrophages are able to secrete pro-inflammatory mediators in specific conditions (33). These studies reveal extremely dynamic macrophage populations and newer technologies such as live imaging undoubtedly will enhance our understanding of the changes occurring in macrophage polarization state upon weight gain. Indeed, Haase et al. showed recently that most adipose tissue macrophages arising *in situ* stained positive for the M2 markers CD206 and CD301 (34). Live imagery and tracking techniques allowed the revelation that newly formed M2 macrophages originates within the CLS before they migrate to the interstitial space. Given that CLS form around dying adipocytes, the presence of M2 macrophages could be viewed as a resolving mechanism. Given that macrophages are able to alter their phenotype and that most studies assess adipose tissue macrophage content at one single time, care must be exercised when interpreting data. For example, one cannot expect to predict a metabolic phenotype based upon observed adipose tissue macrophage polarity.

It remains plausible that the inflammation associated with obesity initially constitutes a physiological rather than pathological process within the adipose tissue. Indeed, in an elegant study, Scherer and colleagues have demonstrated that adipocyte inflammation is essential for adipose tissue expansion and remodeling (35). Using the "adipochaser mouse," they tracked newly formed adipocytes and distinguished them from older "blue" adipocytes expressing β-galactosidase. They determined that acute inflammation promotes adipogenesis and improved adipose tissue function and insulin sensitivity. Conversely, they demonstrated that abrogation of inflammation within the adipose tissue led to defective adipogenesis followed by ectopic lipid accumulation and glucose intolerance (35). These findings highlight a previously unappreciated role for inflammation *per se* in healthy adipose tissue function.

CAN WE TARGET IMMUNITY TO TREAT T2D?

It is important to ensure that the findings made in rodent models are useful in human pathology. It is clear that macrophages also accumulate in adipose tissue of obese humans (7) and have been correlated with insulin resistance (36). Wentworth et al. also showed that pro-inflammatory CD11c⁺ macrophages are positively associated with systemic insulin resistance in obese patients (37). Furthermore, macrophage content is reduced in the adipose tissue following gastric bypass surgery (38). Importantly, in these patients, the macrophage status was switched toward a less pro-inflammatory profile. However, other groups have shown that the accrued macrophages in human adipose tissue present M2 surface markers associated with a more anti-inflammatory phenotype (39, 40). Nonetheless, Zeyda et al. showed that these M2 macrophages possess a strong capacity to produce pro-inflammatory mediators (39). These discrepancies may be explained by different experimental protocols. In most studies, WAT is obtained from subcutaneous depots whereas fewer studies report data obtained from omental WAT. Furthermore, the sex and degree of adiposity of the patients may also account for some of the differences reported. The same reasons may partly explain the inconsistencies observed between mice and human beings. In addition, important metabolic differences between rodents and human beings contribute to the difficulty of translating mouse data into human therapies.

In addition, there are further evidences that inflammation and macrophages should be targeted with the greatest care in T2D. Indeed, Chawla's group recently reported that M2 macrophages can secrete catecholamines in response to cold exposure, activating thermogenesis of brown adipose tissue and lipolysis of WAT (41). They later found that M2 macrophages were directly involved in the "beginning" of adipose tissue (42). In addition, a current article proposed that M2 macrophages strongly promote β-cell proliferation (43). The presented studies tend to suggest that the positive effects of macrophages in obesity and T2D are carried on by repair macrophages exhibiting an M2 phenotype. Hence, there appears to be rationale targeting the enhancement of M2 populations as opposed to depleting M1 macrophages, which may jeopardize the patient's immune function. However, increased M2 macrophage function is also tightly linked to tumor proliferation

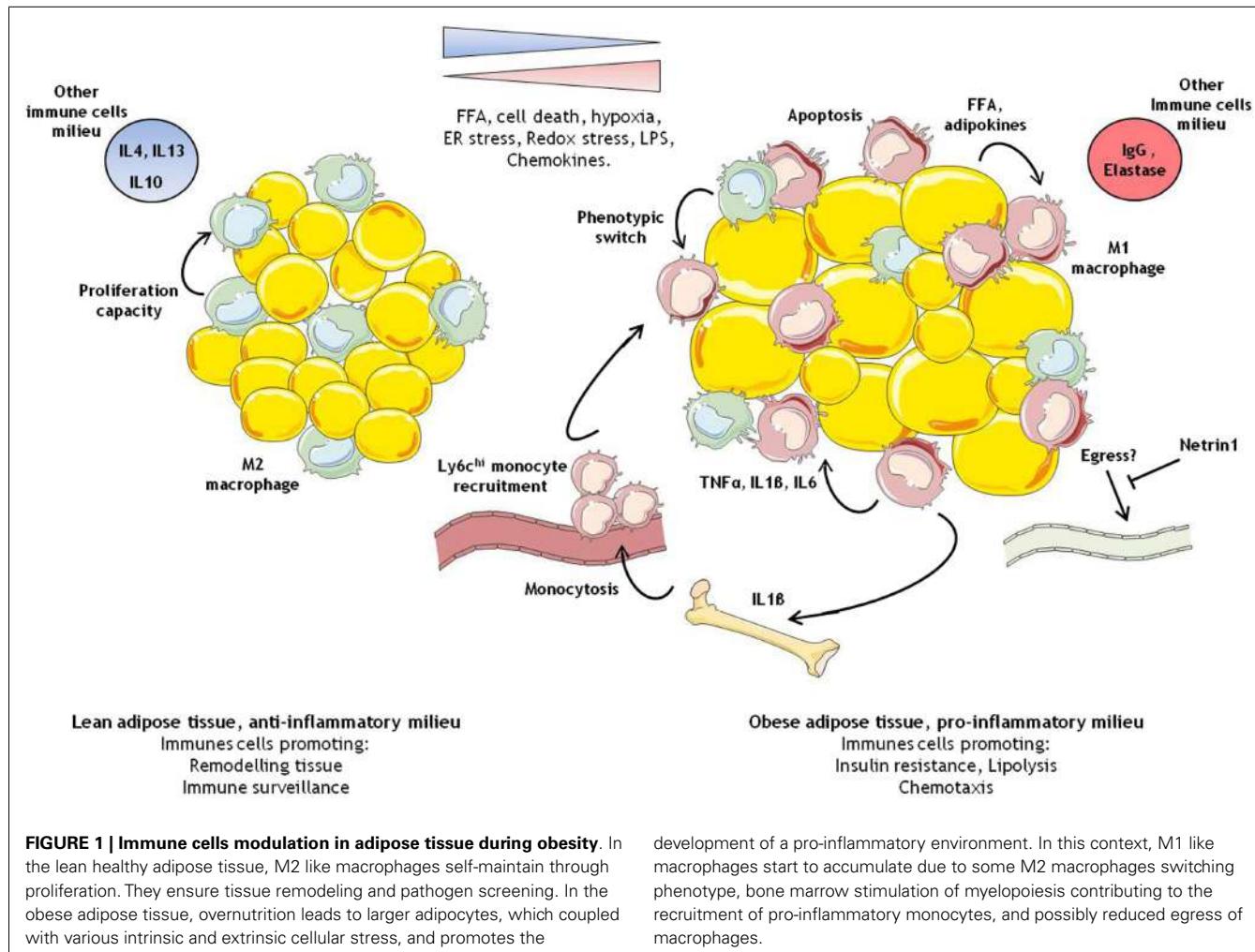


FIGURE 1 | Immune cells modulation in adipose tissue during obesity. In the lean healthy adipose tissue, M2 like macrophages self-maintain through proliferation. They ensure tissue remodeling and pathogen screening. In the obese adipose tissue, overnutrition leads to larger adipocytes, which coupled with various intrinsic and extrinsic cellular stress, and promotes the

development of a pro-inflammatory environment. In this context, M1 like macrophages start to accumulate due to some M2 macrophages switching phenotype, bone marrow stimulation of myelopoiesis contributing to the recruitment of pro-inflammatory monocytes, and possibly reduced egress of macrophages.

so manipulating M2 populations must be considered carefully as obese patients already present with an increased incidence of cancer (2).

CONCLUSION

It is increasingly appreciated that immune cells play a crucial role in the control of whole body metabolism (2). In light of this view, it seems clear that the macrophage accumulation within the adipose tissue is associated with metabolic dysfunction observed in obesity. However, the role of macrophages in the pathogenesis of obesity and diabetes is still conflicting. In addition to the complexity of the immune response itself, different rodent models, the use of different high-fat diets and of different intervention time courses not to add the different environments of each animal facility, have often led to contradictory results raising more confusion within the field. As it was nicely exposed in a recent review by Murray et al., it is important for the coherence of results in this domain that researchers try and decide which models to focus on to allow the field to progress and result in more human translational potential (5).

In this review, we have summarized the recent findings on the role of macrophages in obesity and T2D. M1 macrophage

accumulation within the adipose tissue remains tightly associated with obesity. It is still unclear how much this accumulation contributes to the glucose intolerance and insulin resistance described in obese rodent models. There is growing literature suggesting their presence could be required for physiological adaptations of the adipose tissue (35). Perhaps these findings reflect the dynamic nature of macrophage polarity and the essential role of macrophages in the biology of adipose tissue (Figure 1). However, it is also important to take a “non-glucose/insulin resistant-centric view” with respect to the role of adipose tissue macrophages and to appreciate that they could also contribute significantly to the risk of other associated diseases including cardiovascular disease and cancer. As a whole, these data advocate that macrophages should be targeted with the greatest care in metabolic diseases.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 21 August 2014; accepted: 12 September 2014; published online: 26 September 2014.

Citation: Kraakman MJ, Murphy AJ, Jandeleit-Dahm K and Kammoun HL (2014) Macrophage polarization in obesity and type 2 diabetes: weighing down our understanding of macrophage function? *Front. Immunol.* **5**:470. doi:10.3389/fimmu.2014.00470

This article was submitted to Molecular Innate Immunity, a section of the journal *Frontiers in Immunology*.

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Are “resting” microglia more “M2”?

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Keywords: microglia, M2, alternative activation, neuroinflammation, resting, activation

Over the last decade, the concept and nomenclature of microglial phenotype polarization has been carried over from the peripheral macrophage literature. However, it is not entirely correct to view these two cell types as overlapping. Microglia, although related to macrophages, have several differences and their own unique repertoire of features. In particular, microglia arise from a distinct early yolk sac progenitor population and therefore have different developmental origins than macrophages (1). Furthermore, once in the central nervous system (CNS), microglia are maintained through local self-renewal (2). Under normal conditions, there is no infiltration of peripheral macrophages (3). Microglia also maintain expression profiles distinct from peripheral macrophages (4). This demonstrates that microglia are not simply macrophages that have migrated into the brain; rather, they are a distinct cell type.

These ideas have led to a reassessment of microglia activity, leading many researchers to shift their thinking on glial biology in general. One of the more outdated concepts carried over from macrophages is the idea that microglia in the healthy brain exist in a “resting” state. Through a variety of stimuli these “resting” cells can rapidly be “activated,” yielding microglia that are cytotoxic (5). These “activated” microglia were described in and were thought to play a major driving role in many neurodegenerative diseases (6). Thus, microglia “activation” took on a largely negative connotation. Taking cues from ongoing work in peripheral macrophages, this initial concept of microglia “activation” being generally harmful gave way to more specific ideas suggesting there was not just one

kind of “activation.” Under the influence of either pro- or anti-inflammatory cytokines, microglia could be polarized into an inflammatory or anti-inflammatory phenotype, designated classically and alternatively activated microglia, respectively (7). Others have given different designations not solely based on inflammation but from inhibiting vs. healing functions, and label them M1 and M2, respectively (8). It is now becoming clear that the responses of microglia, like macrophages, are heterogeneous: these responses can include pro- and anti-inflammatory signatures within single cells and small, nearby populations and are driven by the local environment that can supply M1 and M2 polarizing cues simultaneously (9). Therefore, microglial responses are much richer than the dichotomous nomenclature suggests. It is common to represent microglia polarization as a spectrum with each respective phenotype occupying the extremes of the scale (10). However, it is unclear whether the diverse functional responses observed are a product of many different kinds of microglia subsets or simply varying ratios of M1 and M2 microglia. While there is much work that details this spectrum of activation, and much remains to be investigated, an interesting but overlooked area is the phenotype of the “resting” microglial cell.

Classically, those who study microglial function in a healthy, normal brain are not the same groups that study microglia in an “activated” or disease setting. However, it appears that the microglia in each of these settings may be more similar than originally thought. The notion that microglia are truly “resting” has long been cast aside. The advent of *in vivo* techniques, in particular 2-photon microscopy, has revealed

the constant surveillance and activity of microglia, even in the absence of traditional activation signals (11). Therefore, it might be incorrect to view microglia in the normal, healthy CNS as a distinct population that can become “activated.” Rather, it seems likely that even at baseline, microglia are already on the activation spectrum. So the question arises, where on the spectrum are they?

Inflammation in the brain is typically associated with harmful outcomes. Even acute, low level inflammation can impair synaptic function, leading to cognitive dysfunction and behavioral abnormalities (12). Moreover, neuroinflammation has been recognized as a pathological hallmark in most chronic neurodegenerative diseases (13). This demonstrates the sensitivity of the brain to inflammation and the importance of protecting the CNS from insult. This protective role has generally been assigned to M2 microglia. Many investigators have observed protective effects of M2 cells; such as elevated neuronal survival and process extension after treatment with M2 conditioned media (14), or as reported in numerous papers that detail the beneficial effect of direct treatment with M2 inducing agents (15). However, all of these take place during pathology and do not consider the normal protective function of microglia. Thus, given the critical role of basal microglia in maintaining homeostasis, an attractive hypothesis is that under normal conditions, microglia are skewed toward a protective, anti-inflammatory phenotype. In fact many of the normal functions of microglia are reminiscent of M2 cells, although they are not as prominent as fully polarized cells. Even without stimulation, microglia are vital sources of important, neurosupportive cytokines

such as IGF-1 and BDNF (16–18). Neuroprotective cytokine secretion is generally considered an M2 microglial function, but we now know it also occurs in "resting" microglia, albeit at lower levels. Indeed, in models that lack proper M2 inducing signals like IL-4^{-/-} and SCID mice, cognitive impairment is observed, which was attributed to decreased production of these necessary neurotrophins (19, 20).

Furthermore, microglia in a basal state share another important function with fully polarized M2 microglia, namely, rapid and efficient debris clearance (21). This process, which is one of the quintessential defining functions of M2 polarized cells, seems to be a default function for microglia. Although the concept of phagocytosis is not unique to alternatively activated microglia, the speed and quality at which this occurs differs between the phenotypes. In contrast to M1 polarized cells, where a slower and less acidic phagosome is beneficial for downstream immune functions such as antigen-presenting abilities, a rapid, more acidic phagosome aids M2 cells in quick, efficient removal of debris (22). This speed and efficiency can be observed in the highly dynamic process called synaptic pruning, which is characterized as rapid elimination of developing synapses. In the past several years, microglia have been shown to be crucial to this process (23). This concept is thought to be carried over into the adult but at a less dramatic level (24). The role of microglia in normal synaptic maintenance is not fully understood, so it is difficult to directly attribute baseline phagocytic function to an M2 related mechanism. However, due to the speed and efficiency at which this process occurs, we can speculate that this type of phagocytosis shares more similarities with M2 than M1 polarized microglia.

In addition to the neuroprotective and functional similarities resting microglia share with traditionally polarized M2 microglia, skewing to an M2 state can be seen in the receptor profile resting microglia express. For example, unlike macrophages, microglia express very low levels of MHCII (25). Only when microglia polarize to an inflammatory phenotype do they upregulate MHCII expression (26). Contrary to MHCII, DC-SIGN has been observed on microglia in the normal brain. This c-type lectin receptor, which has been

implicated in promoting immune homeostasis, also maintains the immunosuppressive environment in the healthy brain (27). By limiting particular surface receptors while expressing others, microglia are biased toward a particular phenotype, namely anti-inflammatory. However, it appears that this M2 biased phenotype may change with age. For still unknown reasons, there is a loss of signals that keep microglia anti-inflammatory. During normal aging, a reduction in IL-4R α is observed as well as a decreased sensitivity to other anti-inflammatory cytokines (28). This is mirrored by an increase in sensitivity to proinflammatory cytokines, suggesting a switch in the basal phenotype of microglia (29). A similar switch, termed priming, has been observed after microglia were exposed to inflammatory cytokines. Following the initial inflammatory insult, microglia appear to return to their basal state. However, with a second inflammatory stimulus these "primed" microglia produce significantly more inflammatory cytokines than unprimed microglia (30), suggesting that their basal state is altered toward a more M1 phenotype. Age-associated and priming switches may be involved in increased susceptibility to neurodegenerative disorders (31).

Keeping microglia skewed toward a non-inflamed state is critical for normal homeostasis and specific control mechanisms exist that actively prevent microglia from adopting an inflammatory profile. In particular, neurons express several receptors and ligands that signal to their counterparts specifically localized on microglia. CX3CR1, CD200, CD47, TREM2, and several other receptors have been identified that participate in constant cross-talk between microglia and neurons (32). Interestingly, in genetic knockout mice missing CX3CR1, impaired cognition was observed (33). Two explanations appear likely. First, the loss of direct inhibition resulted in increased inflammation, which in turn, caused cognitive dysfunction (33). Secondly, the loss of CX3CR1 resulted in impaired phagocytic ability (23) and subsequent loss of proper synaptic pruning during a critical developmental period, as discussed previously. Although different, both of these explanations share the idea that divergence from proper normal baseline function (namely M2 skewed

functions) results in CNS pathology. Furthermore, this suggests that neurons, under normal conditions, are active in controlling microglial polarization. This environmental control on phenotype skewing poses an interesting question, is basal state polarization present in other tissue specific macrophages? The answer is most likely yes. However, it seems that other tissue macrophage cells such as peritoneal, lung, or splenic red pulp macrophages all exhibit greater diversity in their normal gene expression and most likely, basal function when compared to microglia, even though they share common yolk sac progenitor (1, 34). Therefore, it is hard to directly compare between the cell types. This demonstrates the large role the environment plays in skewing cells to the needs of a specific tissue. In the CNS, an anti-inflammatory state is most beneficial so the environment favors slight M2 skewing. It would be interesting to characterize potential skewing in other organs and how that relates to normal resident macrophage function.

The idea that microglia exist as a skewed population in normal, non-pathological tissue highlights this phenotype as an innate characteristic of microglia. The classical view of macrophage biology is that the adaptive arm of the immune system, primarily T cells, controls phenotypes via release of cytokines such as IFN- γ or IL-4 (35, 36). Unfortunately, it is difficult to prove innate or adaptive control in peripheral tissue due to the normal presence of T cells. However, the healthy brain is largely devoid of T cells, which accentuates the idea that M2 phenotype skewing is a default, innate function of microglia (37). Interestingly, similar ideas have been proposed for macrophages (38).

In conclusion, to properly understand and discuss microglia, we have to do away with terms such as "activation" and "resting." By using these outdated concepts, we fail to acknowledge the complex plastic nature of microglia. In addition to not correctly representing the non-pathological, normal functions of microglia, the term "activation" is vague and provides no specific information about the many possible microglial phenotypes. Microglia are always "active," so the true distinguishing feature is where they exist on the phenotype spectrum. Healthy, normal

brain microglia do not sit precisely in the center between inflammatory and anti-inflammatory cells. Rather, these sentinel microglia are slightly shifted toward an anti-inflammatory phenotype, which is beneficial to brain homeostasis. The field of microglial biology does not need to battle over nomenclature for baseline microglia. No single term can adequately encompass all microglial functions at all times. But it is important to recognize that these cells are a plastic population that can dynamically shift between a spectrum of phenotypes and should not be boxed into fixed, rigid "activation" states.

AUTHORS CONTRIBUTION

Jonathan D. Cherry researched the literature and drafted the manuscript. M. Kerry O'Banion and John A. Olschowka critically reviewed and edited the work. All the authors read and approved the final manuscript.

ACKNOWLEDGMENTS

The authors thank Dr. Ania Majewska for her editorial assistance as well as scientific insight on the subject matter. This work was supported by the NIH/NIA grant R01AG030149.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 06 August 2014; accepted: 05 November 2014; published online: 18 November 2014.

*Citation: Cherry JD, Olschowka JA and O'Banion MK (2014) Are "resting" microglia more "M2"? *Front. Immunol.* **5**:594. doi: 10.3389/fimmu.2014.00594*

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M1/M2 macrophage polarity in normal and complicated pregnancy

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INTRODUCTION

Macrophages within the maternal–fetal compartment have been a research focus for over 30 years. Both maternal and fetal derived macrophages play an important role in all stages of pregnancy. These cells support a variety of processes essential for successful pregnancy such as remodeling of the uterine connective tissues and blood vessels, regulation of trophoblast (fetal cell) implantation, immune-tolerance toward fetal antigens, immunomodulation of neighboring leukocytes, and initiation of parturition (1–5). All of these functions are manifestations of macrophage polarity or state of activation, which at the most basic level has been referred to as M1 and M2. M1 macrophages display the capacity to present antigen, produce IL-12, IL-23, reactive oxygen species (6, 7), and skew T cell responses toward a TH1 or cell mediated immune response (8). In contrast, M2 macrophages participate in tissue remodeling, have immunosuppressive qualities, and promote TH2 or antibody mediated immune responses (6). In essence, M1/M2 activities arise from arginine metabolism via two enzymatic pathways (iNOS and arginase) that down regulate each other (7, 9). The factors that influence which pathway dominates are based on the surrounding signals that the macrophage receives as well as the available arginine pool (7, 10). Thus, macrophage function, which is a manifestation of the cell's polarization or activation state, is ultimately decided by the surrounding milieu.

The maternal–fetal interface is a unique environment in that it comprises three distinct compartments: the placenta (fetal origin), and maternal origin (endometrium or decidua, and myometrium), which are infiltrated with fetal cells. Furthermore, these compartments undergo dramatic changes in architecture and leukocyte composition as gestation progresses. These changes are necessary for placental development and tolerance of foreign

Tissue macrophages play an important role in all stages of pregnancy, including uterine stromal remodeling (decidualization) before embryo implantation, parturition, and post-partum uterine involution. The activation state and function of utero-placental macrophages are largely dependent on the local tissue microenvironment. Thus, macrophages are involved in a variety of activities such as regulation of immune cell activities, placental cell invasion, angiogenesis, and tissue remodeling. Disruption of the uterine microenvironment, particularly during the early stages of pregnancy (decidualization, implantation, and placentation) can have profound effects on macrophage activity and subsequently impact pregnancy outcome. In this review, we will provide an overview of the temporal and spatial regulation of utero-placental macrophage activation during normal pregnancy in human beings and rodents with a focus on more recent findings. We will also discuss the role of M1/M2 dysregulation within the intrauterine environment during adverse pregnancy outcomes.

Keywords: macrophages, M1/M2, normal pregnancy, complicated pregnancy, uterine atherosclerosis, spiral artery, chorioamnionitis, spontaneous abortion

fetal antigens. Macrophages are present in all compartments and during all stages of pregnancy (implantation, placentation, fetal growth, and parturition) (11). Successful pregnancy requires that the macrophage activation state remain regulated throughout pregnancy. Indeed, inappropriate macrophage polarization within the maternal–fetal compartments is associated with spontaneous abortion or miscarriage (12), inadequate remodeling of the uterine vessels during placentation (12–14), and intrauterine parasitic infections (15). In this review, we provide an overview of M1/M2 dynamics relevant to the maternal–fetal interface, the temporal and spatial changes in macrophage M1/M2 within the maternal and fetal compartments during normal pregnancy, and imbalanced M1/M2 dynamics associated with complicated pregnancies.

M1 AND THE MANY SHADES OF M2

Macrophages display divergent phenotypes that were originally described as M1 or M2 polarity (7). M1 refers to the classically activated macrophage whereby the cell displays the capacity to present antigen, produce IL-12, IL-23, and reactive oxygen species (6, 7). M1 polarized macrophages are more effective at antimicrobial killing and skewing T cell responses toward a TH1 or cell mediated immune response (8). External stimuli known to promote M1 polarization include toll-like receptor (TLR4) agonists such as lipopolysaccharide (LPS) from *E. coli*, IFN- γ , TNF- α , and GM-CSF (7, 16). In contrast, M2 polarized cells (alternatively activated cells) display abundant expression of mannose and scavenger receptors and produce IL-10 and TGF- β (6). M2 macrophages participate in tissue remodeling, have immunosuppressive qualities, and promote TH2 or antibody mediated immune responses (6). Stimuli that induce M2 polarization include IL-4, IL-10, IL-13,

IL-33, TGF- β , and G-CSF (7, 16–19). Because of the broad range of activities carried out by M2 macrophages, Mantovani et al. (6) later proposed that these cells be further divided into M2a (induced by IL-4 and IL-13), M2b (macrophages exposed to immune complexes or toll-like receptor agonists), and M2c (induced by IL-10 and glucocorticoid hormones). These subcategories were based on both the type of agonists responsible for triggering their differentiation and the distinct functional profile induced by these agonists. For example, M2a cells display the alternatively activated phenotype typically attributed to M2 cells. M2b confers a Type II phenotype, because it promotes TH2 responses and produces both pro- and anti-inflammatory cytokines (TNF- α , IL-1, IL-6, IL-10^{high}, and IL-12^{low}) (6). M2c confers an M2 macrophage that produces IL-10, TGF- β , and extracellular matrix components (6).

At the most fundamental level, M1/M2 polarity is driven by arginine metabolism and available arginine within the extracellular milieu. In essence, M1 is a product of iNOS pathway, which drives the conversion of arginine to citrulline and NO, whereas M2 is a product of the arginase pathway in which arginine is hydrolyzed into ornithine and urea (7, 20). iNOS and arginase pathways are antagonistic, not only do they compete for arginine

but also arginase directly inhibits NO synthase 2 (NOS2) (10). Although M2 is defined by the arginase pathway (7, 9), in Mantovani et al.'s designation, M2b develops independent of arginase activity, while M2a and M2c still require arginase activity (6).

A distinctive feature of the iNOS pathway is that its activation requires extracellular arginine, even if an adequate level of intracellular arginine is already present (21). Without sufficient arginine iNOS is blocked at the translational level and NO is not produced even in the presence of IFN- γ or LPS. Under these conditions, the macrophage does not acquire an M1 phenotype, but still produces TNF- α in response to IFN- γ or LPS (21). This condition bears a resemblance to M2b macrophages (6). This dependency upon extracellular arginine reserves may be particularly important during pregnancy since placental tissues are rich in arginase I and II (22), and therefore, placental extracellular arginine levels may actually be too low for induction of M1 macrophages.

Several studies have attempted to characterize the functional state of macrophages at the maternal–fetal interface during different stages of a healthy pregnancy (Table 1). These include transcriptome analysis (23, 24), methylation profiling (25), characterization of cell surface markers (26–28), and

Table 1 | Summary of recent studies that characterized M1/M2 status of macrophages within the maternal–fetal interface during healthy pregnancy.

First author	Year	Gestation at sampling (weeks)	Methods	Results	Reference
Laskarin	2005	6–8 Term	Immunohistochemistry, costaining for CD14 decidual and placental macrophages (M ϕ) and mannose receptor (M2 biomarker)	Confirmed that decidual and placental M ϕ express mannose receptor <i>in vivo</i> CD14 $^{+}$ decidual M ϕ surround endometrial glands in early pregnancy 50% Of CD14 $^{+}$ M ϕ in term decidua and villi (Hofbauer) express mannose receptor	(29)
Repnik	2008	9–18 19–23 Term	Flow cytometry of decidual M ϕ	Decidual M ϕ from early/mid gestation have higher CD80, CD86, and HLA-DR expression than term M ϕ M ϕ in decidua basalis express CD105, DC-SIGN, and mannose receptor	(26)
Gustafsson	2008	7–11	RNA Microarray analysis of CD14 $^{+}$ M ϕ isolated from decidua and peripheral blood	The majority of genes upregulated in decidual M ϕ were functionally related to immunomodulation and tissue remodeling consistent with an activated M2 phenotype	(23)
Houser	2011	6–12	Flow cytometry and RNA microarray of CD14 $^{+}$ M ϕ subsets isolated from decidua basalis and peripheral blood	Identified 2 decidual M ϕ subsets: CD11c ^{HI} and CD11c ^{LO} CD11c ^{LO} decidual M ϕ express genes associated with extracellular matrix formation, muscle regulation, and tissue growth CD11c ^{HI} decidual M ϕ express genes associated with lipid metabolism and inflammation	(24)
Svensson	2011	7–12	Phenotypic characterization of decidual M ϕ by flow cytometry RNA microarray on <i>in vitro</i> differentiated M ϕ cultures	Identified 2 decidual M ϕ subsets: ICAM-3 ^{HI} and ICAM-3 ^{LO} ICAM-3 expression on decidual M ϕ positively correlated with CD11c expression as reported by Houser and colleagues M-CSF/IL-10 <i>in vitro</i> differentiated M ϕ and decidual M ϕ showed similar cytokine secretion patterns (\uparrow IL10, IL6, TNF, and CCL4)	(27)
Kim	2012	Term	Methylome analysis of decidual M ϕ , Hofbauer cells, neonatal, and maternal blood monocytes (Mo)	Both decidual M ϕ and Hofbauer cells exhibited hypermethylation of genes encoding classical M ϕ activation Both decidual M ϕ and Hofbauer cells exhibited hypomethylation of genes encoding alternative M ϕ macrophage activation	(25)

immunohistochemistry (29). When viewed in the context of M1 and M2, the results from these studies suggest that M2 macrophages or M2 subgroups are the predominant phenotype in the decidua with a smaller subset of macrophages bearing some characteristics of M1 or M2b (24). One drawback is that most of these studies analyzed macrophages out of their *in situ* context so the local factors responsible for triggering the differentiation of these macrophage subsets are still unknown.

The role of phosphatidylserine receptor Tim-3 in macrophages from the maternal–fetal interface has gained recent attention (30). Tim-3 is recognized as a regulator of pro- and anti-inflammatory innate immune responses (31), and Tim-3 expression is enhanced in M2 immunoregulatory macrophages (32, 33). Therefore, Tim-3 may play an important role in maintaining macrophage mediated immune tolerance at the maternal–fetal interface (31). Indeed, Tim-3 facilitates phagocytic activity of apoptotic bodies at the maternal–fetal interface, and blockade of Tim-3 on uterine macrophages led to increased production of IFN- γ and TNF- α by inflammatory granulocytes with subsequent rejection of the fetus (30). To date, it is unknown if loss of Tim-3 or dysregulation of Tim-3 occurs in adverse pregnancy outcome.

Hofbauer cells are macrophages that reside within the mesenchymal stroma of the chorionic villi (34), which are thought to originate from fetal hematopoietic cells (35). In human placenta, Hofbauer cells have been detected by 4 weeks gestation, and their numbers increase during the first trimester and then decline by term (36). Based on their hypermethylation patterns, Hofbauer cells display a commitment to M2 in healthy pregnancy (25). Other phenotypic features that suggest these cells are pro-M2 include constitutive expression of DC-sign (CD209), high levels of CD163, CD68, CD45, hyaluronan receptor LYVE-1, and HLA antigens A, B, and C, IL-10, and TGF- β (37–39). Hofbauer cells may have a role in placental angiogenesis, remodeling of the extracellular matrix, and modulation of inflammation, which are M2 characteristics (39–41). Although not a particular feature of macrophage function, it has been suggested that Hofbauer cells participate in the regulation of stromal fluid balance, ion exchange, and transfer of serum proteins to the vascular system (36).

ANATOMY OF THE MATERNAL–FETAL INTERFACE IN RELATION TO MACROPHAGES

The various compartments of the maternal–fetal interface (placenta, decidua, and myometrium) are anatomically distinguished by the composition of the connective tissue and the leukocyte populations present in each compartment. For example, macrophages residing in the myometrium may be receiving signals from T-cells, dendritic cells, and myocytes, whereas macrophages within the decidua receive signals from decidualized stromal cells, uterine NK cells, T-cells, and trophoblasts. In addition, decidual macrophages respond to immunoregulatory compounds such as HLA-G, TGF- β , and vasoactive intestinal peptide (VIP) that are produced by adjacent trophoblasts (40–42).

The mammalian uterus has two distinct tissue layers: the endometrium and myometrium. Macrophages are found interspersed in both layers of the uterus. The luminal surface of the endometrium consists of simple cuboidal epithelium that is supported by underlying connective tissue. The endometrium

contains a complex vascular supply and a diverse population of leukocytes that changes with stage of the menstrual cycle and stage of pregnancy (43). The myometrium is composed of interlacing bundles of smooth muscle. In human beings, the myometrium exhibits minimal change during pregnancy (44). In rodents, however, the myometrium below the site of placental attachment expands and develops into the metrial triangle (44). Prior to embryo implantation, the endometrium begins to undergo structural changes referred to as decidualization, which is initiated by increased progesterone levels (44, 45). The process of decidualization in human beings stabilizes by 12 weeks gestation (46). The physical changes that occur during decidualization include proliferation of luminal epithelium, development of secretory glands with large apical protrusions (pinopodes) and microvilli, and transformation of fibroblast-like endometrial stromal cells into larger rounded cells (47, 48). In addition, endometrial cells produce a variety of compounds that attract leukocytes to the decidualized tissue such as prolactin, colony stimulating factor 1 (CSF-1), macrophage inhibitory factor (MIF), IL-15, insulin growth factor binding protein-1 (IGFBP-1), and cyclooxygenase-2 (COX-2), and cell adhesion molecules (ICAM-1, VACM-1, LFA-3, H-CAM) (41, 45, 48, 49). Both CSF-1 and MIF influence the recruitment of macrophages into the decidua (11, 43, 50, 51).

Following embryo implantation, the decidua differentiates into two distinct regions: the decidua basalis, which refers to the portion of the uterus attached to the placenta, and decidua parietalis, which refers to the rest of the endometrium lining the main cavity of the pregnant uterus. In human beings, macrophages within the decidua basalis coexist with trophoblasts, CD56^{bright}/CD16^{low} NK cells, and T-cells, 25% of which are CD4⁺/CD25⁺/FOXP3⁺ regulatory T-cells (Tregs) (11, 44). Within the decidua basalis, CD14⁺ macrophages and NK cells are often observed in aggregates in association with spiral arteries (11, 52). Decidual macrophages are also observed in close association with trophoblasts (40). Factors released by neighboring trophoblasts may induce decidual trophoblasts to display cell surface expression of CD14 and CD16, exhibit increased phagocytic capacity, and secrete more IL-1 β , IL-10, and IP-10 (53).

The microenvironment within the myometrium slightly differs from the decidual basalis in that macrophages and T-cells are the predominant leukocyte populations, and there are few CD56^{bright}/CD16^{low} NK cells (54). In rats and mice, macrophages are more predominant in the myometrium than in the decidua, which has been attributed to higher CSF-1 concentrations within this compartment (55). Trophoblasts are also present within the inner third of the myometrium in both human beings and rats, but not in mice (14, 56). Macrophages recruited into the myometrium are important for the normal induction of labor and post-partum uterine remodeling (5, 57).

Placentation in human beings, non-human primates, and rodents is described as hemochorial because there are no barriers between trophoblast cells and maternal blood (58). Hemochorial placentation requires extensive tissue remodeling in which the endometrium that becomes the decidua basalis is obliterated and the uterine arteries are transformed from high resistance, low-flow arteries to low resistance, and high-flow vessels (14). During remodeling of spiral arteries, the endothelium and smooth

muscle cells are replaced by invading extravillous trophoblasts and fibrinoid deposits (59). This complex process depends on a coordinated crosstalk between decidual stromal cells, uterine NK cells, and CD14⁺/CD68⁺ macrophages (59–62). Under optimal circumstances, the transient invasion of uterine NK cells and CD14⁺/CD68⁺ macrophages into the spiral arteries pave the way for the second stage of remodeling that involves invasion of trophoblasts into the vessels (52, 59).

M1 OR M2 IN NORMAL PREGNANCY, IT IS IN THE TIMING

It has been recently proposed that pregnancy is actually an active and highly regulated immunologic process (63). Successful implantation requires a transient inflammatory phase that is initiated by cytokines and prostaglandins within seminal fluid (64). During the peri-implantation period, the polarization pattern of decidual macrophages is skewed toward M1 (65). However, as trophoblasts establish attachment into the endometrial lining and invade the uterine stroma, decidual macrophages begin to transition to a mixed M1/M2 profile (65). This mixed population may represent M1/M2 or possibly a blend of M2 subtypes (pro-inflammatory M2b mixed with anti-inflammatory M2a and M2c). This mixed polarization pattern continues through the first trimester and the early phase of the second trimester of pregnancy when the uterine vasculature is undergoing extensive remodeling in order to establish an adequate placental–fetal blood supply (63). After placental development is complete, the decidua shifts toward a pro-M2 environment, which prevents rejection of the fetus and allows fetal growth until parturition. Parturition, which is another inflammatory event, is preceded by an influx of macrophages into the myometrium and decidua (3–5). This inflammatory process promotes the contraction of the uterus, expulsion of the baby, ejection of the placenta, and uterine involution (57).

A2V, which is an isoform of the alpha subunit of vacuolar ATPase (V-ATPase), is a key immune regulator important for implantation (66). At least in normal murine pregnancy, A2V is expressed in sperm, embryo, and endometrium (65), and loss of a2v results in reproductive failure (67). In the reproductive tract, a2V promotes a transient pro-inflammatory effect followed by a balanced immune response that facilitates embryo implantation without rejection. During the pre-implantation period, cleavage of a2V in semen fluid releases a soluble N-terminus portion of the protein (a2NTD). Seminal a2NTD has pro-inflammatory effects such as upregulation of leukemia inhibitory factor (LIF), IL-1 β , TNF- α , and MCP-1 gene expression in the uterus, creating a transient pro-M1 effect. The developing embryo continues to produce a2V, which continues to stimulate uterine MCP-1. This in turn attracts new macrophages into the endometrium leading to a more balanced M1/M2 ratio and protection against rejection of the fetus. In the BALB/c mouse, these studies clearly demonstrate that a2V mediated induction of MCP-1 is important for proper regulation of M1/M2 during early pregnancy (65, 67). Additional factors present within the decidualized tissue that influence macrophage polarization of invading macrophages also contribute to tolerance.

As pregnancy progresses, the M1/M2 ratio decreases (23, 27) and the population of decidual macrophages become more heterogeneous. This, in part, is caused by the uneven spatial distribution of neighboring leukocytes and trophoblasts within the

decidua, which provide different cues to resident macrophages. Events that account for these changes include the contribution of immunoregulatory molecules from trophoblasts such as HLA-G (68, 69), TGF- β (40), and VIP (41). These compounds have direct effects on macrophages or on Tregs, which express pro-M2 factors such as IDO and IL-10 (1). In contrast, CD56^{bright}/CD16^{low} NK cells, which are intimately associated with decidual macrophages invading the spiral arteries, produce pro-M1 IFN- γ (70) and TNF- α (11). Houser et al. identified two distinct macrophage subsets in first trimester decidua based on the degree of CD11c expression (24). Regardless of the degree of Cd11c expression, these cells uniformly expressed CD68 and CD14. CD11c^{HI} macrophages expressed less phagocytic receptors such as CD209 (DC-SIGN) and CD206 (mannose receptor) compared to CD11c^{LO} macrophages. CD11c^{HI} and CD11c^{LO} macrophages also had different transcriptional profiles. For example, CD11c^{HI} macrophages express genes involved in invasion, mobility, inflammatory processes including lipid metabolism, and anti-apoptotic effects, whereas CD11c^{LO} macrophages express genes that regulate growth and development, as well extracellular communication including networking. CD11c^{LO} macrophages may be the subpopulation of cells that actively suppress CD56^{bright}/CD16^{low} NK cell mediated killing of invading trophoblasts (2). On the other hand, the transcriptional profile of CD11c^{HI} macrophages suggests that these cells participate in the remodeling of the uterine arteries that occurs during this stage of pregnancy.

During the growth phase of pregnancy, the predominant immunological feature is the induction of an anti-inflammatory state. It is presumed that the immunosuppressive qualities observed in decidual macrophages studied from first trimester pregnancies continue to be present as pregnancy advances (71). This notion is supported by several studies that evaluated macrophages isolated from term decidua. First, the methylation pattern of these cells show silencing of genes that encode M1 markers such as TLR9, IL1B, IL12RB2, CD48, and FGR, whereas genes associated with M2 polarity such as CCL13, CCL14, A2M, HNMT, and IL10 were hypomethylated (25). Second, decidual macrophages express M2 polarity characteristics such as reduced expression of co-stimulatory molecule CD86 with spontaneous production of high levels of IL-10 and IDO (72). Third, decidual macrophages express C-type lectin receptors such as CD206 throughout pregnancy (73), which is more in line with an M2 phenotype (71).

Normal parturition is an inflammatory process characterized by an enhanced expression of IL-1 β , IL-6, and IL-8 with an accumulation of leukocytes in the cervix, fetal membranes, decidua, and myometrium (74). Macrophages are one of the predominant cell types in the decidua and myometrium during normal parturition. CD68⁺ macrophages begin to invade the decidua a few days before parturition (3, 4), which coincides with a global increase in the production of chemokines such as CCL2 (MCP-1), CCL4 (MIP-1 β), CCL5 (Rantes), CXCL8 (IL-8), and CXCL10 (IP-10) within the decidua (75). Immediately before parturition, a wave of macrophages invades the myometrium (4), resulting in an increase in pro and anti-inflammatory cytokines such as IL-1 β , IL-6, IL-12, and IL-10. To date, the polarity profile of macrophages invading

the maternal–fetal interface before or during labor has not been determined.

Although the density of Hofbauer cells within the villous mesenchyme fluctuates with gestational age (36), the limited characterization of these cells suggests that they display a commitment to M2 related functions. DNA methylation profiling of Hofbauer cells obtained from term placentas show that pro-M1 genes such as TLR9, IL1B, IL12RB2, CD48, and FGR are silenced (25). In contrast, pro-M2 genes such as CCL2, CCL13, CCL14, CD209, and A2M are hypomethylated, and thus, available for expression (25). Hofbauer cells constitutively express anti-inflammatory cytokines such as IL-10 and TGF- β 1 (38), which may in part be related to the immune suppressive effects of neighboring mesenchymal stem cells that have the capacity to shift macrophages from an M1 phenotype to M2 (76). It is also likely that arginase I and II activity from surrounding villous trophoblasts (22) reduces extracellular arginine pools within the villous mesenchyme that would be required for translation of iNOS and differentiation into M1.

M1/M2 IMBALANCES DURING EARLY PREGNANCY

It is noteworthy that some obstetric complications associated with decidual M1/M2 imbalances occur during the early inflammatory phase of pregnancy. Under these circumstances, it is likely that the surrounding environment supports M1 polarization over M2. Some examples include spontaneous abortions and disorders involving inadequate remodeling of the uterine arteries.

Spontaneous abortions that occur within 12 weeks gestation are associated with an increased influx of macrophages in the decidual stroma (12, 77). In one study, decidual macrophages from spontaneous abortions had increased Fas-L expression, which coincided with an increased rate of trophoblast apoptosis (12). It was proposed that the increased FasL-expressing population of macrophages reflected M1 activation (12); however, this is yet to be proven. Jaiswal et al. recently demonstrated that a shift toward M1 polarization at the maternal–fetal interface enhances abortion in CBA × DBA/2 mouse matings treated with LPS (67). The underlying mechanism involved a significant decrease in placental a2V coupled with a decrease in uterine MCP-1 expression.

Inadequate remodeling of the uterine spiral arteries is associated with a spectrum of obstetric complications such as pre-eclampsia, intrauterine growth restriction, pre-term birth, pre-term premature rupture of membranes (PPROM), late sporadic miscarriage, and premature separation of the placenta from the uterus (78). During optimal spiral artery remodeling, there is a transient influx of NK cells and activated CD68 $^{+}$ macrophages into the vessel wall. This leukocytic influx, which is described as stage 1 remodeling, is thought to initiate the disruption of the organized smooth muscle layer and endothelium needed for subsequent trophoblast invasion into the vessel (59). Pro-M2 stromal macrophages in the outer periphery of the vessel assist in controlling inflammation by phagocytizing potentially pro-inflammatory cellular debris and secreting anti-inflammatory TGF- β 1 (40). Excessive M1 or M2b activity during the early phase of remodeling can prevent the resolution of this inflammatory phase, thus, disrupting trophoblast invasion into the arterial wall and preventing complete remodeling of the vessel (52, 59). Indeed,

reduced trophoblast invasion into utero-placental spiral arteries is associated with an excess of activated macrophages in and around these arteries (79, 80). Macrophages activated by exogenous TNF- α , tryptophan depletion (which reduces production of IDO), GM-CSF, or M-CSF promote apoptosis of extravillous trophoblasts *in vitro* (80, 81).

M1/M2 IMBALANCES DURING LATE PREGNANCY

Decidual inflammation is also presumed to produce M1 or M2b excess in the pathogenesis of uterine atherosclerosis. Uterine atherosclerosis is a late pregnancy lesion of the decidua that is characterized by the accumulation of lipid filled CD68 $^{+}$ foamy macrophages in the subendothelial layer of uterine spiral arteries (14, 82). This lesion is also associated with increased local TNF- α (83). In rare instances, acute atherosclerosis has also been observed in the myometrial segments of the spiral arteries (78, 82). During early pregnancy, acute atherosclerosis can also be seen in cases of defective spiral artery remodeling (78). In this instance, the lesions are present in the lower section of the arteries where they feed into the intervillous space (82).

Staff and Redman proposed that acute atherosclerosis is an end result of various inflammatory pathways triggered by immunologic, genetic, and/or hemodynamic influences (sequela to impaired spiral arterial remodeling and perturbed laminar blood flow) that may be working singly or in combination (82). This hypothesis is based on the observation that acute atherosclerosis is found in a wide range of pregnancy complications such as pre-eclampsia, fetal growth restriction, and certain autoimmune diseases (systemic lupus erythematosus and antiphospholipid syndrome). It has also been suggested that infectious organisms implicated in promoting cardiovascular disease such as *Chlamydia pneumoniae* may play a role in initiating or activating uterine atherosclerosis (84). Recent work in our laboratory suggests that the periodontal pathogen, *Porphyromonas gingivalis*, which is implicated in promoting cardiovascular disease (85), may indeed have such an effect.

M1/M2 IMBALANCES ASSOCIATED WITH INTRAUTERINE INFECTION

Porphyromonas gingivalis is a common periodontal pathogen of human beings that is also implicated in low-birth weight, fetal growth restriction, pre-eclampsia, and spontaneous pre-term birth (86–93). In prototypical M2 Balb/c mice, intrauterine infection with *P. gingivalis* induces pro-M1 or M2b inflammatory responses such as increased TNF- α and IFN- γ with suppression of IL-10, and fetal growth restriction (94, 95). Using a rat model of intrauterine infection, we observed that the local presence of *P. gingivalis* within the uterus produced lesions suggestive of an M1 > M2 or M2b > M2a, M2c imbalance. Namely, infected dams exhibited acute arteritis within the endometrium and metrial triangle characterized by perivascular necrosis, hyaline degeneration with varying degrees of thrombosis (96, 97). Moreover, the spiral arteries had increased densities of CD68 $^{+}$ macrophages, with increased stromal TNF- α and a concomitant decrease in extravillous trophoblast invasion into the placental bed. Since this was an acute study (4 days duration), we did not observe lipid filled macrophages surrounding affected spiral arteries. Nevertheless,

these results provide a compelling argument that certain bacterial infections may promote M1/M2 imbalances that in the placental bed have a negative impact on spiral artery remodeling.

To date, there is no evidence that pro-M1 activity is actually involved in the pathogenesis of chorioamnionitis and pre-term delivery. Rather, acute chorioamnionitis may actually promote M2 polarization. Decidual macrophages in gestation day 16 FVB/NJ mice sustain an M2 polarity phenotype even after LPS treatment (98). C57BL/6 mice, which are resistant to chorioamnionitis during intrauterine infection with *Ureaplasma parvum* (99), do not develop pro-M1 responses, instead C57BL/6 mice exhibit a pro-M2 profile within the placenta and decidua (100). This is particularly intriguing since C57BL/6 mice are known for their prototypical M1 immune responses (8). Human chorioamniotic and umbilical cord macrophages express more nuclear IL-33 (also known as full length IL-33) during acute chorioamnionitis (101). Nuclear IL-33 acts as a transcriptional regulator that has been shown to suppress production of LPS-stimulated pro-inflammatory cytokines *in vitro* (102). Moreover, extracellular or mature IL-33 released from activated or dying cells promotes M2 polarization of macrophages at the site of inflammation (103). At best, pro-M2 activation during chorioamnionitis may minimize detrimental inflammation associated with chorioamnionitis, or it may enhance infection by suppressing antimicrobial responses.

Manipulation of macrophage polarity at the maternal–fetal interface by infectious agents can impact pregnancy outcomes. The obligate intracellular parasite *Toxoplasma gondii* can invade the placenta and fetus, resulting in spontaneous abortion, stillbirth, fetal neurological, and ocular damage (104). A virulent strain of *T. gondii*, TgCtwh3, actively induces M2 polarization in infected macrophages (15). TgCtwh3 rapidly invades the placenta producing a heavy parasite burden with minimal inflammation but a high rate of trophoblast apoptosis (15). On the other hand, less virulent strain TgCtwh6 triggers pro-M1 responses in infected macrophages (15). TgCtwh6 can still invade the placenta, but infection is contained and associated with a profound lymphocytic inflammatory response with trophoblast apoptosis that is less extensive than TgCtwh3 (15).

HOFBAUER CELLS IN COMPLICATED PREGNANCY

Placental Hofbauer cells are responsive to paracrine signals from surrounding cells within the chorionic villus (105). Alterations in Hofbauer cell densities within the villi have been reported during chorioamnionitis (106, 107), but there is no evidence that these cells shift to M1 polarity phenotype during chorioamnionitis. Instead, HIV infected Hofbauer cells or cells from chorioamnionitis cases continue to display M2 characteristics such as production of IL-10, TGF- β , expression of DC-SIGN, CD163, and mannose receptor/CD206 (38, 39). In another study, Hofbauer cells from chorioamnionitis patients and healthy controls had similar gene expression profiles, which were not considered to be committed to either M1 or M2 (108).

Interestingly, it was reported that hyperglycemia can shift Hofbauer cells toward M1 activation (109). Hofbauer cells isolated from diabetic women displayed characteristics that could be attributed to either M1 or M2b phenotype (decreased CD163, CD209,

IL-10 with increased CD68, CCR7, and IL-1 β) (109). Further, when rat Hofbauer cells were cultured in high-glucose conditions *in vitro*, these cells expressed increased levels of NOS2 gene expression and NO, clear markers of M1 activation (109). But a caveat of that experiment was that Hofbauer cells were cultured in RPMI media, which contains supraphysiologic levels of arginine (7). High-arginine concentrations in RPMI are not representative of the tissue microenvironment, especially during inflammation (7). Further, high-arginine levels will promote iNOS activation in macrophages *in vitro* (7).

Folate receptor β (FR- β) is preferentially expressed on M2 polarized macrophages, and is considered a biomarker for immunoregulatory M2 macrophages (110). Decreased expression of FR- β and CD163, but not CD68, has been observed in Hofbauer cells isolated from women with severe pre-term pre-eclampsia (111) suggesting that in this syndrome, Hofbauer cells may have shifted toward M1 polarity. This would be consistent with Aziza et al.'s study (112) that reported increased iNOS with concurrent decreased eNOS in placentas from women with pre-eclampsia.

Villitis of unknown etiology (VUE) is an inflammatory lesion of the chorionic villi that is associated with intrauterine fetal growth restriction and perinatal morbidity and mortality (105). VUE is characterized by influxes of Hofbauer cells, maternal T-cells (more CD8 $^{+}$ than CD4 $^{+}$), and increased expression of chemokines (CXCL9, CXCL10, CXCL11, CXCL13, CCL4, CCL5, CXCR3, CCR5) within the placental villi (113, 114). It has been suggested that the increased expression of chemokines and their receptors within the villous mesenchyme of VUE patients provides the pro-migratory signals that promote increased migration of Hofbauer cells and maternal T-cells into the villous (115). Hofbauer cells do migrate in response to fibroblast secreted MCP-1 *in vitro* (106). Whether or not changes in villous mesenchyme promote M1 polarization of Hofbauer cells remains to be determined.

CONCLUSION

Macrophages serve important roles in the development of hemochorial placentation, maintenance of pregnancy, and initiation of parturition. As cells that are highly responsive to altering their polarization pattern *in vivo*, the mixed M polarity phenotypes present in the decidua reflect the heterogeneous nature of the maternal–fetal interface. Both spatial and temporal regulation of M1 and M2 polarization is required for successful pregnancy. Similarly, ill-timed or ill-placed macrophage polarization at the maternal–fetal interface is associated with pregnancy complications and poor outcomes. Despite recent insights into M1/M2 dynamics at the maternal–fetal interface, there are still critical knowledge gaps concerning the *in situ* context of macrophage polarity, the mechanisms that promote dysregulation, and its impact on healthy pregnancy and obstetric disease.

ACKNOWLEDGMENTS

This project was funded in part by R15 HD81439-01, National Institute of Child Health and Human Development. The funders had no role in the decision to publish or preparation of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 14 August 2014; accepted: 11 November 2014; published online: 24 November 2014.

Citation: Brown MB, von Chamier M, Allam AB and Reyes L (2014) M1/M2 macrophage polarity in normal and complicated pregnancy. *Front. Immunol.* **5**:606. doi: 10.3389/fimmu.2014.00606

This article was submitted to Molecular Innate Immunity, a section of the journal *Frontiers in Immunology*.

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Ontogeny and polarization of macrophages in inflammation: blood monocytes versus tissue macrophages

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The explosion of new information in recent years on the origin of macrophages in the steady-state and in the context of inflammation has opened up numerous new avenues of investigation and possibilities for therapeutic intervention. In contrast to the classical model of macrophage development, it is clear that tissue-resident macrophages can develop from yolk sac-derived erythro-myeloid progenitors, fetal liver progenitors, and bone marrow-derived monocytes. Under both homeostatic conditions and in response to pathophysiological insult, the contribution of these distinct sources of macrophages varies significantly between tissues. Furthermore, while all of these populations of macrophages appear to be capable of adopting the polarized M1/M2 phenotypes, their respective contribution to inflammation, resolution of inflammation, and tissue repair remains poorly understood and is likely to be tissue- and disease-dependent. A better understanding of the ontology and polarization capacity of macrophages in homeostasis and disease will be essential for the development of novel therapies that target the inherent plasticity of macrophages in the treatment of acute and chronic inflammatory disease.

Keywords: tissue-resident macrophages, M1M2, microglia, adipose tissue macrophages, Kupffer cells, obesity, neurodegenerative disease, hepatic steatosis

Macrophages are a heterogeneous population of immune cells that have a range of roles in both the induction and resolution of inflammation. Tissue-resident macrophages promote tissue homeostasis and exhibit unique transcriptional profiles and characteristics depending on the tissue in which they reside (1). For instance, alveolar macrophages regulate pulmonary surfactant turnover while osteoclasts promote bone resorption, and red-pulp macrophages (RPMs) in the spleen promote red blood cell clearance and regulate iron recycling. In addition, multiple macrophage subtypes occur within a given tissue, and they can perform distinct functions depending on their anatomical location (2). For example, in the bone there are at least two types of tissue macrophages. TRAP⁺F4/80⁻ osteoclasts promote bone resorption while F4/80⁺CD169⁺TRAP⁻ tissue-resident macrophages promote red blood cell development by providing a niche that promotes erythropoiesis and engulfing the extruded nuclei of red blood cell (RBC) progenitors at late stages of development (Figure 1A). Depletion of CD169⁺ macrophages results in impaired recovery of mice from hemolytic anemia (3). Recent studies suggest these two macrophage populations in the bone are replenished by distinct subsets of monocytes in response to stress. Mac3⁺F4/80⁻ monocytes regulate osteoclast activity but are not thought to be precursors of osteoclasts under homeostatic conditions (Figure 1A). However, these cells can differentiate into osteoclasts under inflammatory conditions. Alternatively, in the presence of increased levels of extracellular heme resulting

from conditions of stress such as hemolytic anemia, a subpopulation of monocytes in the bone marrow and spleen develop into F4/80⁺CD11b^{hi} progenitors termed pre-RPMs and subsequently into RPMs and F4/80⁺VCAM⁺ bone marrow macrophages that resemble RPMs to re-establish iron homeostasis (Figure 1A) (4).

In the developing embryo, hematopoiesis begins in the yolk sac where primitive erythrocytes and macrophages develop in the absence of hematopoietic stem cells (HSCs). Subsequently, HSCs arise in the aorto-gonado-mesonephric (AGM) region and eventually migrate to the fetal liver where development of all hematopoietic lineages from HSCs occurs. In the neonate, hematopoiesis moves from the fetal liver to the bone marrow where it persists throughout adulthood. The traditional view of macrophage origin contended that tissue-resident macrophages develop from bone marrow-derived monocytes. It was subsequently believed that tissue-resident macrophages derive from Ly6C⁻ "resident" monocytes that traffic to tissues under homeostatic conditions. However, studies utilizing Runx1^{CreER} and Csf1^{CreER} mice, which allow for tamoxifen-inducible activation of Cre during the early stages of yolk sac hematopoiesis crossed with Rosa²⁶ mice to label yolk sac-derived (YS) cells (5) have indicated that some tissue macrophages arise from the yolk sac and seed tissues in the embryo where they repopulate throughout adulthood (6, 7). Studies utilizing Flt3^{Cre} mice to mark HSC-derived macrophages confirm that YS tissue macrophages (Flt3^{Cre-}) are distinct from HSC-derived macrophages (Flt3^{Cre+})

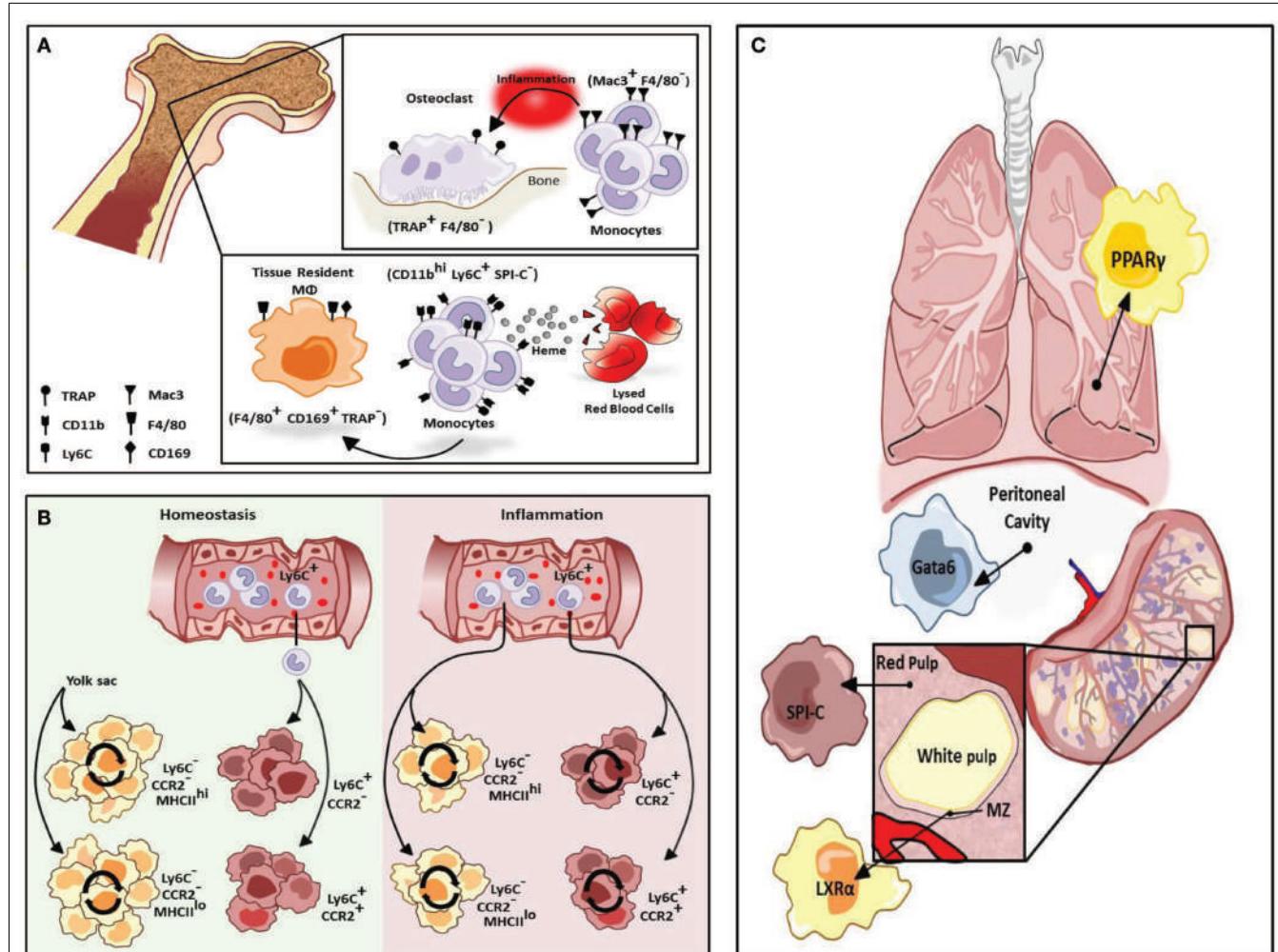


FIGURE 1 | Differentiation of resident macrophages in various physiological states. **(A)** Two tissue macrophage populations reside in bone, F4/80⁻ TRAP⁺ osteoclasts and F4/80⁺ CD169⁺ TRAP⁻ tissue-resident macrophages (MΦs). Osteoclasts are developmentally dependent on Mac3⁺F4/80⁻ monocytes but only during inflammation. Alternatively, tissue-resident macrophages in bone can be maintained by a heme-induced subset of monocytes (CD11b^{hi} Ly6C⁺ SPI-C⁻). **(B)** Cardiac tissue-resident macrophage populations are primarily yolk sac-derived (YS) however a minor subset of the population is derived from fetal liver and HSC-derived progenitors. In homeostasis, primary yolk sac-derived

cardiac resident macrophages are maintained through self-renewal but in response to cardiac insult, Ly6C^{hi} monocytes contribute to all four macrophage populations. **(C)** Selective transcriptional control plays an important role in the development of different types of macrophage populations. While red-pulp macrophages are dependent on SPI-C activity for development and maintenance, marginal zone (MZ) macrophage differentiation is mediated by LXR α . Alternatively, Gata6 is mandatory for the differentiation and proliferation of peritoneal macrophages while GM-CSF-dependent induction of PPAR γ regulate alveolar macrophage development.

thus challenging the traditional view of macrophage ontogeny. Kupffer cells, microglia, and cardiac tissue macrophages are primarily YS and are maintained throughout adulthood. These cells are independent of the transcription factor Myb, which is required for the development of HSCs, suggesting these populations of tissue-resident macrophages develop and persist independent of contribution from HSCs (8).

While Langerhans cells are first seeded by yolk sac macrophages, these cells are largely replaced by fetal liver-derived monocytes at later stages of embryonic development as demonstrated by adoptive transfer of fetal liver monocytes to host embryos (9). In addition, studies utilizing the CX₃C chemokine receptor 1 (CX₃CR1) promoter, which marks fetal-derived resident

macrophage populations to drive GFP, Cre or a tamoxifen-inducible Cre together with adoptive transfer studies have demonstrated that peritoneal macrophages and alveolar macrophages are also largely fetal liver-derived. While cardiac tissue macrophages are also largely YS, two minor populations also display contribution from fetal liver and bone marrow progenitors (10–12). Other tissue-resident macrophages including those from spleen, pancreas, and kidney also exhibit mixed contribution from fetal and adult HSC-derived precursors (13), while intestinal macrophages appear to be entirely derived from circulating monocytes (14, 15). While these studies make a distinction between YS and fetal liver-derived macrophages, a recent report describes an erythro-myeloid progenitor (EMP) population that develops in the yolk sac and

later migrates to the liver. These progenitors are distinct from HSC-derived hematopoiesis, suggesting that YS and fetal liver-derived tissue-resident macrophages have a common origin in the yolk sac (16).

Unlike previous assumptions that macrophages are terminally differentiated cells, YS macrophages appear to have stem cell-like properties in that they can proliferate and self-renew (9). In the lung, tissue-resident macrophages are maintained locally in the steady state. Moreover, following irradiation and bone marrow transplantation, residual lung-resident macrophages can re-establish normal homeostasis when the development of donor-derived macrophages is compromised (13). These cells expand in response to colony-stimulating factors, M-CSF and GM-CSF, in an interleukin 4 (IL-4)-independent manner. It is therefore likely that, following irradiation, the expansion of resident macrophages is delayed giving the monocyte-derived macrophages a developmental advantage. Alternatively, in response to helminth infection, Th2-dependent production of IL-4 promotes the proliferation of lung-resident macrophages (17) and this proliferation is independent of colony-stimulating factor-1 (CSF-1) (18). However, while Langerhans cells are embryonic-derived cells that exhibit hallmarks of self-maintenance, these cells are eventually replaced by circulating precursors following non-myeloablative transplantation (19). Similarly, YS cardiac resident macrophages are maintained through local proliferation, while monocyte-derived cells can replace all populations of resident cardiac macrophages following depletion or in response to damage (**Figure 1B**) (10, 20).

The development and maintenance of tissue-resident macrophages is also under tissue-selective transcriptional control. The transcription factor Gata6 is responsible for the transcriptome profile of resident peritoneal macrophages as well as for their proliferation under homeostatic conditions and in response to inflammation (**Figure 1C**) (21) and this programming is regulated reversibly by retinoic acid-induced Gata6 expression (22). Reflecting the fetal origin of peritoneal macrophages, retinoic acid induces expression of Gata6 in fetal-derived macrophages but not bone marrow-derived macrophages (BMDMs) due to epigenetic silencing of the Gata6 locus in BMDMs (22). Alternatively, the development of alveolar macrophages from fetal monocytes shortly after birth is regulated by the GM-CSF-dependent induction of peroxisome proliferator-activated receptor γ (PPAR γ), resulting in a transcriptosome profile unique to alveolar macrophages (**Figure 1C**) (23). The development of splenic RPMs in response to excess heme requires the transcription factor SPI-C (**Figure 1C**) (4), however, marginal zone (MZ) macrophages develop normally in SPI-C knockout animals. MZ macrophages play a critical role in the recognition and uptake of blood-borne antigens. While the differentiation of RPMs requires SPI-C, the differentiation of both populations of MZ macrophages is under the regulatory control of the nuclear receptor, liver x receptor α (LXR α) (**Figure 1C**) (24), and LXR α -deficient mice exhibit abnormal responses to blood-borne antigens.

Unlike previous assumptions that Ly6C $^{-}$ monocytes are precursors of tissue-resident macrophages, it has recently been shown that these monocytes do not infiltrate tissues but rather

have a “patrolling” function in promoting endothelial integrity. Alternatively, Ly6C $^{+}$ “inflammatory” monocytes are actively recruited to inflamed tissues by the chemokine, chemokine ligand 2 (CCL2) where they promote inflammation and pathogen clearance. Analogous populations of monocytes in humans have been described based on gene expression analysis and are classified as CD14 $^{\text{hi}}$ CD16 $^{-}$, which are the primary subset in healthy individuals and resemble Ly6C $^{+}$ murine monocytes while the minor CD14 $^{\text{lo}}$ CD16 $^{+}$ population of human monocytes shares gene expression profiles with murine Ly6C $^{-}$ monocytes (25). Furthermore, the CD14 $^{\text{lo}}$ CD16 $^{+}$ population of human monocytes, like Ly6C $^{-}$ monocytes, also play a role in patrolling the endothelium (26). Ly6C $^{-}$ monocytes derive in the circulation from the Ly6C $^{+}$ population and exhibit an extended half-life compared with Ly6C $^{+}$ monocytes (9). Intriguingly, recent studies indicate that a subset of Ly6C $^{+}$ monocytes can traffic to tissues under homeostatic conditions where they fail to differentiate into tissue-resident macrophages, rather they acquire antigen, which they carry to the draining lymph nodes (27).

Under non-inflammatory conditions, tissue-resident macrophages largely exhibit an M2 phenotype that promotes tissue homeostasis and repair. However, upon infection, M1 macrophage activation is induced by the engagement of pattern recognition receptors (PPRs) by pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), in cooperation with interferon γ (IFN γ) (28). These cells produce cytotoxic oxygen and nitrogen radicals and pro-inflammatory cytokines resulting in increased microbicidal activity. M2 macrophage activation is induced by the Th2 chemokines IL-4 and interleukin-13 (IL-13) (29, 30). Alternatively, during the resolution of inflammation, the balance of macrophage activation toward an M2 phenotype occurs in order to promote clearance of debris, inhibit the production of inflammatory mediators, and restore tissue homeostasis. M2 macrophages produce anti-inflammatory cytokines and express endocytic receptors, and these cells promote the clearance of apoptotic cells, proliferation, and wound healing (28). However, the relative contribution and level of plasticity of tissue-resident versus monocyte-derived macrophage populations is only recently beginning to be elucidated.

The M1/M2 macrophage subsets are most commonly distinguished based on the catabolism of L-arginine. While classically activated macrophages express increased levels of inducible nitric oxide synthase (iNOS), which converts L-arginine to L-citrulline and nitric oxide, alternatively activated macrophages express arginase I (ArgI), which catabolizes L-arginine to L-ornithine, a precursor of polyamines and proline. While Th1 and Th2 cytokines promote M1 and M2 macrophage activation, respectively, these phenotypes are observed in T cell deficient mice suggesting that T helper cells are not required to drive macrophage polarization *in vivo* (29, 30). Conversely, M1 and M2 macrophages are capable of promoting Th1 and Th2 differentiation, respectively, suggesting these polarized macrophage phenotypes play an important role in driving the immune response to environmental insult (29). Recent attempts have been made to re-classify macrophage subpopulations in response to a range of stimuli and increasingly complex combination of markers (31). However, *in vivo*, macrophages are exposed simultaneously to a diverse array of

signals, so it remains to be determined to what extent distinct subpopulations exist *in vivo*.

The origin and plasticity of macrophages in chronic inflammation remains poorly understood and is likely variable depending on the tissue and the underlying cause of inflammation. Tissue-resident macrophages as well as monocyte-derived macrophages appear to have the capability of adopting both M1 and M2 phenotypes, however, the relative contribution of these subsets of macrophages to the progression and resolution of chronic inflammation remains enigmatic. Here, we will review current progress in understanding the complex relationships between tissue-resident macrophages and infiltrating monocyte-derived macrophages in homeostasis and chronic inflammation and their contributions to M1/M2 polarized phenotypes in homeostasis and disease with a focus on chronic inflammation in the brain, adipose tissue, and liver.

MICROGLIA: RESIDENT CNS MACROPHAGES

Microglia, parenchymal tissue-resident macrophages in the central nervous system, plays a central role in mediating tissue homeostasis in health and disease (32). Fate mapping studies conducted independently by Ginhoux et al. and Prinz et al. demonstrated the embryonic origin of microglia from c-kit⁺ EMP prior to the formation of blood–brain barrier and vascularization of the embryo (7, 33, 34). The colony-stimulating-factor 1 receptor (CSF1R) and its ligand, CSF-1, play a central role in macrophage development and maintenance. However, while CSF1R is essential for the development and maintenance of microglia, mice deficient for CSF-1 exhibit only a partial reduction in microglial cells. Recent studies have identified an additional ligand for CSF1R, interleukin-34 (IL-34), which plays an essential role in the development and maintenance of microglia. While mice deficient for IL-34 exhibit significantly decreased numbers of microglia and Langerhans cells, the development of other tissue macrophage populations remain largely intact (35, 36). After birth, the microglia undergo massive expansion in response to CSF-1 and IL-34 in order to populate the developing nervous system. Cortical, optical, and spinal cord microgliogenesis is sustained by the transcription factors Pu.1 and interferon regulatory factor-8 (IRF-8), and is independent of Myb, which is required for the development of hematopoietic stem cells (34, 37). Microglia are sustained and self-renew within host tissues throughout adulthood independent of progenitors from the bone marrow (38).

In a healthy brain, microglia are dispersed relatively uniformly throughout the parenchymal tissue. Microglia are characterized by morphological features that reflect their functional capacity. In a healthy brain, microglia are in a quiescent state or have a “down-regulated” phenotype exhibited by a ramified shape, similar to Langerhans cells that have short fine processes and thus increased surface area for tissue surveillance (39). This down-regulated phenotype is characterized by an attenuated innate immune function correlated with decreased expression of CD45, MHCII, and Fc receptors (39–41). Initially, it was believed that steady-state microglia are static, however, recent studies have characterized the quiescent phenotype as a more dynamic state in which microglia are performing housekeeping functions by constitutively surveying the parenchymal tissue (42). This dynamic

state is characterized by increased expression of oxidative genes and favors homeostatic tissue remodeling and steady-state wound healing (43).

Recent studies by Butofsky et al. (44) demonstrate that resident microglia exhibit a distinct expression profile that is not observed in microglial cell lines and is distinct from M1 or M2 polarized microglia, but rather includes genes associated with nervous system development. The induction of this unique profile is highly dependent on transforming growth factor β (TGF- β) – *in vitro* in human microglial cultures, and these cells are absent in TGF- β knockout mice. The down-regulated phenotype of microglia is critical for normal neuronal growth, and intimate interactions between neurons and microglia are important for optimal synaptic growth and maintenance (Figure 2) (45). The interaction between neurons and microglia is fostered by the chemokine CX₃C chemokine ligand 1 (CX₃CL1; also known as fractalkine) and CD200 membrane proteins expressed on healthy neurons that interact with their respective transmembrane protein receptors on microglia, CX₃CR1, and CD200R, respectively (32, 46–48). These microglial receptors carry immunoreceptor tyrosine-based inhibitory motifs (ITIMs) such that, upon ligand–receptor interaction, the ITIM based receptors suppress downstream immune signaling through the recruitment of Src homology 2 domain-containing phosphatase 1 (SHP-1) (32, 48–50). These cell–cell mediated interactions act synergistically with constitutively released neurotropic elements in order to create an environment fostering the down-regulated phenotype of microglia.

Whether or not bone marrow-derived cells can contribute to the microglial population remains under debate (reviewed in Prinz et al.). Bone marrow transplantation of irradiated recipient mice demonstrated that macrophages in the perivascular space and choroid plexus can be repopulated by donor cells (51, 52). While microglia are largely radio-resistant cells, upon irradiation and bone marrow transplantation, bone marrow-derived progenitor cells that are distinct from monocytes have been shown to seed the brain and differentiate into microglial-like macrophage cells (45). However, it is not clear whether BMDMs maintain the range of functions exhibited by YS microglia. In addition, irradiation results in the breakdown of the blood–brain barrier, thus whether the seeding of the brain with peripheral bone marrow-derived cells can occur under steady-state conditions in the presence of an intact blood–brain barrier is not known.

Like macrophages in the periphery, *in vitro* studies demonstrate that M1 and M2 activation can be induced in microglia by LPS and IL-4, respectively (40, 53). However, whether this phenotypic switching of resident microglia occurs *in vivo* remains unclear. Experimental models of Alzheimer’s disease (AD), aging, and multiple sclerosis (MS) (54, 55) have demonstrated microglial heterogeneity analogous to systemic tissue-resident populations. These studies suggest that early in disease progression, microglial cells develop an altered inducible “activated” state that is functionally different from steady-state microglia. This activated state is then further subdivided into a classical M1 and alternative M2 state (32, 40). Morphologically, activated microglia exhibit an amoeboid shape in contrast to the quiescent ramified shape of steady-state microglia (42). While the quiescent state of microglia

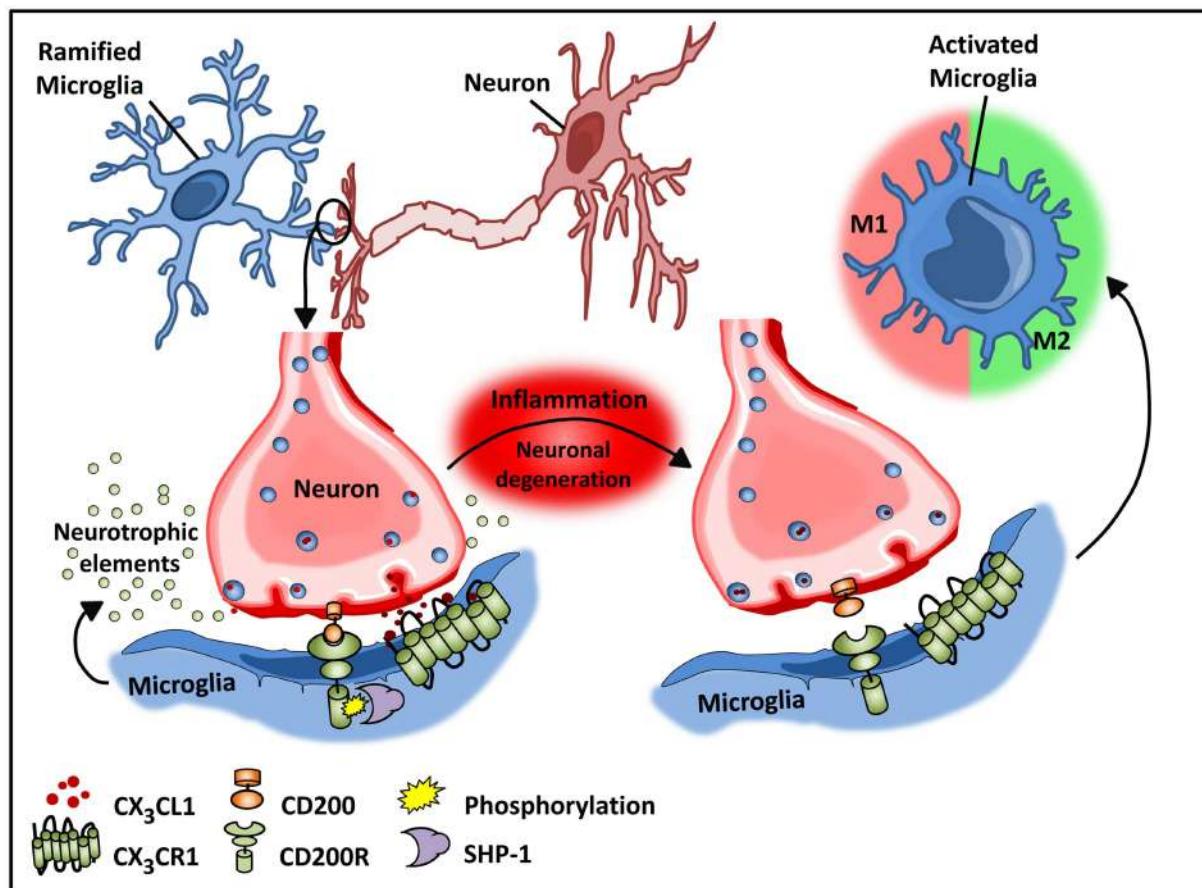


FIGURE 2 | Microglial–neuronal interactions in health and disease.

Healthy neurons expressing chemokine fractalkine ($\text{CX}_3\text{CL1}$) and CD200 membrane proteins intimately interact with their respective transmembrane protein receptors on microglia, $\text{CX}_3\text{CR1}$, and CD200R to sustain a down-regulated microglial phenotype. Microglial receptors have

immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which upon ligand–receptor activation suppresses downstream immune signaling through the recruitment of phosphatases including SHP-1. Chronic inflammation disrupts this intimate neuronal–glial interaction, thus releasing the microglial cells from a down-regulated inhibited state to an activated phenotype.

is maintained by neuronal–glial interactions, in the course of inflammation, neuronal, and glial interactions are disrupted due to degeneration of neurons (43, 56). Disruption of $\text{CX}_3\text{CL1}$ – $\text{CX}_3\text{CR1}$ interactions releases microglia from tonic inhibition (48), resulting in microglial activation as a consequence (Figure 2). Additionally, a unique “primed” microglial state, which exhibits a functional state somewhat distinct from the activated states, has been described (57). These microglia are thought to function as more of an adaptive response (57). This primed state refers to a mode of preconditioning underlying chronic inflammation whereby microglial cells are activated continuously by being persistently exposed to a pro-inflammatory milieu (32, 57). Importantly this priming state enables the conditioned microglia to mount a heightened response to secondary or future immunological insult in the course of disease progression. Primed microglia exhibit a more robust inflammatory response as compared to those that were not subjected to prior stimulatory challenges (32, 57). Additionally, the heightened inflammatory response to a physiological challenge is believed to promote a switch from a more

anti-inflammatory to a pro-inflammatory reactive phenotype resulting in cellular stress and exacerbated inflammation (57).

Investigations in spinal cord injury (SCI), AD, and the animal model of MS, also known as experimental autoimmune encephalitis (EAE) demonstrate an increase in both M1 and M2 phenotypes, possibly acting synergistically in an attempt to mitigate inflammation (40). While both M1 and M2 transcripts increase in a majority of chronic CNS disease models, the M1 phenotype offsets the M2 phenotype in the early stages of chronic inflammation, disrupting normal neuronal/glia cross-talk, and promoting a pro-inflammatory milieu implicated in the progression of disease (39, 40, 53). With progressive degeneration, the M1 phenotype predominates, as M2 microglia exhibit decreased responsiveness to anti-inflammatory cues (53). Alternatively, the classically activated M1 phenotype is predominately induced by acute pathological conditions such as stroke, traumatic brain injury, or experimentally induced systemic inflammation by LPS (42). The M1 state in chronic and acute manifestations is characterized by the up-regulation of MHCII, CD86, and Fc receptors and increased

production of pro-inflammatory cytokines such as interleukin-6 (IL-6), interleukin-1 (IL-1), and tumor necrosis factor alpha (TNF α) (58). Furthermore aging models hint at a possibility of phenotypic switch in microglial activation from an M2 to M1 state over time, coincident with decreasing neurogenesis and a growing inflammatory niche (32). However, it still remains unclear whether these populations of macrophages are due to phenotypic switching of resident microglia, or the activation states of infiltrating monocyte-derived macrophages. Interestingly, research in microglial senescence sheds light into the notion that microglial degradation precedes neurodegeneration and perhaps neurodegeneration is a secondary factor to aging induced dysregulation of microglial populations (59, 60).

Several studies of chronic inflammation in the CNS have demonstrated a degree of immune infiltration into the CNS, which is presumed to promote tissue remodeling as an attempt to maintain homeostasis (61, 62). The observed monocytic infiltration is attributed to the breakdown and weakening of the blood–brain barrier, which then facilitates the entrance of immune cells (63, 64). However, while monocyte infiltration has traditionally been considered a late event in disease development recent studies by Yamasaki et al. using an EAE model, demonstrated that infiltration of Ly6C $^+$ monocytes occurs at the onset of disease and that these infiltrating cells are responsible for initiating nodal demyelination (65). It was further demonstrated that, while infiltrating monocytes are responsible for initiating nodal demyelination, resident microglia are relatively quiescent upon disease onset (65–67). The infiltrating monocytes promote disease progression though they do not appear to contribute to the resident microglial population (68). Similarly in a model of AD immune cell infiltration and localization of microglial populations in areas of amyloid β (A β) plaque deposits has been observed (69, 70).

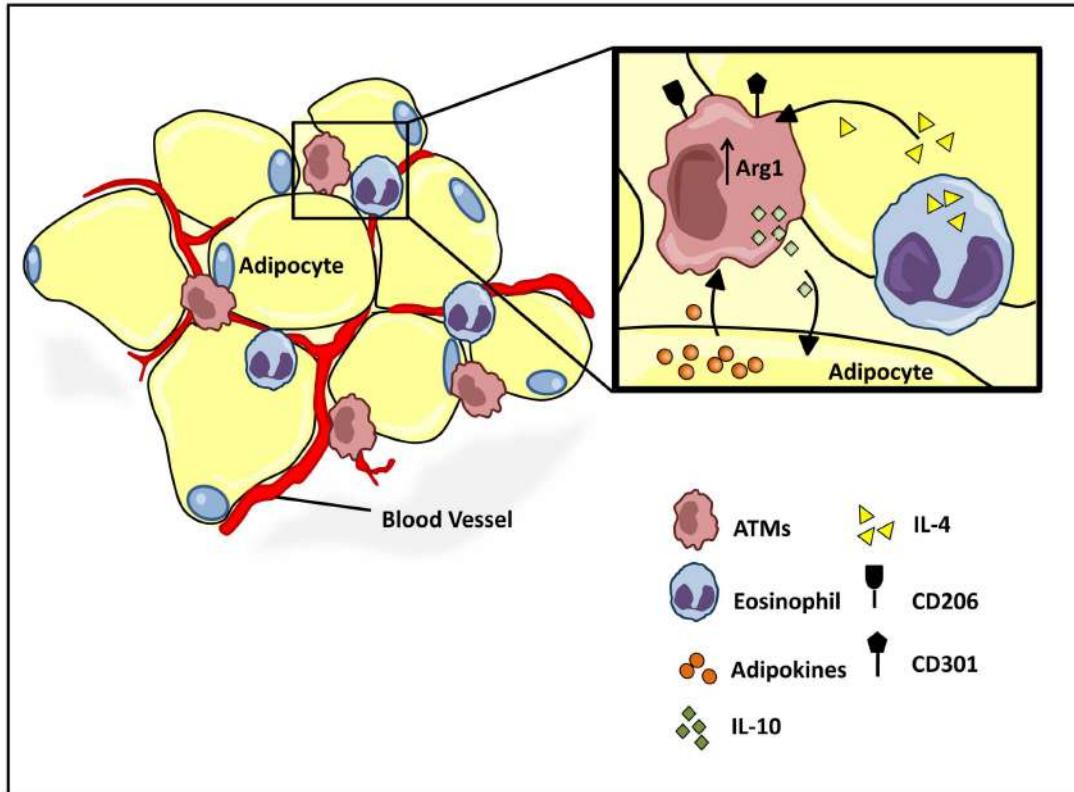
Recently, researchers have been targeting microglial genes, such as TGF- β activated kinase (TAK1) to elucidate how tissue-resident macrophages are behaving during inflammation. While infiltrating monocytes are highly phagocytic and inflammatory, resident microglia are relatively quiescent upon disease onset. However, a recent study by Goldmann et al. demonstrated an essential role of microglia in the onset and progression of disease in an EAE model (71). By targeting TAK1 in microglia, but not peripheral monocytes/macrophages, they found that the lack of TAK1 in microglia inhibited the progression of inflammation in the CNS, highlighting the contribution of both resident microglia and bone marrow-derived monocytes in the onset and progression of EAE. However, in a model of EAE, infiltrating monocytes are not maintained and do not appear to contribute to the resident microglial population (68). Furthermore, recent studies using intravital correlated microscopy in zebrafish show that infiltrating phagocytes undergo cell death and are engulfed by resident microglia during resolution of inflammation (72). Popovich et al. investigated the potential for targeting CX₃CR1 in a model of SCI as the ligand–receptor interactions between CX₃CR1 and CX₃CL1 are fundamental for optimal microglial function (47). Furthermore, Donnelly et al. presented two important findings, targeted CX₃CR1 deficiency/blocking in SCI results in enhanced recovery and decreased monocytic infiltration to injury site (48).

ADIPOSE TISSUE MACROPHAGES

While it has long been clear that undernutrition can impair immunocompetence, more recently, the progression of obesity has also been attributed to a shift in immune function from an M2 to M1 type responses (73). Work by both Spiegelman (74, 75) and Lumeng et al. demonstrate critical interactions between adipose tissue metabolism and the immune system, and the active role of adipose tissue macrophage (ATM) polarization in the progression of obesity. Lean individuals in a non-inflammatory state maintain a relatively low percentage (~10–15%) of resident ATMs (76). Healthy adipose tissue consists of uniformly distributed alternatively activated M2 macrophages, expressing Arg1 and the cell surface antigens CD206 and CD301 (Figure 3) (77–79). The M2 polarized state of the ATM population in healthy adipose tissue is maintained by eosinophils (80), which secrete IL-4 (Figure 3) (81, 82). Polarized M2 ATMs secrete interleukin-10 (IL-10), which regulates glucose homeostasis within adipose tissue and systemic tissues including muscle (Figure 3) (83). In turn adipose resident invariant natural killer T cells (iNKT), expressing nuclear factor interleukin 3 regulated (E4BP4), induces the M2 phenotype through secretion of IL-10 (84). Healthy adipocytes, in turn, secrete anti-inflammatory adipokines such as adiponectin, a hormone that acts synergistically with IL-4 to exert anti-inflammatory effects through the activation of AMP kinase (AMPK) and signal transducer and activator of transcription 6 (STAT6), respectively (78, 85, 86). The alternative M2 state is further sustained by the transcriptional regulators peroxisome proliferator-activated receptor- δ (PPAR δ), PPAR γ , and Krüppel-like factor 4 (KLF4) (82, 87–89).

The intimate interaction between adipocytes and ATMs may reflect a common origin of these two cell types. Studies have shown not only cross-talk between ATMs and adipocytes, but a great deal of plasticity between these two lineages compartments (90, 91). Furthermore, while still controversial (92) several studies have suggested that some adipocytes and adipose progenitors may be hematopoietic stem cell-derived (93, 94). In addition, Coussin et al. have shown that hematopoietic stem cells that can reconstitute lethally irradiated recipient mice exist within adipose tissue (95), and more recent studies have supported the concept that adipose tissue is an extramedullary source of hematopoietic stem/progenitor cells (96, 97). Yet other studies have demonstrated the phagocytic capacity of pre-adipocytes and adipocytes suggesting they can adopt macrophage-like functions (85, 98). Interestingly, a recent study by Eto et al. has identified a novel subpopulation of ATMs present in perivascular regions of adipose tissue (99). This population of ATMs is characterized by the expression of CD206 and the stem cell marker CD34. The investigators further demonstrated that these cells can differentiate into the adipogenic lineage *in vitro*, suggesting the possibility that this subpopulation of ATMs might be a source of adipocytes *in vivo*. Whether these cells are hematopoietic-derived or whether they are precursors of the more abundant CD206 $^+$ CD34 $^-$ ATMs is unclear.

In the late 2000s, Lumeng et al. identified adipose tissue inflammation as an important early event in the development of obesity related complications (82). At the onset of environmental and

**FIGURE 3 | Homeostatic regulation of ATM microenvironment.**

Healthy adipose tissue contains a relatively low and uniformly dispersed population of alternatively activated M2 macrophages, expressing cell surface antigens CD206 and CD301. The M2 polarized state is maintained

by eosinophil and adipocyte derived adipokine secretions, IL-4, and adiponectin, respectively. M2 ATMs maintain a homeostatic adipose milieu with IL-10 secretions, which in turn regulate glucose homeostasis within systemic tissues.

metabolic perturbation, such as chronic exposure to a high-fat diet (HFD), adipose tissue undergoes vast biochemical and morphological remodeling in part facilitated by infiltrating monocytes that then differentiate into ATMs. An excess of nutrients stimulates adipose tissue hyperplasia and hypertrophy as individual adipocytes enlarge with excess triglycerides (TAGs) and simultaneously undergo heightened levels of cellular stress imparted by endoplasmic reticular (ER) stress, hypoxia, release of excess free fatty acids (FFAs), increased reactive oxygen species (ROS) production, and adipocyte necrosis (77, 82, 100). The “stressed adipocytes” create an environmental milieu that promotes a more pro-inflammatory state exemplified with an increase in local IL-6, TNF α , and IL-1 production (101). Early adipocyte death is facilitated by ATMs in an effort to maintain homeostasis, followed by the development of a new range of pre-adipocytes (77, 102). Further studies demonstrated that adipocyte necrosis is not only an early event in diet-induced obesity (DIO), but it is a pivotal event, which results in the release of chemokines that attract circulating monocytes to the site of inflammation (101, 103). It is well established that throughout the course of adipose tissue inflammation, the resident macrophage population increases from ~10 to 50–60% associated with a parallel increase in F4/80 $^{+}$ CD11c $^{+}$ inflammatory macrophages (78, 82). Interestingly, Kosteli et al.

demonstrated that weight loss also leads to a rapid recruitment and accumulation of ATMs in white adipose tissue (WAT) (104). ATM accumulation occurs with lipolysis-induced release of excessive FFAs and subsequent clearance of lipolysis byproducts by WAT macrophages. Contrary to DIO ATM accumulation, weight loss induced ATM recruitment does not exacerbate the existing inflammatory state and ATM populations diminish with the declining rate of lipolysis (105).

At the onset of a physiological imbalance in the adipose niche, such as weight gain, the uniformly dispersed small population of resident macrophages exhibits an alternatively activated CD206 $^{+}$ CD301 $^{+}$ Arg1 $^{+}$ M2 phenotype (79, 82, 87). The ensuing pro-inflammatory milieu created by stressed adipocytes and necrosis attracts circulating monocytes to the site of inflammation as early as 10 weeks from the onset of HFD introduction. Circulating Ly6C $^{+}$ /chemokine receptor-2 (CCR2) $^{+}$ monocytes are recruited and differentiate into the classically activated CD11c $^{+}$ CD11b $^{+}$ F4/80 $^{+}$ M1 ATMs expressing high levels of iNOS and TNF α (78, 106). Cinti et al. demonstrated that the differentiated M1 ATMs are exclusively localized in epididymal white adipose tissue (eWAT) and not inguinal white adipose tissue (iWAT), more specifically within “crown-like structures (CLS)” composed of necrotic adipocytes (107). The authors incorporated a HSL $^{-/-}$

(hormone sensitive lipase KO) model to show that adipocyte hypertrophy itself triggers a pro-inflammatory microenvironment that facilitates infiltration of monocytes (107). The M1 monocyte-derived macrophages are recruited and stabilized by CCR2 and its ligand CCL2 in addition to other factors such as osteopontin and fatty acids (FAs) (78, 106). CCL2 loss of function as well as CCR2 gain of function or overexpression studies have collectively illustrated the fundamental role of CCR2 and CCL2 in recruitment and differentiation of macrophages with an M1 phenotype (78, 85). However, Amano et al. recently demonstrated that local proliferation of ATMs contributes significantly to tissue ATM accumulation. They further demonstrated that this *in situ* proliferation is driven by monocyte chemotactic protein (MCP-1) (108). These studies highlight the contribution of both local proliferation and infiltrating monocytes to the accumulation of ATMs in an obesity induced inflammatory state (108, 109).

While the observed ATM increase associated with obesity is predominantly attributed to macrophages exhibiting an M1 phenotype, Shaul et al. recently suggested that there exists an M1/M2 hybrid ATM population and that the increase in ATMs with obesity exhibit more of this mixed phenotype. The study noted an increase in both M1 and M2 gene transcripts in a mouse DIO model of chronic inflammation suggesting that perhaps both populations may influence the progression of obesity-associated chronic inflammation (103). However, despite transcriptional increases in both M1 and M2 genes, further studies by the authors demonstrated an overall decrease in the M2 populations in the DIO model as exhibited by flow cytometry analysis of eWAT cells expressing macrophage galactose type-C lectin-1 (MGL1, also known as CD301). It is not clear whether this intermediate population of ATMs represents a truly separate population of cells or whether these ATMs represent a transitional population, resulting from the inherent plasticity of ATM phenotypes *in vivo*.

In healthy adipose tissue, tissue remodeling is accompanied by angiogenesis to maintain oxygen supply and critical nutrients to promote adipose tissue homeostasis. However, under pathological conditions, the existing adipocytes enlarge, angiogenesis is limited, and tissue hypoxia ensues, resulting in the recruitment of inflammatory cells. Angiogenesis is induced by vascular endothelial factor-A (VEGF-A) stimulation of vascular endothelial growth factor receptor-2 (VEGFR-2) (110, 111). When VEGFR-2 is blocked early in the onset of obesity, the development of metabolic disorders is elevated suggesting that expanding vascularization can promote diet-induced disease (112). However, Elias et al. demonstrated that overexpression of VEGF-A in adipose tissue in a DIO model promotes the maintenance of a larger M2 population and attenuates adipose tissue inflammation (113), coinciding with an increase in local blood flow and decreased presence of necrotic CLS. The authors further conclude that the correlative increase in VEGF and M2 macrophage populations is likely due to recruitment of M2 macrophages to sites of inflammation in adipose tissue rather than promoting a phenotypic switch between M1 and M2 macrophage populations. Recent studies also demonstrate an increase in brown adipose tissue thermogenesis as a response to cold, particularly in the presence of VEGF overexpression (113, 114). Interestingly, pivotal studies in by the Chawla lab demonstrated that the absence of alternatively activated M2 macrophages

results in impaired metabolic adaptation to cold, while administration of IL-4 promotes adaptation in a macrophage-dependent manner (115), suggesting that the increased thermogenesis in response to cold in the presence of VEGF could also be mediated, at least in part, by M2 macrophages.

Therapeutic strategies in obesity and obesity-associated disease may lie in understanding the molecular underpinnings of tissue-resident macrophage heterogeneity. For instance, loss of KLF4 function has been associated with a loss of wound healing capacities under inflammatory conditions suggesting an important role for KLF4 in maintaining an M2 phenotype *in vivo* (87). In addition to KLF4, differential expression of PPAR δ and PPAR γ in current experimental models, including HFD-induced obesity, illustrate a critical role for activated PPARs in directing monocytic infiltration and polarization of macrophages toward the alternative state in adipose tissue and liver (116, 117). Genetic deletion of PPAR δ and/or PPAR γ in macrophages directs the differentiation of monocytes toward a pro-inflammatory M1 phenotype (88, 118). Conversely, agonist mediated PPAR-activation stimulates monocytic differentiation into an alternately activated M2 phenotype (119). These agonists include synthetic anti-diabetic agents such as Thiazolidinediones (TZD) or natural agents such as abscisic acid (ABA) (116, 119, 120). Recent studies by Satoh et al. have demonstrated an essential role for the pseudokinase, Trib1, in the development of M2-like macrophage populations. Mice lacking Trib1 in the hematopoietic cell compartment exhibit a severe reduction in M2-like macrophages in a number of tissues under homeostatic conditions (121). In addition, these mice also exhibit a significant reduction in eosinophils, suggesting that the defect in M2 polarization could be, in part, secondary to diminished levels of eosinophil-derived IL-4 under homeostatic conditions. Importantly, these mice develop hypertriglyceridemia and insulin resistance when maintained on a HFD, associated with increased expression of pro-inflammatory cytokines (121, 122).

KUPFFER CELLS (LIVER-RESIDENT MACROPHAGES)

One of the largest resident populations of macrophages exists in the liver. These YS macrophages, termed Kupffer cells, are positioned in the liver sinusoids where they efficiently clear microbes and apoptotic cells from the portal circulation (Figure 4) (123). While the proliferative capacity of hepatic macrophages remains controversial, recent studies have suggested that Kupffer cells are established prenatally and are maintained into adulthood independent of replenishment from blood monocytes in a manner dependent on M-CSF and GM-CSF (9). Kupffer cells also play essential roles in tissue homeostasis, tissue remodeling, and regulation of metabolic function in the liver (Figure 4). Due to their constant exposure to blood-borne food antigens and bacterial products of commensal intestinal flora, these cells are also required to prevent the onset of inflammation in response to these non-pathogenic stimuli (124). In contrast, monocyte-derived macrophages in the liver play dual roles in promoting both tissue inflammation and repair. These infiltrating cells are initially CD11b $^+$ F4/80 $^+$ and eventually differentiate into more mature CD11b lo F4/80 hi macrophages.

Following acute hepatic injury induced by acetaminophen, there is a large influx of monocyte-derived macrophages induced

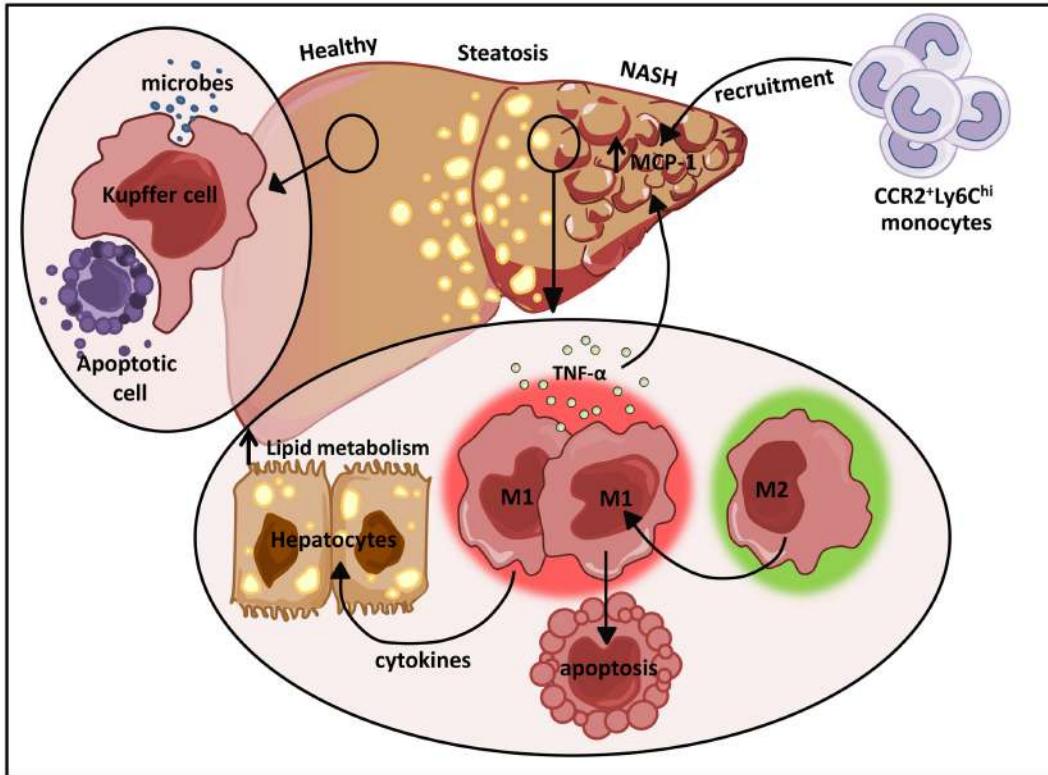


FIGURE 4 |The dual role of M1/M2 Kupffer cells in NAFLD. Kupffer cells play a pivotal role in host defense where they routinely clear microbes and apoptotic bodies from portal circulation. During diet-induced liver injury, tissue-resident macrophages exhibiting a classically activated M1 phenotype predominate and secrete cytokines

that can alter hepatocyte lipid metabolism and induce MCP-1 dependent recruitment of monocytes into the liver. In turn, these infiltrating cells facilitate the development and progression of NAFLD. Restrained induction of M1 Kupffer cell apoptosis further perpetuates liver inflammation.

by CCL2, accompanied by a significant decrease in the resident population of Kupffer cells (125–127). This influx is blocked in CCR2-deficient mice and these mice exhibit a delay in tissue recovery indicating that the infiltrating population of macrophages plays an important role in promoting liver repair following an acute insult (126, 127). However, when both infiltrating hepatic macrophages and resident Kupffer cells are depleted, tissue recovery is dramatically impaired (126, 128). These mice exhibited impaired clearance of necrotic cells and a defect in the restoration of the liver microvasculature (126, 128). During the resolution phase of acute inflammation, the infiltrating monocyte-derived cells differentiate into the more mature reparative macrophages and the resident Kupffer cell population is restored. Genomic analysis of these co-existing populations of cells demonstrated that, while the recovered Kupffer cell population was similar to resident Kupffer cells before injury, these cells were distinct from the resolution phase monocyte-derived liver macrophage population, suggesting that these populations have distinct functions in liver recovery from acute inflammation and that the infiltrating monocyte-derived macrophages do not replace the resident Kupffer cells (126).

Non-alcoholic fatty liver disease is the most common liver disease in the western world, the prevalence of which has increased in

parallel to the increased incidence of obesity (129, 130). This clinicopathological condition is characterized by lipid accumulation in hepatocytes and ranges from the non-progressive form termed steatosis to non-alcoholic steatohepatitis (NASH), the progressive form that is prone to the development of cirrhosis, liver cancer, and liver failure (130, 131). In response to obesity, Kupffer cells are engaged by cytokines, adipokines, and FAs secreted from adipose tissue promoting a polarized M1 phenotype (132). In addition, the release of FFAs into the liver by hypertrophic adipocytes leads to hepatocyte ER stress and ROS production (132). Hepatocyte death triggered by these responses results in the release of danger associated molecular patterns (DAMPs) that further promote liver macrophage polarization (123, 132).

In hepatic steatosis, pro-inflammatory cytokines produced by M1 activated macrophages induce hepatic cholesterologenesis and increase TAG production, resulting in discordant regulation of lipid metabolism and homeostasis (Figure 4) (133–136). Sterol regulatory element-binding proteins (SREBPs) directly activate genes that mediate the synthesis and uptake of cholesterol and FAs (137). Elevation of SREBPs often underlies the pathogenesis of insulin resistance and type 2 diabetes (138, 139). In addition to their initial propagation of fatty liver disease, activated M1 Kupffer cells trigger the more severe form of non-alcoholic fatty liver

disease (NAFLD), NASH. A recent study demonstrated that TNF α produced by M1 hepatic macrophages increased intrahepatic expression of MCP-1 (**Figure 4**), a major chemokine responsible for CCR2-dependent recruitment of Ly6C hi monocytes (135). The vast infiltration of Ly6C hi monocytes further perturbs liver homeostasis amplifying hepatic inflammatory responses (**Figure 4**). The recruited monocyte-derived macrophage population also plays a key role in promoting fibrogenesis. In mice deficient for CCR2 or depleted of Kupffer cells, inflammatory cell infiltration, steatohepatitis, and fibrosis are ameliorated (135).

Progression of NAFLD to NASH occurs in a subset of patients with fatty liver disease, and this progression is largely linked to the activation of toll like receptor 4 (TLR4) by both exogenous and endogenous ligands. Bacteria and bacterial products, such as LPS, which translocate from the gut can activate TLR4 signaling to induce NASH (140, 141). Furthermore, endogenous high mobility group box-1 (HMGB-1) and FFAs have also been shown to be endogenous ligands for TLR4 (142, 143). A number of recent studies have also demonstrated a crucial link between the composition of gut microbiota, obesity, and the development of NASH (144). CD14 promotes the activation of TLR4 by promoting the recruitment of LPS to the TLR4 signaling complex (145). Interestingly, Imajo et al. demonstrated that the increased levels of leptin associated with obesity promotes the enhanced expression of CD14 in Kupffer cells, resulting in increased sensitivity of the Kupffer cells to low doses of LPS (146). Translocated bacterial DNA has also been shown to promote the development of NASH through engagement of TLR9 in Kupffer cells, which stimulates the production of interleukin-1 β (IL-1 β) (147). IL-1 β in turn, promotes hepatocyte lipid accumulation and death, as well inducing hepatic stellate cells (HpSCs) to promote fibrosis.

Liver fibrosis is a common feature of chronic liver disease. It is characterized by accumulation of extracellular matrix (ECM) resulting from both increased synthesis and decreased degradation of ECM proteins (148). During progressive liver injury, HpSC-derived myofibroblast-like cells predominate and initiate collagen deposition (148). Extensive evidence demonstrates a complex interplay between Kupffer cells and HpSCs during hepatic fibrogenesis. In general, alternatively activated M2 macrophages are involved in tissue remodeling through the production of transforming growth factor beta-1 (TGF β -1), a potent inducer of the HpSC fibrotic phenotype (149, 150). However, while arginase produced by M2 cells during a Th2 response was thought to play a role in progression of fibrosis, studies by Pesce et al., in which arginase is depleted in macrophages, highlight a role for arginase-expressing macrophages in the resolution of fibrosis (151). Alternatively, in hepatic fibrosis, Ly6C hi inflammatory monocyte-derived macrophages express higher levels of TGF β -1 and other pro-inflammatory cytokines that activate HpSCs (152). TGF β -1 not only promotes the fibrogenic activity of HpSCs but also enhances expression of tissue inhibitors of metalloproteinases (TIMPs) by HpSCs that block the degradation of ECM. These inflammatory monocyte-derived macrophages also exhibit decreased expression of matrix metalloproteinases, thus directly promoting fibrosis.

Interestingly, a recent report demonstrated a role for a novel population of monocyte-derived macrophages in the resolution

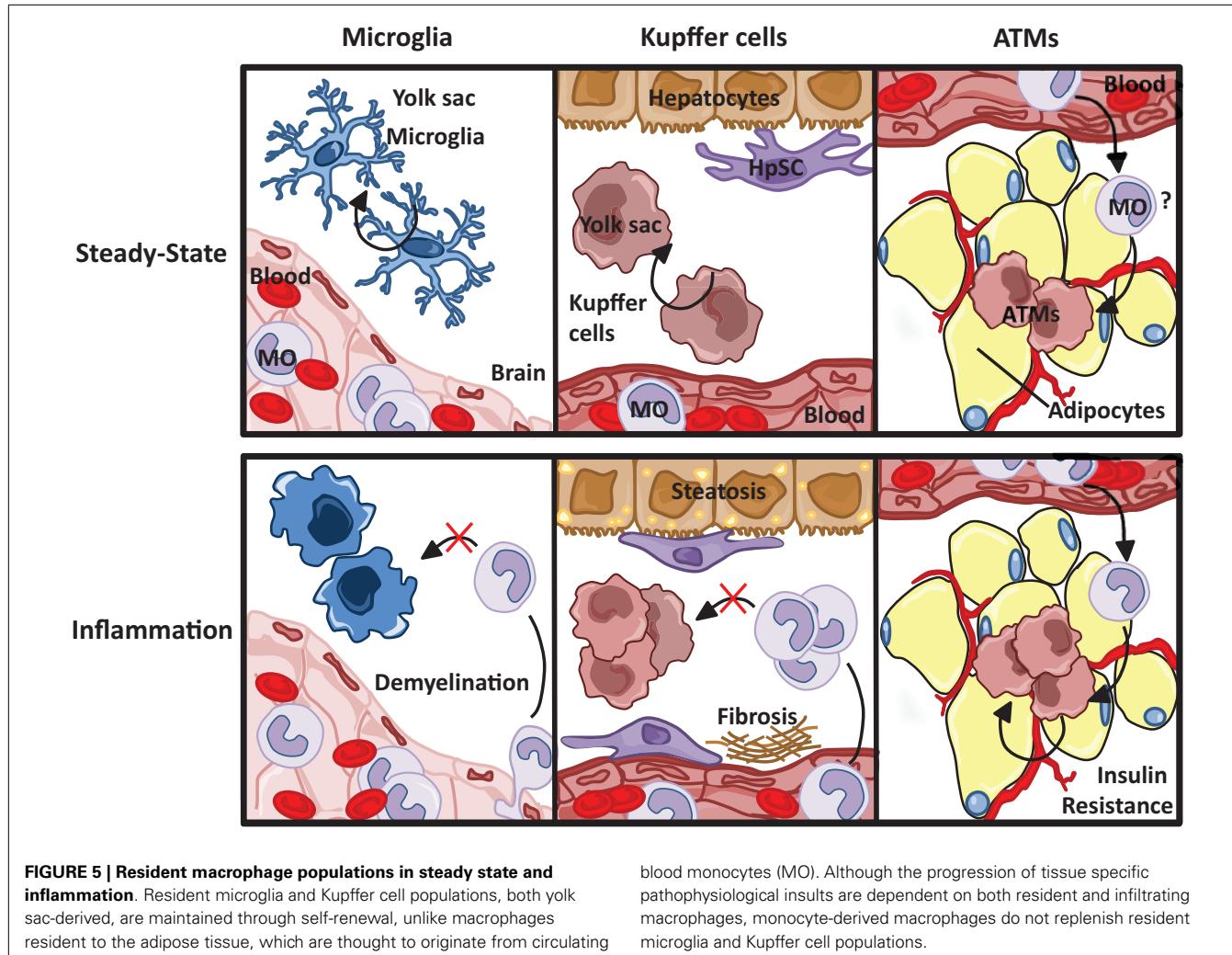
of fibrosis. These cells are derived from Ly6C hi monocytes and exhibit a CD11b hi F4/80 int Ly6C lo phenotype. These cells mediate MMP-dependent degradation of the ECM and the expression profile of this population of resolving monocyte-derived macrophages demonstrates that these cells are distinct from the typical M1/M2 populations of macrophages (152). More recently, it has been reported that alternatively activated M2 Kupffer cells can promote caspase-3-dependent apoptosis of classically activated M1 Kupffer cells, thus providing a protective mechanism against NAFLD (**Figure 4**) (153). The M2 mediated apoptosis of M1 Kupffer cells was shown to be arginase dependent by way of IL-10 (153). Interestingly, alternatively activated M2 Kupffer cells limit hepatocyte apoptosis and steatosis in alcohol induced liver injury through the release of IL-6, a mechanism that may expand to NAFLD (154).

SUMMARY

Based on recent studies, a unifying theme is beginning to emerge between the contribution of tissue-resident macrophages and infiltrating monocyte-derived macrophages in chronic inflammation (**Figure 5**). (1) Both microglia and Kupffer cells are of embryonic origin. Microglia are YS, while contribution of yolk sac and fetal liver has been described for Kupffer cells. However, because it now appears that the yolk sac progenitors seed the liver where they continue to contribute to tissue-resident macrophage populations independent of hematopoietic stem cells, it is likely that these cells share a common progenitor and are ontologically indistinguishable. (2) In the brain and liver, resident macrophages and infiltrating monocyte-derived macrophages play distinct roles in the progression of inflammation. In the brain, microglia are required for the initiation of disease, while infiltrating monocyte-derived macrophages promote demyelination. In the liver, both resident and infiltrating populations contribute to both inflammation and repair, Kupffer cells are largely responsible for the development of fibrosis. (3) In the brain and liver, infiltrating monocytes do not contribute to the resident macrophage pool rather, during recovery, resident cells are replenished through local proliferation. (4) In both the brain and liver, there is evidence that the resident population of macrophages plays a role in promoting apoptosis and/or clearance of the infiltrating monocyte-derived macrophage population during the recovery phase.

However, the relative contribution of resident versus infiltrating populations of macrophages in adipose tissue remains obscure. While ATMs are generally considered to be hematopoietic stem cells-derived, this assumption is based on studies using myeloablation. Further fate mapping studies will be needed to definitively identify the origin of ATMs. In addition, while it is clear that both infiltration of monocytes and proliferation of resident macrophage contribute to the massive expansion of ATMs in obesity, the respective roles of resident versus infiltrating macrophages in the propagation and/or resolution of inflammation is unclear. Finally, it unknown whether infiltrating monocyte-derived macrophages in adipose tissue ultimately contribute to the resident population.

In all cases, the M1/M2 polarization of macrophages in inflammation and disease resolution has been described. However, these



are largely based on studies that do not distinguish between resident macrophage and infiltrating macrophage populations. Given the emergence of technologies that help to distinguish resident cells from infiltrating cells, it will be essential to re-visit the M1/M2 paradigm in the context of macrophage ontogeny to determine the extent of plasticity of individual populations of macrophages.

The recognition that the M1/M2 polarization of macrophages plays a central role in the progression of chronic inflammation in a wide range of diseases and that therapeutic approaches to chronic inflammatory disease could involve the regulation of macrophage polarization renders these outstanding questions highly significant. Taking into consideration not only the polarization state of macrophages, but also their ontogeny and phenotypic plasticity, will be central in the development of therapeutic approaches for the treatment of chronic inflammatory disease aimed at redirecting macrophage polarization and function.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 12 September 2014; accepted: 17 December 2014; published online: 22 January 2015.

Citation: Dey A, Allen J and Hankey-Giblin PA (2015) Ontogeny and polarization of macrophages in inflammation: blood monocytes versus tissue macrophages. *Front Immunol.* **5**:683. doi: 10.3389/fimmu.2014.00683

This article was submitted to Molecular Innate Immunity, a section of the journal *Frontiers in Immunology*.

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Molecular mechanisms that influence the macrophage M1–M2 polarization balance

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As an essential component of innate immunity, macrophages have multiple functions in both inhibiting or promoting cell proliferation and tissue repair. Diversity and plasticity are hallmarks of macrophages. Classical M1 and alternative M2 activation of macrophages, mirroring the Th1–Th2 polarization of T cells, represent two extremes of a dynamic changing state of macrophage activation. M1-type macrophages release cytokines that inhibit the proliferation of surrounding cells and damage contiguous tissue, and M2-type macrophages release cytokines that promote the proliferation of contiguous cells and tissue repair. M1–M2 polarization of macrophage is a tightly controlled process entailing a set of signaling pathways, transcriptional and posttranscriptional regulatory networks. An imbalance of macrophage M1–M2 polarization is often associated with various diseases or inflammatory conditions. Therefore, identification of the molecules associated with the dynamic changes of macrophage polarization and understanding their interactions is crucial for elucidating the molecular basis of disease progression and designing novel macrophage-mediated therapeutic strategies.

Keywords: innate immune response, macrophage polarization, TLR, NLR, SOCS, microRNA

INTRODUCTION

As an essential component of innate immunity, macrophages are capable of differentiating into protean varieties with a range of function (1–3). In respond to various environmental cues (e.g., microbial products, damaged cells, activated lymphocytes) or under different pathophysiologic conditions, macrophages can acquire distinct functional phenotypes via undergoing different phenotypic polarization (4). Macrophage M1 and M2-type responses describe the opposing activities of killing or repairing, and such polarized responses stimulate Th1- or Th2-like responses in macrophages, respectively. First, M1 phenotype is stimulated by microbial products or pro-inflammatory cytokines [IFN- γ , TNF, or Toll-like receptor (TLR) ligands], and the typical characteristics of M1 macrophages include high antigen presentation, high production of IL-12 and IL-23, and high production of nitric oxide (NO) and reactive oxygen intermediates (ROI) (5). In contrast, M2-type responses are the “resting” phenotype and are observed in healing-type circumstances without infections. Such responses can also be further amplified by IL-4, IL-10, or IL-13. M2 macrophages are characterized by the upregulation of Dectin-1, DC-SIGN, mannose receptor, scavenger receptor A, scavenger receptor B-1, CD163, CCR2, CXCR1, and CXCR2 (6). Instead of generating NO or ROI, M2 macrophages produce ornithine and polyamines through the arginase pathway (2, 7). In fact, from the functional point view, NO and Ornithine, correlating to killing (M1) and repairing function (M2) of macrophages, have been regarded by some investigators as the most characteristic molecules of macrophages (8). Second, inflammatory M1 macrophages produce many other pro-inflammatory cytokines like TNF α , IL-1, IL-6, IL-12, Type I IFN, CXCL1-3,

CXCL-5, and CXCL8-10 (9), while M2 macrophages generate anti-inflammatory cytokine such as IL-10 and very low level of pro-inflammatory cytokine such as IL-12 (10). Additional signatures of M2 phenotype, such as YM1 (a member of the chitinase family) and FIZZ1 (found in inflammatory zone 1, RETNLA) are also identified (11). Third, M1 macrophages promote Th1 response and possess strong microbicidal and tumoricidal activity, while M2 macrophages are involved in metazoan parasites containment and promotion of Th2 response, tissue remodeling, immune tolerance, and tumor progression (12, 13). Additional information about polarized activation of macrophages can be found in the previous reviews (1, 14–16).

A coordinate action of various inflammatory modulators, signaling molecules, and transcription factors is involved in regulating macrophage polarization. At cellular level, although M1 and M2 macrophage activities exist without T or B cell influence (17), specialized or polarized T cells (Th1, Th2, Tregs) do play a role in macrophage polarized activation (1). Canonical IRF/STAT signaling is a central pathway in modulating macrophage polarization. Activation of IRF/STAT signaling pathways by IFNs and TLR signaling will skew macrophage function toward the M1 phenotype (via STAT1), while activation of IRF/STAT (via STAT6) signaling pathways by IL-4 and IL-13 will skew macrophage function toward the M2 phenotype (9). Signals initiated by IL-10, glucocorticoid hormones, apoptotic cell-released molecules, and immune complexes can also profoundly affect macrophage functional statue (1). Macrophage polarization is also modulated by local microenvironmental conditions such as hypoxia (18). More importantly, M1–M2 polarization of macrophage is a highly dynamic process and the phenotype of polarized macrophages

can be reversed under physiological and pathological conditions (19, 20). In the course of various pathophysiological settings, the same signaling pathway can be involved in either M1 or M2 polarization of macrophages. The molecular mechanisms that govern the phenotype switch of macrophages, however, remains incompletely understood. Moreover, imbalances of macrophage M1–M2 polarization are associated with various diseases. Disease conditions are frequently associated with polarization of macrophage activation, with classically activated M1 macrophages implicated in initiating and sustaining inflammation and M2 macrophages associated with resolution of chronic inflammation (6). In the past decade, a new class of small non-coding RNAs, termed as microRNAs (miRNAs), have emerged as important regulators in biological processes. An important role of miRNAs in modulating macrophage phenotypic polarization is demonstrated by accumulating evidences in which an excessive or impaired inflammatory response of macrophages is found to be tightly linked to the deregulation of miRNAs. In this review, we focus on recent progress in understanding the molecular basis underlying the

dynamic macrophage polarization, including signaling pathways, transcription factors and miRNAs.

IRF/STAT SIGNALING

As shown in **Figure 1**, IRF/STAT signaling is a central pathway in controlling macrophage M1–M2 polarization. Toll-like receptor signaling, particularly TLR4 stimulated by lipopolysaccharide (LPS) and other microbial ligands, drives macrophages to a preferentially M1 phenotype. Two adaptors, MyD88 and TRIF, mediate the signaling downstream of TLR4. The signaling pathway through the MyD88 adaptor results in the activation of a cascade of kinases, including IRAK4, TRAF6, and IKK β , which finally leads to the activation of nuclear factor kappa B (NF- κ B). As a key transcription factor related to macrophage M1 activation, NF- κ B regulates the expression of a large number of inflammatory genes including TNF α , IL1B, cyclooxygenase 2 (COX2), IL-6, and IL12p40. NF- κ B activity is modulated via the activation of the inhibitor of kappa B kinase (IKK) trimeric complex (two kinases, IKK α , IKK β , and a regulatory protein, IKK γ). When upstream signals converge at the

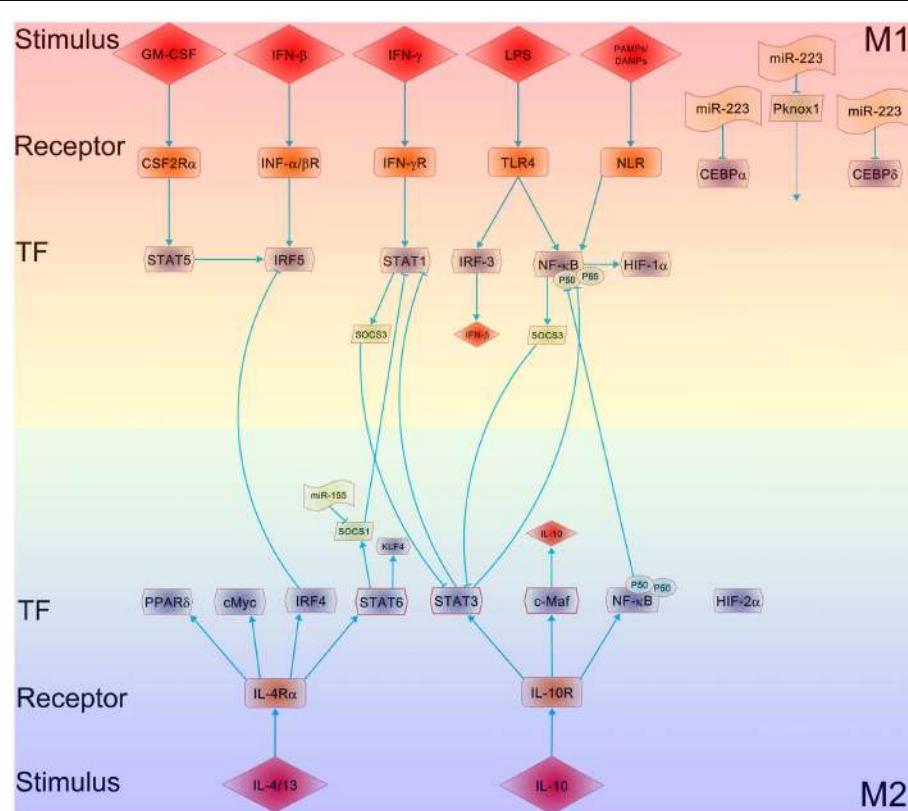


FIGURE 1 | Mechanisms underlying the polarization of macrophages. The major regulatory pathways of macrophage M1–M2 polarization are outlined. The crosstalk between the M1 and M2 macrophage polarizing pathways, particularly the balance between activation of STAT1 and STAT3/STAT6, tightly regulates macrophage polarization and activity. A predominance of NF- κ B and STAT1 activation promotes M1 macrophage polarization, resulting in cytotoxic and tissue-damage proinflammatory functions. In contrast, a predominance of STAT3 and STAT6 activation by IL-4/13 and IL-10 increases M2 macrophage polarization, associated with immune tolerance and tissue

repairing. PPAR δ (and PPAR γ) control distinct aspects of M2 macrophage activation and oxidative metabolism. KLF4, a downstream of STAT6, participates in the promotion of M2 macrophage functions by suppressing the NF- κ B/HIF-1 α -dependent transcription. IL-4 induces not only c-Myc, which controls the expression of a subset of M2-associated genes but also the M2-polarizing IRF-4 axis to inhibit IRF5-mediated M1 polarization. IL-10 promotes M2 polarization through the induction of p50 NF- κ B homodimer, c-Maf, and STAT3 activities. MicroRNAs such as miR-155, miR-223, etc. are involved in modulating macrophage polarization via targeting SOCS1, CEBP, and Pknox1, respectively.

IKK complex, they first activate IKK β via phosphorylation, and activated IKK β further phosphorylates the inhibitory molecule, inhibitor of kappa B (I- κ B). This results in the proteosomal degradation of I- κ B and the release of NF- κ B p65/p50 heterodimer from the NF- κ B/I- κ B complex. The NF- κ B p65/p50 heterodimer is then translocated to the nucleus and binds to the promoters of inflammatory genes. The signaling through the TRIF adaptor pathway activates the transcription factor interferon-responsive factor 3 (IRF3), leading to the expression and secretion of type I interferon, such as IFN α and IFN β . Secreted type I interferons bind to the type I interferon receptor (IFNAR) with consequent activation of the transcription factor STAT1. It has been widely reported that IRF3 and IRF5 are involved in regulating M1 polarization and M1-associated gene induction (21, 22). IFN-stimulated genes include chemokine CXCL9 and CXCL10 (23), which are characteristic of classical M1 macrophage activation. In fact, macrophage polarization is tightly linked to the differential expression of various TLRs on macrophages. The ratio of TLR4/TLR2 is significantly higher in M1 macrophages compared to M2 macrophages (24), while TLR4 deficiency promotes the alternative activation (M2) of adipose tissue macrophages (ATMs) (25). TLR ligands, e.g., imiquimod and CpG, have been used as therapeutic treatments for inflammatory diseases such as asthma by modulating macrophage polarization.

Toll-like receptor and Toll-like receptor-induced cytokine-receptor cascades are broadly inhibited by tyrosine kinases Tyro3, Axl, and Mer. IFN β can activate the receptor for Axl, Tyro3, and Mer and negatively regulate TLR signaling through induction of SOCS1 and SOCS3 (26). A hyperactive signaling mediated by Tyro3, Axl, and Mer receptor is suggested to induce immunosuppression in severe sepsis patients (26). Along the same lines, chronic signaling through the TLR4 pathway has been shown to induce various negative regulators like IRAK-M, ST2, SOCS1, short version of MyD88 (MyD88sh) (27, 28) and SHIPs (29). These negative regulators inhibit TLR-mediated signaling and thus switch macrophages to an immunosuppressive, endotoxin-tolerant phenotype. A switching from an MyD88-dependent to a TRIF-dependent TLR4 pathway in macrophages has also been suggested to shift macrophage phenotype from an inflammatory to anti-inflammatory, endotoxin-tolerant phenotype (30). Thus, interplay of signaling molecules and transcription factors can reverse the phenotype of macrophage polarization.

STAT-mediated activation of macrophages is regulated by members of the suppressor of cytokine signaling (SOCS) family. SOCS family members are inducible inhibitors of cytokine signals and thus play a critical role in limiting inflammation responses. SOCS proteins could be induced by cytokine signaling pathway, and they in turn inhibit the cytokine signaling by several mechanisms. For example, IL-4 and IFN- γ , the latter in concert with TLR stimulation, upregulate SOCS1 (31) and SOCS3 (32), which in turn, inhibit the action of STAT1 and STAT3, respectively. SOCS proteins can be also directly induced by TLR signaling. In macrophages, SOCS proteins not only regulate the sensitivity of cells toward cytokines but also modulate signaling through TLRs. Because SOCS3 is a downstream molecule of Notch signaling (33), it is likely that Notch signaling determines the M1 versus M2 polarization of macrophages through SOCS3 (34). However,

the role of SOCS3 in modulating macrophage M1–M2 polarization is controversial. Although the unique expression of SOCS3 was reported to be essential for classic macrophage activation (32), SOCS3 deficiency also promotes M1 macrophage polarization and inflammation (35).

Macrophages can be driven to M2 phenotype by canonical M2 stimuli like IL-4, IL-13, and IL-10 (36, 37). As shown in Figure 1, IL-4 and IL-13 polarize macrophages to M2 phenotype via activating STAT6 through the IL-4 receptor alpha (IL-4R α), whereas IL-10 promotes M2 phenotype via activating STAT3 through receptor (IL-10R). In IL-4 and IL-13 pathway, receptor binding of IL-4 activates JAK1 and JAK3 (38), leading to STAT6 activation and translocation. Macrophage M2 phenotype is promoted by several transcription factors, including peroxisome proliferator activated receptor γ (PPAR γ) (39, 40) and Krueppel-like factor 4 (KLF-4) (41). Myeloid-specific deficiency of either PPAR γ or KLF-4 resulted in suppressed M2 polarization of macrophages, leading to accelerated lesion formation in apolipoprotein E-deficient (42) or low-density lipoprotein receptor-knockout (43) mice. Moreover, ligation of PPAR γ by specific PPAR γ ligands resulted in a preferential M2 polarization in mice and in human beings (40). Other transcription factors involved in this process include c-Myc and IRF4. Transcriptome analysis of IL-4-stimulated cells consists of various enzymes and transcription factors, including transglutaminase 2 (TGM2), mannose receptor, cholesterol hydroxylase CH25H, prostaglandin-endoperoxide synthase PTGS1 (prostaglandin G/H synthase 1), transcription factors IRF4, KLF-4, and the signaling modulators CISH and SOCS1 (44). During severe respiratory syncytial virus (RSV)-induced bronchiolitis, IL-4R α /STAT6-dependent M2 differentiation of macrophages reduces inflammation and epithelial damage in lungs (45).

PPAR γ play an important role in modulating macrophage M2 polarization induced by IL-4 or IL-13 (46). Studies using PPAR γ -deficient macrophages have shown the role of this nuclear receptor in promoting M2 activation to protect mice from insulin resistance (47). A similar role was also found for the PPAR δ in determination of macrophage polarization (48). Using myeloid-specific transcription factor KLF-4 knockout mice, Liao et al. (41) demonstrated the role of KLF-4 in regulating M2 polarization of macrophages as well as in protecting mice from obesity-induced insulin resistance. In a similar fashion, IRF4 is also involved in regulating the expression of genes associated with macrophage M2 polarization (49). Collectively, all these findings suggest that STAT6, PPAR γ , KLF-4, and IRF4 may coordinate the M2 polarization of macrophages.

IL-10R, a heterodimer of IL-10R1 and IL-10R2, is a receptor for IL-10. Ligation of IL-10R with IL-10 results in the autoposphorylation of IL-10R, leading to the activation of the transcription factor STAT3 and reduction of pro-inflammatory cytokine expression. In macrophages, IL-10 is also reported to respond to TLR activation, glucocorticoid treatment, and C-type lectin signaling (e.g., DC-SIGN and dectin 1 ligation). The components in IL-10-induced macrophage transcriptome include specific Fc receptors, chemoattractants CXCL13 and CXCL4, recognition receptors formyl peptide receptor 1 (FPR1), TLR1, TLR8, and macrophage receptor with collagenous domain (50).

HIF-1 α AND HIF-2 α

Macrophages can rapidly alter their metabolic and functional state to adapt to the microenvironment of surrounding tissues. Microenvironmental conditions in infected, inflamed, or damaged tissues are generally lack of oxygen and nutrients. When macrophages are recruited into inflammatory sites, they encounter the hypoxia condition, which can directly affect the macrophage polarization. Hypoxia executes its effect on macrophages through two isoforms of hypoxia-inducible factor (HIF), HIF-1 α and HIF2 (51, 52). Gene expression profiling of macrophages and monocytes has identified profound changes in response to hypoxia (53, 54). Hypoxia strongly induces the expression of angiogenesis- and metastasis-related genes such as VEGF, FGF2, MMP7, and MMP9. Upregulation of those genes under hypoxia would lead to more recruitment of macrophages into the hypoxic (avascular) areas in pathologies like atherosclerosis, obesity, and cancer where they dampen the inflammation or promote tumor progression. In addition, pro-inflammatory cytokines like TNF α , IL-1 β , MIF, CCL3, and COX2, as well as M2 markers like IL-10 and arginase 1 in macrophages, are also induced by hypoxia (55). The crucial role of hypoxia in regulating macrophage inflammatory response has been confirmed in mice with myeloid cell-specific deletion of HIF-1 α (56), in which HIF-1 α was found to be essential in regulating myeloid cell glycolytic capacity and survival and function in the inflammatory microenvironment. This is in line with the finding that HIF-1 α was induced by NF- κ B (57) and plays an important role in modulating macrophage phagocytosis of bacteria under sepsis conditions (58). Moreover, recent studies also showed that HIF-1 α can mediate the effects of tumor-derived lactic acid (59) and cytokines (Oncostatin M and Eotaxin) (60) on promoting M2-like phenotype. In contrast to these studies, recent study of myeloid-specific HIF-2 α deletion showed the role of HIF2 in mediating macrophage inflammatory responses rather than HIF-1 α (52). In contrast to these studies, a recent (61) suggested that HIF-1 α and HIF-2 α might also drive macrophage polarization by modulating NO homeostasis in a cytokine-induced and transcription-dependent fashion. Specifically, this study showed that inducible NO synthase gene and the arginase 1 gene in polarized macrophages are specifically regulated by HIFs (61). Although HIF-1 α and HIF-2 α displayed physiologically antagonistic functions, their antiphase regulation allows them to coordinately regulate NO production to guide macrophage polarization. Together, these findings suggest HIFs as an important regulator of macrophage polarization, although a detailed dissection of whether the alteration of HIF isoform expression can switch macrophage phenotypes needs further investigation.

OLIGOMERIZATION DOMAIN (NOD)-LIKE RECEPTORS

Stimulated by a diverse set of stimulus, including interferon- γ (IFN- γ), LPS, and other TLR activators, macrophages are polarized toward to M1 state in which oxidative metabolites and pro-inflammatory cytokines are produced. Engagement of the respective receptors by these stimulus results in activation of the adapter proteins such as MyD88, leading to sequential activation of kinases, phosphorylation of transcription factors, and eventual genetic program induction. Pro-inflammatory genes, including IFN- γ ,

tumor necrosis factor- α (TNF- α), IL-1 β , IL-18, chemokines, and proteases, are subsequently produced. Further activation of the M1 pathway occurs through the assembly of the NLR inflammasome and caspase-1 activation, which results in the conversion of IL-1 β and IL-18 into secreted active forms (62). With the NLRP3 inflammasome serving as a sensor of obesity-associated danger signals, the progression of obesity can switch macrophages from “M2-like” to “M1-like” cells (63). In macrophages, the activation of NLR stimulates the cryptopyrin/NLRP3 inflammasome to induce IL-1 β and IL-18 production via caspase-1. Caspase-1 and IL-1 β are induced in adipose tissue with diet-induced obesity (DIO), and Nlrp3- and caspase-1-deficient mice both demonstrate a resistance to DIO-induced inflammation (63). The mechanism of this protective effect may be driven by the alteration of M1 activation of ATMs, as Nlrp3-knockout mice show decreased M1 but increased M2 gene expression in ATMs.

In addition to binding to TLRs, some pathogen-associated molecular patterns (PAMPs) are also recognized by a family of cytosolic nucleotide-binding receptors and NOD-like receptors (NLRs) (64), another groups of PAMP receptors. Some NLRs are involved in the recognition of microbial molecules and/or endogenous factors released from tissue destruction. This recognition can lead to activation of caspase-1 (a pro-inflammatory caspase), and subsequent proteolytic conversion of potent pro-inflammatory cytokines IL-1 β and IL-18 from their precursors pro-IL-1 β and pro-IL-18, respectively. The proteolytic conversion of IL-1 β and IL-18 is mediated by a cytosolic caspase 1-activating protein complex, termed as inflammasome (65).

As the most well-characterized members of the NLR family, NOD1 is ubiquitously expressed and NOD2 is restricted to monocytes, macrophages, dendritic cells, and intestinal Paneth cells (66). Both NOD1 and NOD2 induce NF- κ B activation in a TLR-independent manner (67). Structural analysis demonstrated that NOD1 and NOD2 recognize different core motifs derived from peptidoglycan (PGN), a component of bacterial cell walls. NOD1 activity is triggered by γ -D-glutamyl-meso-diaminopimelic acid, a unique PGN structures from all Gram-negative and some Gram-positive bacteria (68). In contrast, NOD2 is activated by muramyl dipeptide, a PGN motif in all Gram-positive and Gram-negative bacteria (69). Upon ligand recognition, NOD1 and NOD2 undergo conformational changes and self-oligomerization, which is followed by the recruitment and activation of the serine threonine kinase RICK (RIP2, also known as RIPK2), an essential step for the activation of NF- κ B and MAPKs. The ubiquitination of RICK is essential for NOD1/NOD2-mediated signaling because removal of this modification by deubiquitinating enzyme A20 largely dampens NOD1/NOD2-induced NF- κ B activation (70, 71). Although both NOD1 and NOD2 induce similar K63-linked ubiquitination of RICK for NF- κ B activation and upregulation of various inflammatory mediators, NOD2 signaling appears to preferentially utilize the E3 ligase TRAF6 and NOD1-mediated signaling is mainly associated with TRAF2 and TRAF5. Nevertheless, the role of NOD1 and NOD2 in activating NF- κ B-dependent inflammatory responses is not limited to the recognition of PGN motifs. Recent study by Keestra et al. (72) reported that that NOD1 can sense activation state of small Rho GTPases. In this study, NOD1 signaling pathway was triggered by RAC1 and CDC42

activated by bacterial delivery or ectopic expression of SopE, a virulence factor of the enteric pathogen *Salmonella*.

GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR

As the most recently discovered cytokine involved in regulation of macrophage polarization, granulocyte-macrophage colony-stimulating factor (GM-CSF) is produced by a variety of cells including macrophages and parenchyma cells. The main functions of GM-CSF include regulating the proliferation and differentiation of functional hematopoietic cells. The GM-CSF receptor forms a dodecamer structure (73) and recruits JAK2, leading to the activation of STAT5, extracellular signal-regulated kinase (ERK), V-Akt murine thymoma viral oncogene homolog 1 (AKT), and the nuclear translocation of NF- κ B and IRF5 (21). Many of these regulators are part of the IFN- γ and TLR signaling pathways. GM-CSF enhances macrophage antigen presentation, complement- and antibody-mediated phagocytosis, microbicidal capacity, leukocyte chemotaxis, and adhesion. GM-CSF induces cytokine production of IL-6, IL-8, G-CSF, M-CSF, TNF, and IL-1 β in monocytes and macrophages, although the degree of cytokine induction by GM-CSF is less than that by LPS. Global gene expression analyses of macrophages differentiated from GM-CSF-treated monocytes showed that GM-CSF upregulated 340 genes and downregulated 190 genes in macrophages. Macrophage-specific genes including CD14, CD163, C5R1, and FcyR1A, and several cell surface adhesion molecules, cytokine receptors were induced by GM-CSF (74). In this study, a high-resolution transcriptome profiling of human macrophages by RNA sequencing was employed to discover novel marker genes unique for human macrophages. A similar strategy has been used to obtain a high-resolution transcriptome profile of human macrophages under M1 (or M1-like) and M2 (or M2-like) polarization conditions, resulting in a more comprehensive understanding of the transcriptome of human macrophages (75). The GM-CSF deficient mice have normal numbers of macrophages in many tissues but display an impaired maturation of alveolar macrophages and develop pulmonary alveolar proteinosis (76). In human beings, mutations in the GM-CSF receptor, especially in the common beta chain, lead to alveolar macrophage dysfunction, proteinosis, and malignancy (77, 78).

MicroRNAs

MicroRNAs (miRNAs), a class of 19–24 nt non-coding RNAs that induce gene silencing at the posttranscriptional level, have emerged as an important regulatory mechanism for gene expression in many immune cells including monocytes and macrophages (79, 80). Functional miRNAs associated with polarized macrophages have been identified (81). While these functional miRNAs like miR-155 and miR-146 are induced by a variety of inflammatory stimuli, including LPS, TNF α , and IL-1 β , they are instrumental in attenuating TLR4/IL-1R signaling pathways in monocytes and macrophages (79, 82, 83). These findings allow us to postulate that miRNAs may contribute to the switching of inflammatory macrophages to an immunosuppressive phenotype, needed for resolution. For instance, miR-146, miR-125b, miR-155, and miR-9 have been shown to be induced by LPS, and in turn, these miRNAs inhibit TLR4/IL-1R signaling through regulation

of IRAK-1, TRAF6, IKK ϵ , p50NF- κ B, and TNF α at transcriptional and posttranscriptional level (79, 82–86). Our recent study has also shown that a panel of miRNAs including miR-17, miR-20a, and miR-106a are stimulated by LPS through c-Myc pathway, and these miRNAs collectively reduces the expression level of macrophage differentiation related marker, signal-regulatory protein α (SIRP α) (87). It has been reported that miR-98 and miR-21 inhibit the expression of inflammatory genes in monocytes and macrophages via controlling IL-10 level (88, 89). These findings strongly argue that miRNAs can regulate macrophage phenotype in the course of various diseases, for example, during endotoxin tolerance (30, 90).

In the efforts to delineate the role of miRNAs in macrophage activation in inflammatory diseases, Ponomarev et al. (91) found that miR-124 promotes microglia quiescence and suppresses EAE by deactivating macrophages in a C/EBP α -PU.1-dependent manner. This is one of the few studies in which a specific miRNA is found to regulate macrophage plasticity, although it remains unclear how C/EBP α suppresses macrophage M2 polarization. Zhang and co-workers (92) also reported that miR-223 modulates obesity-associated adipose tissue inflammation through regulating macrophage activation. In the study, they found that miR-223 was upregulated in LPS-treated macrophages but downregulated in IL-4-treated bone marrow-derived macrophages (BMDMs). In agreement with the observation of differential expression of miR-223 in various macrophages, the miR-223-deficient macrophages were hypersensitive to LPS stimulation, whereas such macrophages exhibited a delayed responses to IL-4 compared with controls. Moreover, miR-223-deficient mice exhibited an increased adipose tissue inflammatory response but a decreased adipose tissue insulin signaling. They further identified Pknox1 as a genuine target of miR-223. Although Pknox1 as a miR-223 target in regulating macrophage polarization was validated by gain-of-function and loss-of-function analyses in BMDMs, it remains unclear how Pknox1 further regulates macrophage polarization.

A recent work by Banerjee et al. (93) demonstrated that let-7c could regulate bactericidal and phagocytic activities of macrophages, two functional phenotypes implicated in macrophage polarization. In the study, they found that let-7c was expressed at a higher level in M2-type macrophages than in M1-type macrophages. When M2-type macrophages were re-polarized to M1-type macrophages or M1-type macrophages converted to M2-type macrophages, let-7c expression level was decreased or increased, respectively. As LPS stimulation reduced let-7c expression in M2 macrophages, let-7c might play an inhibitory role in modulating macrophage inflammatory responses. In line with this, upregulation of let-7c in macrophages diminished M1 phenotype but promoted M2 phenotype polarization. Their study further identified that let-7c targeted C/EBP- δ , a key transcriptional factor in macrophage pro-inflammatory response to TLR4 stimulation (94, 95).

The modulation of macrophage polarization by miR-155 has also been recently reported (96, 97). The expression of miR-155 was found to be repressed in naive macrophages or LPS-stimulated Akt2 $^{-/-}$ macrophages. In this process, miR-155 targets transcriptional factor C/EBP- β , a hallmark of M2 macrophages. C/EBP- β can regulate Arg1 and its level is increased upon Akt2 ablation. Overexpression or depletion of miR-155 drove macrophages to

M1 or M2 phenotype, respectively, confirming that miR-155 plays a central role in regulating Akt-dependent M1/M2 polarization of macrophages. It has also reported that miR-155 can directly block IL-13-induced macrophage M2 phenotype via suppressing the expression of IL-13R α 1 (96). As an oncomiR, miR-155 also targets SHIP1 to promote TNF α -dependent tumor cell growth (98). Through overexpression of miR-155, we successfully re-polarized tumor-associated macrophages (TAMs) into pro-inflammatory M1 macrophages (97). Taken together, these studies support the hypothesis that miR-155 is a key molecule in causing macrophage polarization toward M1-type activity.

M1–M2 PHENOTYPE SWITCH

Macrophage differentiation is highly dynamic. Responding to microenvironmental cues macrophages can rapidly switch from one phenotype to the other. In fact, activation of NF- κ B or IRF family members in macrophages by TLR4 or other TLRs can drive macrophage to either M1 or M2 polarization under various pathological conditions (99–105). Accumulating evidences have shown that the spatiotemporal activation of NF- κ B is a key regulator of the plasticity of macrophages observed in the courses of various disease progressions. For example, during the early phase of tumorigenesis, NF- κ B activation in M1 macrophages is critical for cancer-related inflammation. However, at the late phase of tumorigenesis, macrophages are re-programed to TAM or M2-like macrophages displaying low NF- κ B activation but increased immunosuppressive capacity (106). A similar situation of macrophage polarization is observed at different stages along the progression of sepsis, in which NF- κ B activation in M1 macrophages drives the initial overt inflammatory phase, while during the late phase of endotoxin tolerance, macrophages are polarized to an anti-inflammatory, tumor growth-promoting (M2) phenotype, and display an impaired NF- κ B activation (107). The studies on RSV infection also show that polarization of macrophages is complicated process and the phenotype of macrophage activation can be varied at the different stage along disease progression. As the most significant cause of lower respiratory tract infection in infants and young children, RSV infection is found to be associated with a mixed “Th1” and “Th2” cytokine storm. At the initial stage of RSV infection, RAV induces the expression of various anti-viral genes like IFN- β in airway epithelial cells, and then promotes the expression of many NF- κ B-dependent pro-inflammatory genes in macrophages through stimulating TLR4, TLR2, TLR3, and retinoic acid-induce gene I (RIG-I), driving macrophages toward anti-viral, pro-inflammatory M1 phenotype. However, to maintain a mild but persisting infection, RSV also induces alveolar macrophages to produce IL-4 and IL-13 that contribute to macrophage M2 polarization and disease resolution through IL-4R α /STAT6-, TLR4-, and IFN- β -dependent signaling pathways (45).

Although under certain conditions like parasite infections and allergy, the functional phenotypes of macrophages *in vivo* largely mirror those of canonical M1 and M2-polarized states, macrophage populations often express mixed phenotypes in the course of various disease settings. Indeed, macrophages with combinations of M1 and M2 markers can be found in neurodegenerative disorders (108), atherosclerotic plaques (109), and some

murine tumors (110). Therefore, the contribution of coexisting macrophages with different phenotypes, the impact of dynamic changes of macrophage plasticity on diseases, and the molecular networks orchestrating the switch of macrophage phenotype are required to be analyzed for a full understanding of the M1–M2 paradigm of macrophage polarization.

FUTURE DIRECTIONS

Tremendous progress has been made in defining the molecular networks underlying M1–M2-polarized activation of macrophages. Molecular determinants of M1–M2 polarization include members of the PPAR, KLF, IRF, STAT, NF- κ B, and HIF families, and miRNAs. However, new molecules that regulate macrophage M1–M2 polarization may still remain unidentified. A novel class of large intergenic non-coding RNAs, termed as lincRNAs, has been recently shown to be involved in both activation and repression of immune response genes (111). Among thousands of lincRNAs identified in the mammalian genome, 159 lincRNAs was found to be differentially expressed following innate activation of THP1 macrophages (112). In these differentially expressed lincRNAs, linc1992 was found to specifically bind to heterogenous nuclear ribonucleoprotein L (hnRNPL) and form a linc1992–hnRNPL complex that regulates TNF α gene transcription. The role of lincRNAs in modulation of macrophage polarization, however, has not been reported so far but certainly needs to be further studied.

Different from the irreversible phenotypic changes seen in lymphocytes after exposure to polarizing cytokines, macrophage polarization is transient and plastic. In order to adapt to the microenvironmental conditions of surrounding tissues, macrophages can rapidly switch their phenotypes. For example, M2 macrophages can be re-polarized into macrophages with M1 phenotype following exposure to TLR ligands or IFNy or overexpression of miR-155 (113, 114), whereas M1 macrophages can be reprogramed to express various genes of M2 macrophage by treating macrophages with reagents that increase IL-10 level (115, 116). Therefore, further exploring the dynamic process of macrophage polarization and the mechanisms that govern this process not only is important for our understanding of the M1–M2 paradigm of macrophage polarization but also provides new therapeutic strategies for various diseases including cancers via targeting imbalances of macrophage polarization.

ACKNOWLEDGMENTS

This work was supported by grants from the National Basic Research Program of China (973 Program, 2012CB517603 and 2011CB504803), the National Natural Science Foundation of China (No. 30988003, 30225037, 30471991, 30570731), and the Natural Science Foundation of Jiangsu Province (No. BK2011013).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 14 August 2014; accepted: 14 November 2014; published online: 28 November 2014.

*Citation: Wang N, Liang H and Zen K (2014) Molecular mechanisms that influence the macrophage M1–M2 polarization balance. *Front. Immunol.* **5**:614. doi:10.3389/fimmu.2014.00614*

This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology.

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SOCS proteins in macrophage polarization and function

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Keywords: suppressor of cytokine signaling proteins, macrophage, M1, M2, inflammation

INTRODUCTION

Macrophages were initially described as “big eaters” due to their phagocytic nature. It is now clear that macrophages have many diverse functions not only in innate immunity and tissue homeostasis but also in metabolism, development, and regeneration. Macrophage functions are driven largely by tissue-derived and pathogenic microenvironmental stimuli that help them adapt to changing conditions within tissues and tailor an appropriate response. The heterogeneity of macrophages has resulted in their classification into subtypes based on their phenotype and function (1). One major classification, based on function, is M1 and M2 macrophages, with destructive and healing properties, respectively (2, 3). As imbalances between M1 and M2 states have been observed in a number of diseases, an understanding of the molecular mechanisms, signaling pathways, and transcription factors controlling their polarization has obvious therapeutic implications. Recent studies have established strong potential for suppressor of cytokine signaling (SOCS) proteins to regulate M1 and M2 macrophage polarization (4–7). Here, the focus will be on the evidence for this, and the consequences of altered SOCS expressions on macrophage function in health and disease. Overall it is proposed that a high SOCS1 to SOCS3 ratio could be a potential marker for M2 macrophages while high SOCS3 expression is associated with M1 cells.

SOCS PROTEINS

Suppressor of cytokine signaling proteins are a family of intracellular cytokine-inducible proteins, consisting of eight members (CIS and SOCS1–SOCS7) (8, 9).

SOCS1 and SOCS3 are most widely characterized regarding their roles in shaping M1 and M2 macrophage polarization (4–6). They show low expression in resting macrophages, but are rapidly induced on activation. All SOCS family proteins contain an Src homology 2 (SH2) domain, a variable length amino-terminal domain and a conserved carboxy-terminal SOCS box motif that interacts with ubiquitin-ligase machinery (8, 9). SOCS are induced by a variety of stimuli that cause M1 and M2 activation, including cytokines, toll-like receptor (TLR) ligands, angiotensin II, immune complexes, and high glucose (9). The most studied signaling pathway regulated by SOCS is JAK/STAT activation. SOCS negatively regulate JAK/STAT signaling through association with key phosphorylated tyrosine residues on JAK proteins and/or cytokine receptors, and by degradation of signaling molecules mediated via the ubiquitin-proteasome pathway (8, 9). SOCS1 and SOCS3 contain a kinase inhibitory region (KIR) that directly suppresses JAK tyrosine kinase activity. SOCS proteins also influence ERK (10), PI3K (11), Notch (12), MAPK (13), and NF-κB (14) signaling cascades that directs M1 and M2 functions.

SOCS1

SOCS1 regulates M1-macrophage activation by inhibiting the interferon gamma-induced JAK2/STAT1 pathway and TLR/NF-κB signaling (9, 15) (Figure 1). To suppress the latter pathway, SOCS1 binds to the p65 subunit of NF-κB and the TLR adaptor molecule Mal/TIRAP as well as IRAK, facilitating its ubiquitin-mediated proteolysis via ubiquitin ligases recruited by the SOCS box (8, 14–17). SOCS1 indirectly inhibits TLR4 signaling through

secondary mechanisms targeting IRF3 and IFN- β induced JAK/STAT pathways (18, 19). Thus, SOCS1 mediates a negative feedback mechanism during TLR4 signaling, via control of both MyD88-dependent and MyD88-independent signaling. SOCS1-deficient mice succumb to severe systemic autoimmune and inflammatory disease (14, 16) and their M1-macrophages display an increased capacity to kill intracellular bacterial pathogens, presumably due to unrestrained IFN- γ /STAT1 and p65 signaling. In line with this, SOCS1 knockout or knockdown M1-activated macrophages show enhanced levels of IL-6, IL-12, MHC class II, and nitric oxide suggesting SOCS1 sustains the properties of M1 macrophages at a less destructive level to prevent overshooting inflammatory responses (4, 18). This explains why SOCS1 promoter hypermethylation, which results in loss of SOCS1 expression leads to enhanced secretion of lipopolysaccharide (LPS)-induced pro-inflammatory cytokines (20). Micro RNA-155 (miR-155) is a critical regulator of innate immunity and TLR signaling (21–23); miR-155 targets and degrades SOCS1 in M1-activated macrophages (21), thus miR-155 induction during activation serves to maximize and extend the inflammatory process.

SOCS1 also regulates M2 macrophage polarization. Expression of macrophage SOCS1, but not SOCS3, is strongly upregulated in an M2 polarizing environment *in vitro* and *in vivo*, where it has an important role in acquisition of M2 functional characteristics, such as a high arginase I/low inducible nitric oxide synthase (iNOS) expression ratio (4). Strikingly, this contrasts with macrophages infiltrating an *in vivo* inflamed M1-activating environment, where macrophages with

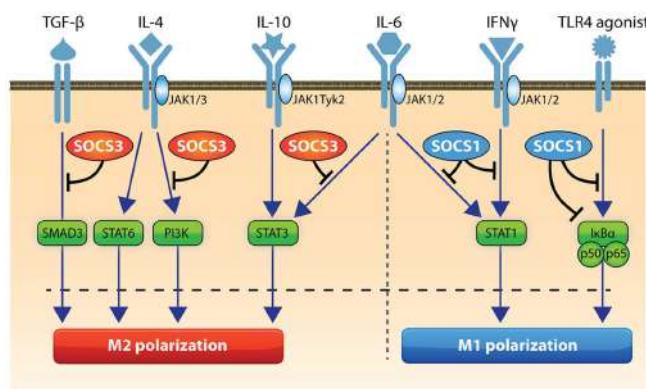


FIGURE 1 | Role of SOCS1 and SOCS3 in macrophage activation. STAT1 and NF κ B drive M1 polarization and SOCS1 can inhibit these pathways. SOCS3 can regulate TLR signaling and inhibits IL-6-induced STAT3 activation and SMAD3 and PI3K activity to action an appropriate destructive effect. STAT3, STAT6, and PI3K can drive M2 activation and SOCS3 inhibits STAT3 and PI3K. Pathways that trigger SOCS1 in macrophages include STAT1 and NF κ B, while SOCS3 expression can be induced by STAT3, NF κ B, NOTCH1, PI3K, and MAP kinase activation.

enhanced SOCS3 but not SOCS1 expression are prominent (5). This suggests that exclusive upregulation of SOCS1, or indeed, a high SOCS1/SOCS3 expression ratio, has potential as a useful and additional *in vivo* biomarker for M2 (see later). Arginase I expression, as an M2 macrophage marker, can be mediated via activation of either STAT6 (24) or PI3K (25). SOCS1 is important in controlling PI3K activity, supporting a mechanism for regulating arginase I expression in M2 cells; SOCS1 also regulates STAT6 phosphorylation (26). Following activation, SOCS1 knockdown or SOCS1-deficient macrophages show a reciprocal upregulation of SOCS3 expression. SOCS3 inhibits PI3K activation (27), and so the expression of high SOCS1 and low SOCS3 in M2 macrophages could result in greater PI3K activity and more arginase I induction in these cells. An elevated expression of SOCS1 is important for the arginase I-induced suppressive nature of M2 macrophages that attenuate lymphocyte proliferation (28). Moreover, siRNA-mediated knockdown of SOCS1 results in the induction of iNOS in IL-4-pretreated cells stimulated with IFN/LPS (4). Thus, SOCS1 regulates the iNOS/arginase I expression ratio in both M1 and M2 macrophages and helps fine-tune key signaling pathways to mount an appropriate response to changes within the microenvironment.

SOCS2

An important role for SOCS2 in driving M2 polarization and limiting M1 polarization has been shown, with IL-4 activation of macrophages, resulting in enhanced SOCS2 expression (27). Macrophages from SOCS2^{-/-} mice display increased secretion of IFN- γ , IL-1 β , and TNF- α in response to LPS in parallel to an increased pro-inflammatory cytokine mRNA expression (29). These BMDMs have higher basal levels of p65-NF- κ B compared with macrophages from wild-type mice (29). In another study, SOCS2-deficient macrophages were hyper responsive to IFN- γ , produced more NO and dealt with infection more efficiently (30). SOCS2 has also been described as a feedback inhibitor of TLR-induced activation in dendritic cells (31).

SOCS3

In contrast with SOCS2, a key role for SOCS3 in M1 polarization is proposed (Figure 1). The majority of macrophages activated within an *in vivo* pro-inflammatory conditioning environment show strong upregulation of SOCS3 expression and this cell population co-express the M1 marker, iNOS (5, 6). Without SOCS3, both human and rodent macrophages have a reduced ability to develop pro-inflammatory features but instead display immunoregulatory characteristics (5, 6). Notably, mice

with a targeted deletion of SOCS3 in macrophages and neutrophils demonstrate a reduced IL-12 response and succumb to toxoplasmosis (32). SOCS3 binds to and inhibits gp130-related cytokine receptors and consequently this abrogates IL-6-induced STAT1 gene expression and IL-6-induced STAT3 anti-inflammatory effects (33–35). Therefore, in SOCS3-deficient macrophages, IL-6 signals in a similar manner to the immunosuppressive cytokine IL-10, through prolonged STAT3 activation and dampening of LPS signaling (33). As a result, mice deficient in SOCS3 in myeloid cells are resistant to endotoxic shock (35) with reduced production of pro-inflammatory cytokines. However, one report in the same mice suggests SOCS3 deficiency promotes M1 macrophage activation in spite of enhanced STAT3 activation (7). The reasons for this discrepancy in findings are unclear but could relate to differences in dose and purity of the LPS used in the different studies, as well as and the genes and time-points analyzed after macrophage activation (7, 35). Moreover, the conflicting results for the role of SOCS3 in M1 polarization in isolated macrophages *in vitro* (5–7) could result from the different technologies and species used (siRNA-mediated knockdown in rat and human macrophages, which avoids the risk of compensatory effects of other SOCS genes (5, 6) versus cells from macrophage-specific SOCS3 knockout mice) (7). Resolving these issues should establish the importance of SOCS3 in modulating macrophage function *in vivo*.

Studies of SOCS3-deficient macrophages confirm that SOCS3 positively regulates TLR4 signaling and M1 activation by inhibition of IL-6R-mediated STAT3 activation, as well as TGF- β -mediated SMAD3 activation, which is critical for the negative regulation of TLR-induced TNF- α and IL-6 production (5, 6, 33, 36). Since SOCS3 blocks PI3K that feeds and inhibits TLR responses, this could be an alternative mechanism by which SOCS3 augments TLR signaling in M1 macrophages (6). Forced activation of Notch signaling enhances both M1 polarization and anti-tumor activity via SOCS3 induction (12). In line with this, macrophage-specific SOCS3 knockout animals are resistant to tumor transplantation due to reduced secretion

of tumor-promoting TNF- α and IL-6, together with elevated MCP2/CCL8 that is anti-tumorigenic (37).

Regulation of SOCS3 in innate cells influences downstream T cell fates. The presence of SOCS3 in macrophages is important in fine-tuning downstream T effector cell priming due to both influences in expression of presenting molecules and altered secretion of T cell polarizing cytokines (6, 7). Mouse SOCS3-deficient dendritic cells display an analogous reduced potential to drive T effector cell responses and a tolerogenic phenotype as a result of enhanced TGF β production and expansion of Foxp3-positive regulatory T cells (38). These dendritic cells reduce the severity of experimental autoimmune disease. Therefore, regulation of intracellular signaling pathways by SOCS3 in innate cells is critical for the decision of adaptive responses such as T cell fates. The depletion of macrophage SOCS3 in a clinical situation would thus be predicted to dampen both pro-inflammatory innate and adaptive immune responses.

The above studies suggest that macrophage SOCS3 is associated with M1 macrophages and pro-inflammatory responses and is a potential therapeutic target in inflammatory diseases. However, a word of caution should be introduced as this may not be the case in all inflammatory conditions. In diseases, where STAT3 activation exerts a profound inflammatory and pathogenic response (39, 40) then the effects of SOCS3 targeting may not be beneficial. For example, in an IL-1/STAT3 model of chronic arthritis where SOCS3 was deleted in hematopoietic and endothelial cells, animals exhibited more severe disease. Thus, the pathology needs first to be assessed before SOCS3 manipulation as a therapy is considered (37).

MACROPHAGE SOCS EXPRESSION AND PATHOLOGY

The heightened expression of macrophage SOCS1 and SOCS3 proteins have been demonstrated in many pathologies *in vivo* where this has been proposed, through the molecular mechanisms described above, to enhance or inhibit pathogenesis.

SOCS AND GLOMERULONEPHRITIS

Macrophages are an important feature in glomerulonephritis pathology.

Macrophages infiltrating inflamed glomeruli in experimental models are rapidly polarized to express either SOCS1 or SOCS3, but rarely both, with most exclusively expressing SOCS3 (5, 6). The proportion of these SOCS3-expressing macrophages correlates strongly with the severity of immune-mediated injury. Local delivery of IL-4 to inflamed glomeruli has a major effect on reducing the number of SOCS3-expressing glomerular macrophages, and this is reflected by a decrease in the severity of nephritis, supporting a role for SOCS3 in driving M1-mediated injury (5).

SOCS AND ATHEROSCLEROSIS

Human atherosclerotic plaques exhibit a high expression of macrophage SOCS1 and SOCS3 in unstable inflammatory shoulder regions as compared to stable fibrous area (41). SOCS1 and SOCS3 expression is increased in aortic lesion macrophages from apoE(−/−) mice (42). In human tissue, the percentages of SOCS1-positive, M2 macrophages are decreased in morphologically stable atherosclerotic plaques, whereas percentages of SOCS3-positive, iNOS positive, macrophages are increased in unstable, rupture-prone plaques, suggesting targeting macrophage SOCS3 would be beneficial to dampen inflammation and plaque vulnerability (43). The differing expression ratio of SOCS1:SOCS3 in atherosclerotic plaques again suggests that the ratio could be an indicator of the inflammatory status of human macrophages *in vivo*. SOCS1 was atheroprotective in mouse models (44) while the absence of macrophage SOCS3 of apoE(−/−) mice attenuates disease, confirming a causal link between macrophage SOCS3 and atherosclerosis (45).

SOCS AND INFLAMMATORY BOWEL DISEASE

Beneath the gut epithelia, lamina propria macrophages phagocytose bacteria and maintain an M2 phenotype in the steady state. Approximately 10% of these macrophages express SOCS3 in healthy individuals, whereas in inflammatory bowel disease (IBD) patients this increases to 40%, again suggesting SOCS3 expression relates to M1-activated macrophages (46). Peroxisome proliferator-activated receptor- γ (PPAR γ) agonists demonstrate efficacy in ameliorating intestinal

inflammation associated with IBD. PPAR γ expression is upregulated in M2 but not M1 macrophages. In macrophages lacking PPAR γ , a significant upregulation of SOCS3 was noted and this could be important if treating IBD with PPAR γ agonists (47).

SOCS AND TUMORS

In human tumors, SOCS3 expression identifies macrophages with enhanced tumor killing, whereas SOCS1 expressing macrophages (M2) favor tumor survival (48). Macrophage-specific deletion of SOCS1 leads to reduced susceptibility to melanoma growth and colon carcinogenesis through increased anti-tumor responses (49) and a switch to M1 polarization of tumor-associated macrophage. In contrast, mice with a macrophage-specific deletion of SOCS3, subcutaneously implanted with melanoma cells, did not show a difference in tumor size, although the number of metastasis increased in these mice (37). These SOCS3-deficient macrophages produce less IL-6 and TNF- α upon stimulation with tumor lysates due to aberrant STAT3 activity, again showing a positive link of SOCS and macrophage polarization (37).

SOCS AND OBESITY

SOCS3 restrains macrophage responses to IL-6 and leptin that are systemically upregulated in obesity (50). SOCS1 inhibits insulin signaling and macrophage cytokine secretion, resulting in insulin sensitivity in spite of an obese state (17). Moreover, an increase in SOCS1 expression in mouse macrophages inhibits LPS- and palmitate-induced TLR4 signaling and in so doing prevents systemic inflammation and hepatic insulin resistance (17).

CONCLUSION AND PERSPECTIVES

Given the broad role of SOCS in regulating macrophage functions in health and disease, the modulation of macrophage-specific SOCS1 and SOCS3 expression provides new opportunities for therapeutic manipulation of immune and inflammatory responses. However, it is not only macrophages that are affected by SOCS proteins. Other cell types upregulate and react to SOCS proteins to shape cellular functions. Targeting SOCS specifically in macrophages is therefore important as an

efficient means of changing the inflammatory response.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 04 June 2014; paper pending published: 26 June 2014; accepted: 12 July 2014; published online: 28 July 2014.

Citation: Wilson HM (2014) SOCS proteins in macrophage polarization and function. *Front. Immunol.* **5**:357. doi: 10.3389/fimmu.2014.00357

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Myeloid colony-stimulating factors as regulators of macrophage polarization

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The scope of functional heterogeneity in macrophages has been defined by two polarized end states known as M1 and M2, which exhibit the proinflammatory activities necessary for host defense and the tissue repair activities required for restoration of homeostasis, respectively. Macrophage populations in different tissue locations exist in distinct phenotypic states across this M1/M2 spectrum and the development and abundance of individual subsets result from the local and systemic action of myeloid colony-stimulating factors (CSFs) including M-CSF and GM-CSF. These factors have relatively non-overlapping roles in the differentiation and maintenance of specific macrophage subsets. Furthermore, there is now evidence that CSFs may also regulate macrophage phenotype during challenge. Cell culture studies from multiple laboratories demonstrate that macrophages developed in the presence of GM-CSF exhibit amplified response to M1 polarizing stimuli while M-CSF potentiates responses to M2 stimuli. As a consequence, these factors can be important determinants of the magnitude and duration of both acute and chronic inflammatory pathology and may, therefore, be potential targets for therapeutic manipulation in specific human disease settings.

Keywords: macrophage activation, macrophage colony-stimulating factor, granulocyte macrophage colony-stimulating factor, inflammation, cytokines

THE MACROPHAGE POLARIZATION PARADIGM: CLASSICAL (M1) AND ALTERNATIVE (M2) ACTIVATION PHENOTYPES

The mononuclear phagocyte system is known to exhibit substantial functional heterogeneity (1–3). Though the scope of such heterogeneity is dramatically large, the prevailing concept of heterogeneity is organized around two polarized endpoints known as classical and alternative activation, also often termed M1 and M2, respectively (4–6). These polarized states represent the capacities to initiate inflammatory response, carry out anti-microbial function, and promote Th1/Th17 adaptive immune responses for M1, or, for M2, to phagocytize debris, promote wound healing, antagonize destructive inflammation, and suppress adaptive immunity. These functional activities are mediated by molecular features that include, for M1, the production of proinflammatory cytokines, co-stimulatory molecules including CD80/86, effector enzymes such as iNOS, and NADPH oxidase or, for M2, anti-inflammatory cytokines, immunosuppressive arginase, and scavenger receptors (CD163) (5, 6).

Importantly, there are multiple subsets of macrophages with relatively non-overlapping functional responsibilities and this additional heterogeneity must be superimposed upon the M1/M2 paradigm (2, 7–9). Resident macrophage populations exhibit many common features but are not identical, often deriving from distinct origins and showing a broad spectrum of functional potential (10). It is noteworthy that most resident tissue macrophages are capable of self-replenishment and generally do not derive from circulating monocytes (9, 11). There are two major categories of monocytes found in the circulation. In the mouse, these are characterized as Ly6C^{hi}/CCR2^{hi} and Ly6C^{lo}/CX3CR1^{hi}.

The former is likely the source of most infiltrating inflammatory macrophages or dendritic cells, while the latter is a patrolling and longer lived cell population that provides maintenance of the vascular endothelium (7). The understanding of macrophage subsets and their developmental origin has advanced dramatically through the application of fate mapping and other transgenic strategies (2, 11–13). Moreover, the molecular features responsible for distinct functional activities (anti-microbial, inflammatory, reparative, etc.) have been defined through detailed analysis of isolated cell populations and cell type-specific transgenic manipulation (5, 6, 14). These macrophage subpopulations exhibit many molecular similarities that reflect their common myeloid origin but each has very distinct responsibilities and their inter-conversion appears to be limited under steady-state conditions (9). Moreover, each of these subset populations can be induced to exhibit M1 or M2 like functional polarization.

The concepts of classical and alternative activation or even M1 and M2 markedly oversimplify the spectrum of macrophage phenotypes that exist within vertebrate organisms (6, 15). The number of individual phenotypes will, in part, depend upon the number of gene products that are measured and the degree to which gene expression events are independent (16). Furthermore, the spectrum of environmental cues encountered by macrophages is highly complex both in number and exposure sequence. Hence, the combinatorial spectrum of possible phenotypes is extremely large. As a simplifying principle, the number of specific molecular endpoints used to define phenotype can be limited to those activities and molecules requisite to the functions of interest (e.g., anti-microbial, reparative, etc.). This will enable comparison of

macrophage populations within different physiologic and pathologic circumstances and evaluation of how the specific molecular characteristics may vary with cell subset and stimulus environment.

Finally, it is also important to recognize that inflammation is a dynamic process that is well recognized to proceed in stages with an early proinflammatory function followed by a transition to healing and restoration of tissue homeostasis (1, 17, 18). While this process includes the temporally distinct recruitment of multiple cell populations that can provide different aspects of the evolving functional features, there is also reason to believe that infiltrating inflammatory monocytes may cycle through different states of functional activity that are, at a minimum, reminiscent of the M1 and M2 categories (19, 20).

REGULATION OF POLARIZATION

Classically and alternatively activated (M1 or M2) macrophage phenotypes are defined by the specific molecular characteristics induced in response to prototypic pro- and anti-inflammatory stimuli [e.g., IFN γ , Toll-like receptor (TLR) agonists, IL-4/IL-13, IL-10, etc.] (5, 15, 18). Such molecular correlates are now used with increasing frequency to identify populations of M1 or M2 like macrophages in different physiologic settings. Thus, M1 macrophages express high levels of IL-12 or IL-23, TNF α , IL-1 α/β , chemokines eliciting neutrophil, inflammatory monocyte, and proinflammatory T lymphocyte infiltration (CXCL1-3, 8, CCL2, CXCL9, and CXCL10), CD80/86, CD64, MARCO, iNOS, and reactive oxygen species (hydroxyl radicals and H₂O₂) induced by M1 polarizing stimuli. Correlates of the M2 phenotype include IL-10, TGF β , arginase, YM1, FIZZ1 (in the mouse), the mannose receptor (CD206), and scavenger receptors, such as CD163 induced following exposure to IL-4, IL-13, TGF β , IL-10, and other agents.

In this context, M1 and M2 phenotypes are often presented as relating to the actions of Th1 and Th2 cell subsets, respectively, because of the Th1 and Th2 lymphokine-based modulation of their defining features (15, 21). It is evident, however, that polarized macrophage function existed prior to the development of adaptive immunity in evolution (22, 23). Moreover, mice with T- and B-cell deficiency still possess the potential for polarized function demonstrating that the M1 and M2 concept can exist in the absence of adaptive immunity and its products (4). Nevertheless, the normal vertebrate immune system operates with a full spectrum of immune cell populations and their products clearly influence the macrophage polarization process. Indeed, it is likely that macrophages will encounter both M1 and M2 polarizing stimuli simultaneously within the inflamed tissue microenvironment. The complexity of response to this may reasonably explain the large spectrum of macrophage phenotypes encountered in cell populations *in vivo* and the variability in markers of polarization.

Macrophage populations may also exhibit a predisposition for polarization toward the M1 or M2 phenotype. For example, different strains of mice and rats have been shown to have skewed patterns of activation potential that ultimately correlate with their innate and adaptive immune functionality (4, 24, 25). Furthermore, it is well recognized that macrophages in different anatomic

or physiologic settings exhibit dramatically different capacities for polarization. Importantly, there are agents that, by themselves, do not induce expression of common polarization markers but which can alter response to stimulation with classical M1 or M2 stimuli. Because most, if not all macrophage populations, can respond to either M1 or M2 stimuli, the tone of a response may be set by differential regulation of sensitivity to polarizing stimuli. Hence, agents that promote priming of macrophages for enhanced or diminished response to classical or alternative activation are likely to be important determinants of the character and temporal patterns of macrophage functional change in the course of response to injury and infection.

The myeloid colony-stimulating factors (CSFs) M-CSF and GM-CSF are known to modulate macrophage phenotype (26, 27). While both agents were first identified as inducers of myeloid cell differentiation and proliferation in cultured bone marrow progenitors, many studies illustrate their importance in the magnitude, duration, and character of many forms of inflammatory response (26–30). Though GM-CSF is associated with classical or M1 activation while M-CSF is linked with alternative or M2 activation, neither factor is a potent stimulus of definitive polarization markers, when compared with prototypic polarizing stimuli (e.g., IFN γ , TLRs, IL-4, IL-10, etc.) (31–33). Instead, GM-CSF and M-CSF appear to induce a state in which macrophages are primed for M1 and M2 endpoints, respectively.

MOLECULAR AND CELLULAR PHENOTYPES PRODUCED BY M-CSF AND GM-CSF *IN VITRO*

While receptor signaling mechanisms mediating responses to M-CSF and GM-CSF are appreciated in basic detail (5, 6, 26), understanding of how these agents modulate functional polarization remains obscure. The potential for CSFs to regulate the responses of mature myeloid cell populations is well recognized. For example, the capacity of GM-CSF, but not M-CSF, to generate DCs in culture clearly demonstrated distinct functional roles (34). Verreck et al. initially demonstrated that GM-CSF or M-CSF treatment of myeloid cells in culture was able to selectively alter the magnitude of M1 or M2 polarized phenotypes following appropriate stimulation (31, 32). While GM-CSF cultured macrophages stimulated with LPS ± IFN γ produced large amounts of IL-23 or IL-12 and little IL-10, M-CSF cultured macrophages were unable to generate either IL-12 or IL-23 but did produce significant amounts of IL-10 under the same conditions. GM-CSF-treated cells produced appreciably higher levels of other proinflammatory cytokines including TNF, IL-18, IL-1 β , and IL-6 in comparison to those grown in M-CSF. Furthermore, M-CSF-treated cells were more phagocytic but less competent in antigen presentation when compared with GM-CSF treated cells. For the most part, the growth factor-treated cells did not exhibit the full M1 or M2 phenotypes in otherwise unstimulated state but rather showed polarized sensitivity for corresponding response to IFN γ /TLR signaling. These findings led the authors to conclude that GM-CSF and M-CSF were promoting the development of monocyte-macrophages predisposed to exhibit differential M1 and M2 phenotypes, respectively. These findings were confirmed and extended by Fleetwood et al. using bone marrow derived macrophages from mice cultured in either GM-CSF or M-CSF

(33). Interestingly, though GM-CSF is frequently used to generate DCs from bone marrow progenitors, the cells arising from such cultures more closely resemble macrophages than dendritic cells based upon whole genome profiling (35). Though there are many similarities between human being and mouse macrophages in different polarized states, there also appear to be many differences (14, 35–37). Despite these findings, there remain important questions about the strict relationship between these two factors and the molecular/functional phenotype definitions for M1 and M2. Thus, studies in both mouse and human cells show that the effects of GM-CSF and M-CSF on gene expression do not map exactly with M1 and M2 marker expression, even following stimulation (35, 38, 39).

In the context of the hypothesis that macrophage predisposition is an important determinant of polarized phenotype expression, there are certainly other natural ligands that have the capacity to alter macrophage sensitivity to specific polarization stimuli (40–45). Because inflammatory responses *in vivo* will always occur in a complex stimulus environment, these additional agents are likely to co-operate with or antagonize the actions of the CSFs. PPAR γ , in particular, has been reported to be required for development of alternatively activated macrophages in the context of insulin resistance and metabolic inflammatory disease (46). The tyrosine kinase receptor CD136 (RON, MST1R) can also modulate sensitivity for M2-like activators, in part by altering the sensitivity to TLR stimulation (24, 47). In contrast to these agents, NOTCH and its ligand RBP-J, are reported to promote M1-like responses via alterations in intracellular signaling factors including IRF8 and SOCS3 (40). Indeed, the sensitivity of myeloid cell populations to polarizing stimuli appears to be controlled in part by alterations in the abundance or activity of the signaling pathway components that mediate responses to pro- and anti-inflammatory stimuli (41–44, 48–50). Thus, signaling adaptors, protein kinases, protein phosphatases, and transcription factors including members of the IRF, SOCS, Tec, and KLF families have all been implicated in controlling either M1 or M2 polarization. The mechanisms through which stimulus sensitivity is altered by ligand/receptor pairs, such as the CSFs, as well as others mentioned above remains to be fully elucidated but intracellular signaling factors are likely to be important targets.

ROLE OF M-CSF AND GM-CSF IN MACROPHAGE FUNCTIONAL POLARIZATION *IN VIVO*

M-CSF and GM-CSF have distinct effects on the development and expansion of myeloid cell populations in different anatomic settings (26, 27). Based upon studies of mice with targeted deletion of ligand and/or receptor genes, M-CSF is known to be required for the production and maintenance of many (though not all) tissue macrophage populations (51). In this regard, distinctions between receptor and ligand deficient mice revealed the existence of a second ligand (IL-34), which is now known to be necessary for the maintenance of a subset of tissue macrophage populations (microglia and Langerhans cells) (52). M-CSF and IL-34 function in homeostatic maintenance of tissue-resident macrophage populations through promoting viability and proliferation and both drive predisposition to M2 character (53). While M-CSF

is found in the serum of healthy individuals and can be produced constitutively by epithelia, fibroblasts, endothelial cells, and by macrophages themselves, its expression can be elevated by inflammatory conditions in many cells including macrophages as well as T and B lymphocytes (27). In contrast to M-CSF or IL-34, GM-CSF deficiency has little impact on steady-state tissue macrophage populations with the exception of those found in the lung (26, 27). While GM-CSF is believed to be important for the development of infiltrating inflammatory DCs, recent findings show that M-CSF but not GM-CSF sensitivity is requisite for these cell populations (13). Importantly, GM-CSF, unlike M-CSF, is not detectable in most tissues at rest but expression is frequently induced during inflammatory or immune stimulation in many tissues and cell types (26, 27, 54).

While studies using animals with global or cell-type restricted deficiencies in M-CSF or GM-CSF ligand/receptor function do provide insight into their relative contributions to macrophage phenotypes during inflammatory responses *in vivo*, the interpretation of such experiments should be viewed with caution due to the impact of such deficiencies on development and/or abundance of specific myeloid subsets. These studies are effectively complemented by transiently manipulating M- or GM-CSF levels using specific ligand delivery or ligand/receptor antagonism. Results from multiple studies indicate that both M-CSF and GM-CSF can modulate the magnitude and character of inflammatory response in multiple tissue specific disease models (2, 26, 54–56). These include autoimmune encephalomyelitis (MS), atherosclerosis, arthritis, nephritis, lung inflammation, and cancer. In most cases, however, the mechanisms through which these endpoints are achieved have not been elucidated. Certainly, both factors have the capacity to promote survival and/or expansion of macrophage populations both systemically and in specific tissue locations and the decreased number of macrophages observed with CSF antagonism would likely result in reduced intensity and/or duration of disease (26). Multiple approaches including delivery of M-CSF, antibody-mediated depletion of the ligand, antibody-induced antagonism of receptor, or the use of receptor tyrosine kinase inhibitors have provided evidence supporting both positive and inhibitory roles for M-CSF in inflammatory diseases (26, 55). Interestingly, several recent reports demonstrate that antagonistic targeting of M-CSF can have appreciable benefit in tumor therapy as a consequence of altering the tumor-associated macrophage phenotype from M2 to M1 (49, 57–59).

There are several important considerations that can provide some speculative insight into the role for these CSFs in regulating the nature of macrophage polarization *in vivo*. First are the patterns of CSF expression within tissues both at rest and during inflammatory responses. While the expression of both factors can be amplified during response to various forms of tissue injury, it is apparent that M-CSF is produced constitutively in many tissues and is critical for the maintenance of resident tissue populations throughout the body. GM-CSF, in contrast, is rarely detectable except at times of injury and does not appear to be a critical determinant of macrophage numbers with a few exceptions (i.e., the lung). Second, resident

macrophage populations are generally found to exhibit an M2-like phenotype under resting conditions, consistent with the need to minimize tissue damaging inflammatory reaction (10). This is certainly consistent with the ability of M-CSF to predispose toward an M2 phenotype in cell culture experiments involving both human monocytes and mouse bone marrow-derived macrophages. Third, GM-CSF has been shown to be a critical determinant of inflammatory injury in several model systems (60–62). Particularly, T-cell derived GM-CSF was recently shown to be critical for disease phenotype in experimental autoimmune encephalomyelitis, generated by IL-23 action on Th17 cells (60, 61). In this instance, GM-CSF selectively targeted infiltrating macrophages within the CNS. Finally, several studies examining responses to M-CSF and GM-CSF *in vitro* show that the M1/M2 phenotype can be reversibly modulated by GM-CSF/M-CSF exposure in cell culture and GM-CSF predisposition may be dominant (33, 39, 63) (Hamilton, unpublished). This is also supported by increased bioavailability (half-life) of GM-CSF, which might also contribute to its dominance relative to M-CSF. We would suggest then that M-CSF provides the default condition and will promote an M2 (healing) phenotype both at rest and in the absence of other forms of stimulation. Induced expression of GM-CSF (e.g., during adaptive T-cell driven immune responses) will provide the mechanism for retaining or re-expressing an M1 phenotype when conditions require (e.g., continued infection or injury). It is clear, however, that the specific effects of M-CSF and GM-CSF on macrophage polarization in cell culture models are unlikely to fully predict their effects *in vivo*. This reflects complexities associated with the variable nature of inflammatory injury, stimulus exposure, and distinct features of specific tissue microenvironments.

UNANSWERED QUESTIONS

Macrophage heterogeneity or phenotype polarization is an area of high current interest and the impact of myeloid CSFs on this process *in vivo* is evident but poorly understood. Hence, we pose the following outstanding questions regarding the roles that M-CSF and GM-CSF may play, particularly through modulating sensitivity to M1 and M2 promoting stimuli with the expectation that answers will help to clarify the process and provide insights to therapeutic application. (1) What mechanisms are involved in skewing responses to polarizing stimuli? Can we identify specific CSF-induced patterns of gene expression that are requisite to generating macrophages predisposed for more potent responses to cytokines and pattern recognition receptors? Can we correlate outcomes obtained *in vitro* with those obtained *in vivo*? (2) What are the sources and timing of M-CSF and GM-CSF expression within specific tissues during different forms of inflammatory response? Which myeloid cell populations are the targets of the CSFs and how is this co-ordinated with the need to enhance or diminish specific aspects of function over the full course of inflammatory response? (3) Finally, we must begin to consider not only the mechanisms through which M-CSF and GM-CSF operate but also how these stimuli are integrated with the host of other agents encountered within inflammatory settings that also have marked influence on M1/M2 skewing?

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Citation: Hamilton TA, Zhao C, Pavicic PG Jr and Datta S (2014) Myeloid colony-stimulating factors as regulators of macrophage polarization. *Front. Immunol.* **5**:554. doi: 10.3389/fimmu.2014.00554

This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 29 August 2014; *accepted:* 18 October 2014; *published online:* 21 November 2014.



Regulation of macrophage polarization by RON receptor tyrosine kinase signaling

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The M1 and M2 states of macrophage polarization are the two extremes of a physiologic/phenotypic continuum that is dynamically influenced by environmental signals. The M1/M2 paradigm is an excellent framework to understand and appreciate some of the diverse functions that macrophages perform. Molecular analysis of mouse and human macrophages indicated that they gain M1 and M2-related functions after encountering specific ligands in the tissue environment. In this perspective, I discuss the function of récepteur d'origine nantais (RON) receptor tyrosine kinase in regulating the M2-like state of macrophage activation. Besides decreasing pro-inflammatory cytokine production in response to toll-like receptor-4 activation, macrophage-stimulating protein strongly suppresses nitric oxide synthase and at the same time upregulates arginase, which is the rate limiting enzyme in the ornithine biosynthesis pathway. Interestingly, RON signaling preserved some of the characteristics of the M1 state, while still promoting the hallmarks of M2 polarization. Therefore, therapeutic modulation of RON activity can shift the activation state of macrophages between acute and chronic inflammatory states.

Keywords: macrophages, polarization, RON signaling, tumor promotion, immune therapy

INTRODUCTION

Macrophages perform the essential function of preserving tissue homeostasis following infection or tissue damage in all animals in the absence of B and T cells. Macrophages originate from bone marrow-derived monocytic cells through a process of differentiation, directed by the activation of specific transcription factors (1–3). Circulating monocytes are recruited to tissues, where they differentiate into functionally distinct subsets of cells with distinct phenotypic characteristics. In response to the tissue microenvironment, these cells can either produce pro-inflammatory cytokines to kill the offending foreign pathogen and polarize T-cells to mount an adaptive immune response, or participate in tissue repair by increasing their phagocytic activity and producing growth factors for tissue healing and regeneration. The cellular plasticity is a hallmark of macrophages and the complex signaling pathways that contribute to these biochemical and functional differentiation is beginning to be understood (4, 5).

The two states of macrophage activation, “Classical” (M1) and “Alternative” (M2) occupy two extremes of a phenotypic continuum in which macrophages respond to secreted factors to evoke distinct functional responses (6, 7). These functional responses are regulated by a combination of signaling pathway modulators and transcription factors. As an example, combination of IFN- γ with toll-like receptor (TLR) pathway activation produces a complete M1 phenotype in macrophages mediated by the activation of STAT1 and NF- κ B transcription factors. By contrast, IFN- γ alone causes a partial M1 response mediated by STAT1 transcription factor at sites of infection. Similarly, macrophage M2 phenotype and response are fine-tuned at tissue-specific sites by the activation of distinct sets of chemicals, such as IL-10 in the gut, or IL-4 and fatty acids in the adipose tissues (8, 9).

The activation of macrophages (AAM) into M1- or M2-type is dictated by the cytokine milieu of the tissue microenvironment. Monocytes are primed to differentiate in response to macrophage colony stimulating factor (M-CSF) or by granulocyte macrophage colony stimulating factor (GM-CSF) (2, 3). Further priming is dictated by a balance between IFN- γ and IL-4, the former pushing the macrophages into an M1 state and the latter into an M2 state. The primed macrophages receive additional signals in the form of TLR stimulation to display the full complement of classical and alternative activation functions. Whereas, M1-primed macrophages produce pro-inflammatory cytokines and inducible nitric oxide synthase (iNOS) upon TLR activation, M2-primed macrophages produce arginase, IL-10, and growth factors such as transforming growth factor- β (TGF- β), vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF) among others. Interestingly, these macrophage phenotypes can be reversed and brought back to the state of naïve macrophages by growing them in the absence of any priming factors for a couple of days, indicating reversibility of the response (10, 11).

INVOKING THE M1 PHENOTYPE OF MACROPHAGES

M1 polarization can be evoked by treating naïve macrophages with a combination of IFN- γ and LPS. Macrophages express a variety of microbial pattern recognition receptors (PRRs), such as TLRs that recognize pathogen – or danger associated molecular patterns to clear the offending signal (12, 13). A large number of inflammatory cytokines including TNF- α , IFN- γ IL-12p40, IL-6 are produced rapidly after TLR-4 activation. The delayed TLR-4 response is triggered by the recruitment of TIR-containing adaptor protein (TRIF) and TRIF-related adaptor molecule (TRAM) to induce IFN- β and trigger the interferon response (14). Together,

the NF- κ B, p38 MAPK, and IFN- β pathways regulate the output of the TLR-4 signaling. A key enzyme of arginine metabolism, iNOS metabolizes arginine to make nitric oxide (NO), a potent anti-microbial agent. Production of NO is a hallmark of M1 macrophages and IFN- γ regulates NO production via transcriptional upregulation of iNOS (4, 15).

Built within these signaling circuits are negative feedback loops that circumscribe the intensity and duration of the LPS response. Proteins, such as dual specificity phosphatases (DUSP) and suppressor of cytokine signaling (SOCS) are induced to dephosphorylate upstream activators of the MAPK pathway and inhibit signaling downstream of the interferon receptor, respectively (6, 16). As part of body's defense mechanism, M1 polarization is critical to mount an effective innate immune response against the offending pathogen. However, the body can sustain extensive tissue damage if the pro-inflammatory responses are allowed to persist. Therefore, these multiple feedback loops are turned on downstream of TLR-4 and cytokine-signaling pathways that quickly reduce the output from these pro-inflammatory signaling circuits (17, 18).

INVOKING THE M2 PHENOTYPE OF MACROPHAGES

Tissue resident macrophages assume an M2 phenotype by default. This phenotype, also defined as alternative AAM or M2-type of macrophages can be induced by IL-4 + IL-13 and by other signaling molecules (6, 9). In this state, macrophages metabolize arginine into ornithine by the expression of arginase-1 that diverts arginine from the production of NO and citrulline. (15). The M2 macrophages also produce growth factors and extracellular matrix remodeling enzymes that promote processes related to tissue repair and healing. Additionally, their phagocytic activity is increased to help in clearing tissue debris. The M2 activity is sustained by factors produced by injured tissues such as TGF- β and adenosine (4, 19).

SIGNALING PATHWAYS MEDIATING MACROPHAGE POLARIZATION

The signaling circuitry leading to changes in gene expression pattern during macrophage polarization is complex (16). Different subsets of tissue-specific macrophages are dependent on different signaling pathways to polarize and sustain their polarized state. As an example, c-jun N-terminal kinase pathway (JNK) is required for the adipose tissue-associated macrophages to assume M1-phenotype (20). Polarization of macrophages by the phosphatidyl inositol-3 kinase (PI3K) pathway is mediated by the activation of AKT1 and AKT2 kinases. Genetic ablation experiments revealed that AKT1 and 2 regulate macrophage M1 and M2 phenotype in a reciprocal pattern (21, 22). In the absence of AKT1, macrophages produce pro-inflammatory cytokines resembling the M1 phenotype, whereas in the absence of AKT2 the cells express markers of M2 polarization such as Arg-1, Fizz-1, and IL-10 (21, 23, 24). Interestingly, preliminary data support that this reciprocal regulation of macrophage M1/M2 phenotypes is mediated by a micro-RNA, mir-155, and its target transcriptional regulator CAAT-enhanced binding protein- β play an important role (23). JAK/STAT pathway downstream to IFN- γ is a strong inducer of M1 polarization,

although to reach the full spectrum of the M1-phenotypic state, dual activation of the TLR-4 and IFN- γ pathways are required (25, 26).

Signals that promote M2 polarization are diverse and a variety of molecules from cytokines to growth factors can influence this transition (6). The IL-4/IL-13 combination is a physiological mediator of the M2 state that impinges on the transcription factor STAT-6 to induce cell surface expression of M2 markers and metabolic reprogramming (9). In the absence of IL-4/IL-13, M-CSF and IL-10 can push macrophages to assume an M2 phenotype mediated by the transcription factor STAT-3 and SP-1, respectively. In this state, the macrophages become highly phagocytic, produce growth factors that promote repair of wound or tissue damage, and promote Th-2 immune response. Fc γ receptors in combination with LPS promotes Th-2 response, upregulates antigen presentation, turns off IL-12, and induces IL-10 production by activating the Syk and PI3K pathways that cross-talk with TLR signaling. Finally, glucocorticoids promote macrophage adherence, spreading, phagocytosis, induction of complement proteins, and secretion of IL-10 by directly engaging the macrophage transcription machinery.

Integrating the function of M1 and M2 macrophages in a physiological setting raises several questions:

1. Are the M1 and M2 macrophage states mutually exclusive in a tissue environment?
2. Can a cell transition from one state to the other directly, or are there other intermediate states?
3. Can a macrophage assume characteristics of both M1 and M2 states? How do they arise? Do they represent a fleeting intermediate, or can cells in this state be stabilized?

Answers to these questions are not fully known. However, M1- and M2-polarized macrophages are found as mixed populations in the tissue microenvironment. Depending on the inflammatory stimuli, one state may dominate over the other. Both M1 and M2 states are functionally and phenotypically heterogeneous. The translatability of macrophage M1/M2 polarization in normal homeostasis and in diseases have remained elusive beyond the fact that M1 macrophages favor bacterial and viral elimination, whereas M2 macrophages give protection against helminthes and other parasites and participate in tissue repair. Observations *in vitro* cannot always be readily applied to *in vivo* situations and many of the macrophage responses discussed above are yet to find validation *in vivo* (27).

HOW FUNCTIONAL STATES OF MACROPHAGES ARE MODULATED BY GROWTH FACTOR SIGNALING

So far in our discussion, the macrophage phenotypic and functional states have been described in the context of inflammation or interaction between pathogens and immune cells. However, M2-polarized macrophages also perform essential functions in the resolution of tissue inflammation, remodeling of the tissue microenvironment during wound healing and repair of tissue damage. In the next section, the effect of growth factor signaling on macrophage polarization, and its implication in human cancer is discussed.

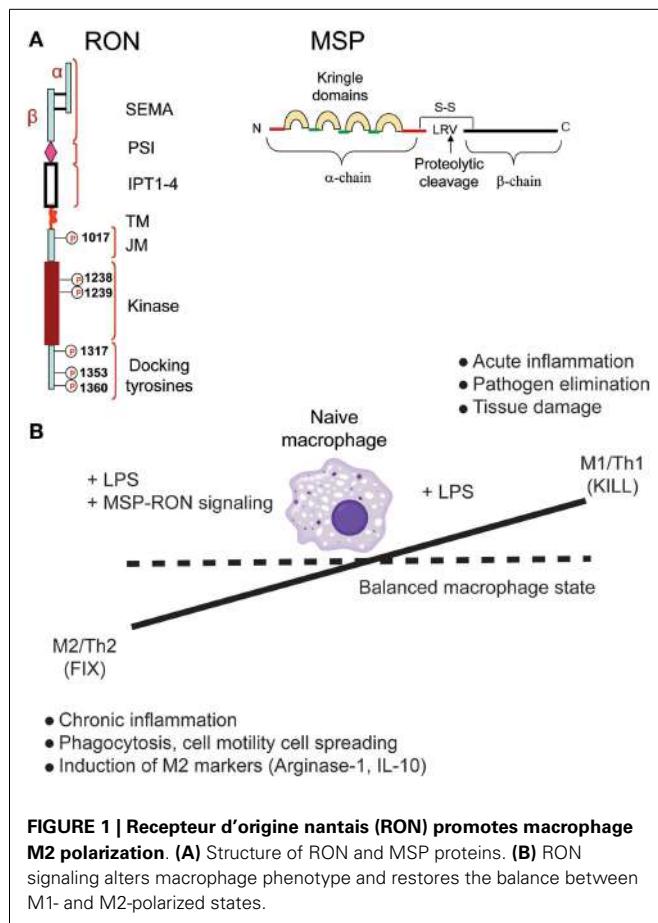


FIGURE 1 | Recepteur d'origine nantais (RON) promotes macrophage M2 polarization. (A) Structure of RON and MSP proteins. **(B)** RON signaling alters macrophage phenotype and restores the balance between M1- and M2-polarized states.

Among the growth factor receptors known to modulate macrophage behavior and function is the receptor tyrosine kinase “recepteur d'origine nantais” (RON) (28, 29). The ligand for RON, macrophage-stimulating protein (MSP) regulates macrophage motility and its phagocytic activity (28). MSP is produced by the liver and circulates in an inactive form in the serum. MSP activation occurs as a result of proteolytic processing of Pro-MSP resulting in active MSP. Several trypsin like proteases such as matriptase, hepsin, and hepatocyte growth factor-A (HGF-A) cleave inactive pro-MSP into an active form (30) (Figure 1A). These proteases are known to be activated at sites of inflammation and can be a source of active MSP that can turn on RON signaling on macrophages and epithelial cells at these sites.

Genetic ablation of RON kinase activity in mice leads to viable and fertile progenies with no apparent developmental defects. However, studies showing that RON knockout (RON-KO) mice are sensitive to LPS challenge suggested that RON signaling negatively regulates downstream effects of TLR-4 activation (31, 32). Further studies by many groups have led to a general model in which RON signaling promotes some of the functional and phenotypic traits of M2-like macrophages, in particular, a strong suppression of pro-inflammatory cytokine production in response to LPS (33, 34) or LPS + IFN- γ (35), suppression of iNOS (36), induction of arginase-1 (37, 38), and expression of scavenger receptors (34). Interestingly, treatment of peritoneal macrophages

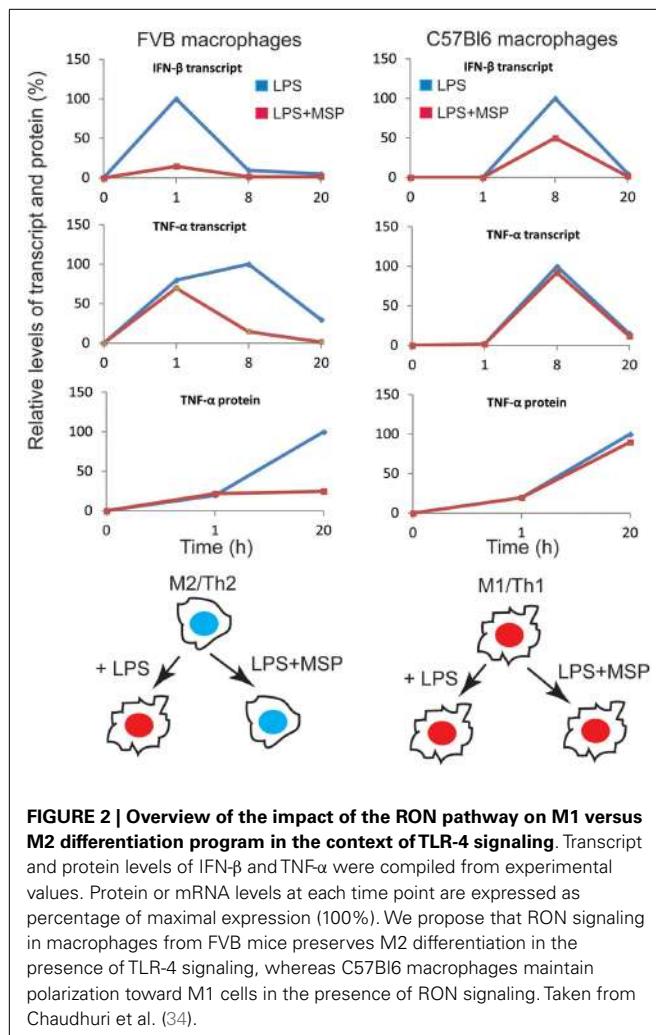
with MSP alone induced activation of MAPK and PI3K pathways, but failed to induce any of the hall marks of M2 polarization such as expression of arginase-1, scavenger receptors, or IL-10 (34). When MSP was combined with LPS stimulation, macrophages exhibited hallmarks of M2-polarized state (Figure 1B). Further, global gene expression analysis and measurement of cytokines in the conditioned media indicated that RON signaling had minimal effect on TLR-4-mediated early NF- κ B activation – the effect being significantly pronounced at later time points (34).

Taken together, these observations support that RON signaling has dual effect on macrophages. On the one hand, it enhances macrophage motility and survival without any input from TLR signaling, but on the other hand, it significantly modifies the TLR-4 signaling output when LPS is present along with MSP. Interestingly, the reprogramming of the TLR-4 signaling circuits by RON is sensitive to mice genetic background (34).

IMPACT OF GENETIC BACKGROUND AND DIFFERENTIAL EFFECT OF RON ON MACROPHAGE POLARIZATION

Studies employing different mice strains have revealed that host genetic background significantly influences the metabolic reprogramming and behavior of macrophages when exposed to LPS or IFN- γ (39). Mills et al. first reported that whereas macrophages from C57Bl/6 mice produced citrulline and nitric oxide (NO) in response to LPS or IFN- γ , those from BALB/c background produced ornithine (40). Both NO and ornithine are the products of differential arginine metabolism mediated by the expression of enzymes iNOS and arginase-1, respectively, and this “fork in the arginine metabolism” is recognized as one of the hallmarks of M1 and M2 polarization (4, 39). Gene expression profiles comparing bone marrow-derived macrophages from different mice strains further revealed that in response to LPS, timing and intensity of expression of genes differed significantly between mice strains (41).

Interestingly, RON signaling modulated the TLR-4 responses of macrophages between C57Bl/6 (M1-polarized) and FVB (M2-polarized) mice differently (34). Whereas MSP strongly suppressed LPS-induced production of pro-inflammatory cytokines in macrophages from FVB background, the effect was minimal in the C57Bl/6 background. A clue to the mechanism came from analyzing the effect of RON signaling on LPS-induced gene expression in macrophages. LPS induced the transcriptional targets of the NF- κ B and MAPK pathways early on, and RON signaling had no effect on most of the early response genes. The late response genes, dominated by the transcriptional targets of interferon signaling, selected NF- κ B target genes, and genes associated with tissue repair and immune tolerance was modulated variably by RON. RON suppressed most of the targets of the interferon pathway (50% of the downregulated genes at the later time point were targets of interferon signaling), as well as few selected targets of the NF- κ B pathway (TNF- α), but enhanced the expression of tissue repair (EGF, PDGF, MMP9) and immune tolerance genes (IL-10, IL-19, CTLA-2A). The kinetics of IFN- β expression in response to TLR-4 activation was rapid (1 h) in macrophages from FVB mice, whereas it was significantly delayed (8 h) in C57Bl/6 background. This early upregulation of IFN- β in FVB mice was blunted by RON signaling resulting in a strong inhibition of the expression of



interferon regulatory factors (IRFs) and target genes of the interferon pathway at later time points. The IFN- β expression however, was minimally affected by RON signaling in C57Bl/6 macrophages partly because of the delayed expression of IFN- β in this strain background, and partly as a result of high expression of TNF- α , IL-12, and IL-6, which by themselves can modulate the expression of IFN- β independently of LPS (34). The final outcome of the interplay between RON and LPS signaling in these two mouse strains resulted in the stabilization of M2-polarized state in FVB mice even in the presence of a strong M1-polarizing signal, but failed to alter the phenotype of macrophages from C57Bl/6 background (**Figure 2**).

In addition to affecting the polarized behavior of macrophages, RON kinase-deficient FVB mice formed less number of tumors, which developed with a delayed kinetics in two models of chemical induced carcinogenesis (34, 42). This inhibitory effect was lost in the C57Bl/6 RON-KO background (34). Additionally, depletion of CD8 $^{+}$ T-cells in a transplantable fibrosarcoma model in FVB mice suppressed the rejection of tumors in the RON-KO background suggesting that lack of RON in the innate immune compartment facilitates generation of a CTL response against

the tumor (34). These observations support the hypothesis that ablation of RON function in the innate immune compartment accentuates tumor-specific T-cell responses.

In the last few years, tumor-associated macrophages have received significant attention due to their pro-tumorigenic properties, such as producing tumor-promoting and pro-angiogenic factors and suppression of the adaptive immune response within the tumor microenvironment (43). Polarization of macrophages into an M2-type is one of the mechanisms that subverts the sentinel function of the innate immune cells and make them pro-tumorigenic.

How can this immune-modulatory property of RON be reconciled with normal tissue homeostasis? Maintaining macrophages in M1/M2-like polarized state is important under certain physiological conditions. For example, during wound healing or during repair of damaged tissues, macrophages serve two important functions. First, it is ready to mount an immune response to eliminate pathogens, if the wound site gets infected, and during the same time limit the intensity and duration of the localized immune response to prevent further tissue damage. Second, it needs to produce growth-promoting and tissue-rebuilding factors to accelerate healing and reverse damage. In this context, the tissue repair or wound healing pathways of macrophages are co-opted by the tumor to promote its own survival against immune attack and activation of RON in the tumor microenvironment may facilitate this conversion.

CONCLUSION AND THERAPEUTIC IMPLICATIONS

M1 and M2 polarization of macrophages is dynamically controlled by changes in the tissue microenvironment. These two functional states participate in two important activities – protection against foreign pathogens and promotion of tissue restoration and healing after injury. Therefore, tight regulation of these two states is critical to the health of the organism (**Figure 1B**). Sustained activation of M1 state can lead to excessive tissue damage as a result of excessive inflammation, whereas prolonged activation of the M2 state can cause chronic inflammation leading to cancer. Therapeutic targeting of certain diseases may involve artificial manipulation of macrophage polarization. As an example, inhibiting RON function in tumor-associated macrophages can restore tumor immunity allowing enhanced efficacy of cancer immunotherapy drugs. Similarly, enhancing RON activity in tissue-associated macrophages can lead to efficient wound healing and restoration of tissue damage. However, such artificial manipulation of immune cell functions has to be tightly controlled to prevent systemic damage to the organism.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 20 August 2014; paper pending published: 22 September 2014; accepted: 13 October 2014; published online: 31 October 2014.

Citation: Chaudhuri A (2014) Regulation of macrophage polarization by RON receptor tyrosine kinase signaling. Front. Immunol. 5:546. doi: 10.3389/fimmu.2014.00546

This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology.

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Complement, C1q, and C1q-related molecules regulate macrophage polarization

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Complement is a critical system of enzymes, regulatory proteins, and receptors that regulates both innate and adaptive immune responses. Natural mutations in complement molecules highlight their requirement in regulation of a variety of human conditions including infectious disease and autoimmunity. As sentinels of the immune system, macrophages are specialized to respond to infectious microbes, as well as normal and altered self, and dictate appropriate immune responses. Complement components such as anaphylatoxins (C3a and C5a) and opsonins [C3b, C1q, mannose binding lectin (MBL)] influence macrophage responses. While anaphylatoxins C3a and C5a trigger inflammasome activation, opsonins such as C1q and related molecules (MBL and adiponectin) downregulate inflammasome activation and inflammation, and upregulate engulfment of apoptotic cells consistent with a pro-resolving or M2 macrophage phenotype. This review summarizes our current understanding of the influence of the complement system on macrophage polarization with an emphasis on C1q and related molecules.

Keywords: macrophage, complement, C1q, adiponectin, inflammasome, cytokine, phagocytosis, efferocytosis

COMPLEMENT SYSTEM

The complement system comprises over 35 cell associated and soluble molecules, which play a critical role in our innate immune response. Activation of complement begins with a recognition step. Recognition proteins of the complement system include C1q, mannose binding lectin (MBL), and ficolins (ficolin-1, -2, -3). These proteins are innate pattern recognition receptors (PRRs) and are capable of recognizing a wide range of structures including foreign organisms, either via binding directly to their pathogen associated molecular patterns (PAMPs) or when coated with antibody in an immune-complex (C1q) [reviewed in Ref. (1)]. In addition, these PRRs also recognize and bind to structures associated with cellular damage/debris such as apoptotic cell associated molecular patterns (ACAMPs) like phosphatidylserine, damage associated molecular patterns (DAMPs) like oxidation neo-epitopes, and fibrillar protein structures (2–8). Activation of complement via the classical pathway (C1q), lectin pathway (MBL/ficolins), or alternative pathway (C3 “tickover” autoactivation/properdin) begins a coordinated cascade of enzymatic cleavage events generating complement protein fragments that carry out effector functions. These include opsonization for enhanced phagocytosis, either directly or via production of C3b, triggering inflammation through production of anaphylatoxins C3a and C5a, and lysis of target cells through deposition of C5b-9, the membrane attack complex (MAC), in the complement terminal pathway (Figure 1).

COMPLEMENT DEFICIENCIES IN HUMAN DISEASE

Genetic deficiencies in complement components highlight its important role not only in clearance of pathogens but also in removal of dying cells/cellular debris and prevention of autoimmunity. For example, genetic deficiencies in lectin, alternative and

terminal pathway components like MBL, factor D, properdin, C3, C5, C6, C7, C8, and C9, increase susceptibility to infections, in particular, by encapsulated bacteria [reviewed in Ref. (9)]. The strong link between late complement component deficiencies (C5–9) and recurrent neisserial infections indicates a critical role for C5b–9 MAC deposition in direct bactericidal defense. However, deficiency in early classical pathway component C1q is strongly associated with development of the autoimmune disease systemic lupus erythematosus (SLE), likely due to impaired opsonophagocytosis, and compromised removal of immune complexes and apoptotic cells [reviewed in Ref. (10)]. Weaker associations with SLE are also seen with deficiencies in other classical pathway components C1r/s, C2, C4, and C3. Thus, complement opsonization via C1q recognition plays a critical role in maintaining normal tissue homeostasis and prevention of autoimmunity. While excessive or inappropriate complement activation is associated with almost all inflammatory or inflammation-related diseases including cancer, Alzheimer's disease (AD), and metabolic disease [reviewed in Ref. (11, 12)], associations of these diseases with (generally very rare) complement deficiencies in humans have not been reported. However, polymorphisms in MBL2 are common, and provide extensive evidence for links between MBL levels and cardiovascular disease [reviewed in Ref. (13)].

COMPLEMENT RECEPTORS ON MACROPHAGES

Macrophages are key players in carrying out complement effector functions. Complement components including C1q, MBL, C3b, C4b, C3a, and C5a interact with a variety of receptors on macrophages, leading to modulation of cytokine production/inflammatory responses and increased opsonophagocytic clearance of targets. For example, monocytes and macrophages

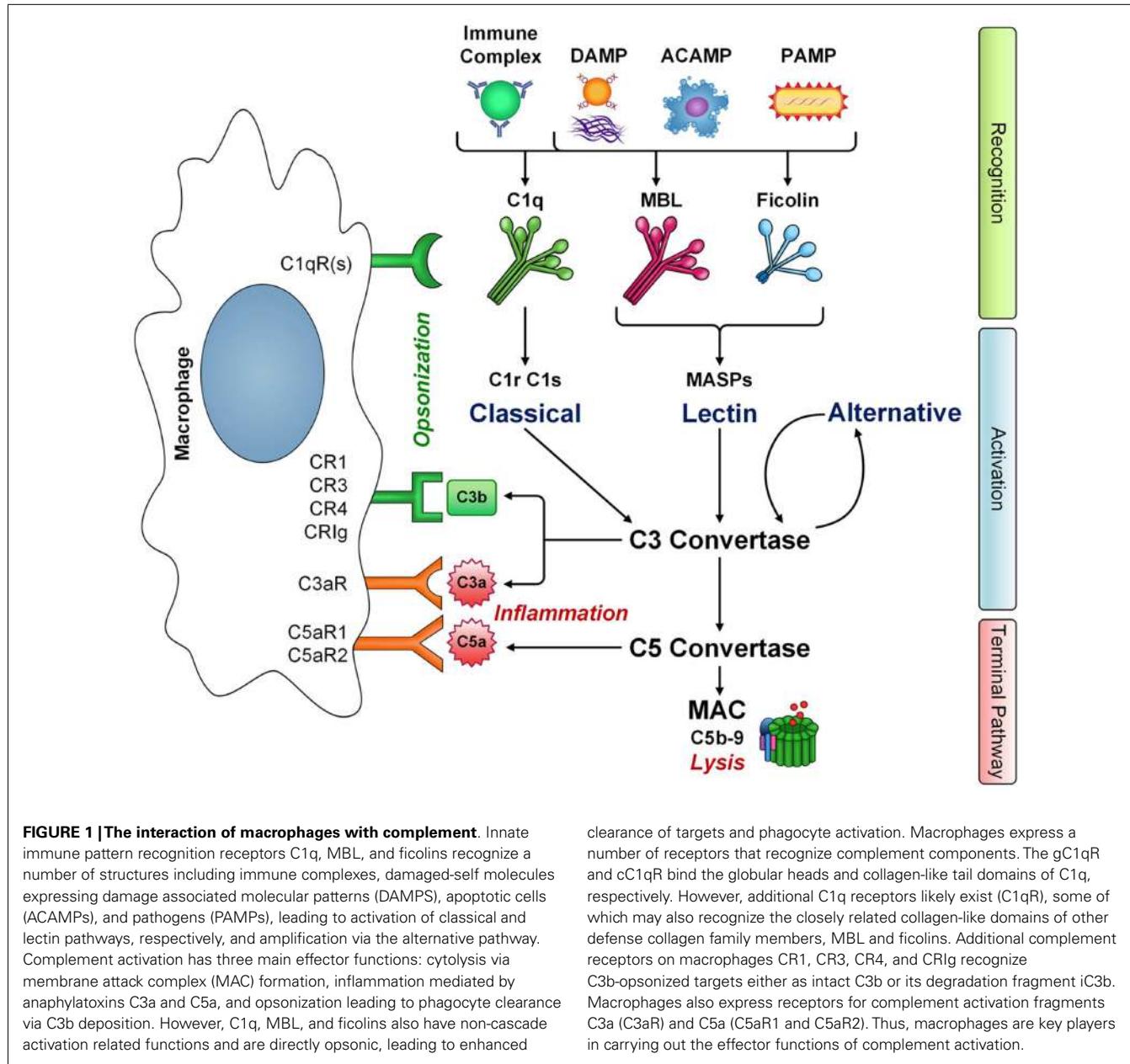


FIGURE 1 | The interaction of macrophages with complement. Innate immune pattern recognition receptors C1q, MBL, and ficolins recognize a number of structures including immune complexes, damaged-self molecules expressing damage associated molecular patterns (DAMPs), apoptotic cells (ACAMPs), and pathogens (PAMPs), leading to activation of classical and lectin pathways, respectively, and amplification via the alternative pathway. Complement activation has three main effector functions: cytosis via membrane attack complex (MAC) formation, inflammation mediated by anaphylatoxins C3a and C5a, and opsonization leading to phagocyte clearance via C3b deposition. However, C1q, MBL, and ficolins also have non-cascade activation related functions and are directly opsonic, leading to enhanced

clearance of targets and phagocyte activation. Macrophages express a number of receptors that recognize complement components. The gC1qR and cC1qR bind the globular heads and collagen-like tail domains of C1q, respectively. However, additional C1q receptors likely exist (C1qR), some of which may also recognize the closely related collagen-like domains of other defense collagen family members, MBL and ficolins. Additional complement receptors on macrophages CR1, CR3, CR4, and CRlg recognize C3b-opsonized targets either as intact C3b or its degradation fragment iC3b. Macrophages also express receptors for complement activation fragments C3a (C3aR) and C5a (C5aR1 and C5aR2). Thus, macrophages are key players in carrying out the effector functions of complement activation.

express complement receptors CR1 (CD35), CR3 (CD11b/CD18), and CR4 (CD11c/CD18). CR1 binds complement opsonins C1q, C3b, and C4b, which are deposited on target cells/surfaces (14) and reviewed in Ref. (15). Binding to CR1 promotes phagocytosis of targets, along with degradation of C3b to its inactive fragment iC3b, preventing C5-convertase activity and thus inhibiting the complement terminal pathway. CR3 and CR4 bind iC3b and promote the phagocytosis of targets. Genetic deficiencies in these receptors are also a risk factor for the development of SLE [reviewed in Ref. (10, 16)]. A subset of tissue macrophages expresses the complement receptor CRlg. Gene expression of this receptor is associated with activated macrophages (17), and the protein is found in human liver Kupffer cells and in subsets of various resident tissue macrophages including alveolar and

synovial macrophages (18). CRlg binds to C3b, and its degradation product, iC3b, and was shown to be important in clearance of C3b-opsonized pathogens from the circulation.

Beyond CR1, macrophages express other receptors capable of binding C1q. These include the ubiquitously co-expressed molecules gC1qR and cC1qR. gC1qR binds to the globular heads of C1q (19), whereas cC1qR (calreticulin) interacts with both the collagen-like tail and globular head domains of C1q (4). C1q opsonized targets are internalized more rapidly than in the absence of C1q via interaction of the collagen-like tail with a receptor on phagocytes (20). In addition, C1q bound to a variety of targets modulates macrophage inflammatory responses via its collagen-like domain (5, 21). The collagen-like domain of C1q is a feature shared with other so-called “defense collagens.” This family of

molecules includes MBL and ficolins, which also trigger enhanced phagocytosis [reviewed in Ref. (1)] and modulate cytokine production (22). While the phagocytic receptor is not definitively identified for all targets, involvement of the cC1qR has been implicated in the C1q- and MBL-mediated removal of apoptotic cells by macrophages (6). An additional family of proteins, termed C1q/TNF-related proteins (CTRs), contains C-terminal globular domains homologous to C1q (23, 24). Adiponectin is perhaps the best characterized member of the CTRP family, and interacts with macrophages, regulating inflammatory responses similar to C1q (25). However, the receptors for the metabolic actions of adiponectin were shown to be distinct from the C1qR, and include AdipoR1 and AdipoR2 (26).

COMPLEMENT ANAPHYLATOXINS

Complement anaphylatoxins C3a and C5a are soluble complement fragments produced from C3 or C5 by activation of the C3- or C5-convertase enzyme complex, respectively. They carry out their biological functions via interactions with three receptors. These include the C3a receptor (C3aR) (27), which binds C3a but not its degradation product C3a-desArg, the C5a receptor (C5aR1) (28), which binds C5a, and C5a receptor-like 2 (C5aR2, C5L2) (29), which binds C5a but has greater affinity for C5a-desArg. C5aR2 lacks signaling capabilities (30), and thus, is considered a decoy receptor, capable of sequestering the bioavailability of C5a/C5a-desArg and limiting their ability to activate via the C5aR1. C5aR2 may also bind C3a-desArg, but this is controversial (31). Cellular expression of the anaphylatoxin receptors is widespread but particularly includes immune cells like monocytes and macrophages (32, 33). Interestingly, LPS, associated with M1 macrophage polarization increases gene expression of C5aR1 in macrophages (34) while IL-4, associated with M2 macrophage polarization downregulates C5aR1 expression (35).

MACROPHAGE POLARIZATION

Macrophages are grouped as M1 and M2 in accordance with the Th1/Th2 nomenclature, and this terminology describes two macrophage phenotypes: the pro-inflammatory/classically activated macrophage (M1) and the pro-resolving/alternatively activated macrophage (M2). The expression of a variety of genes has been associated with macrophage polarization, most notably, the machinery required for enzymatic breakdown of arginine in pro-resolving and pro-inflammatory macrophages. By expressing arginase, pro-resolving macrophages generate ornithine, which promotes proliferation and repair, whereas pro-inflammatory (M1) macrophages express inducible nitric oxide synthase and generate nitric oxide (NO), an important molecule in host defense against invading pathogens, which also inhibits cell proliferation [reviewed in Ref. (36)]. Intermediates in the two enzymatic pathways act antagonistically, inhibiting the other when they are activated (37). Consistent with a role in promotion of inflammation, M1 macrophages are also often associated with an increased production of pro-inflammatory cytokines such as TNF α and IL-1 β . In contrast, M2 macrophages, which are pro-resolving, are often associated with increased production of anti-inflammatory cytokine IL-10.

Although the idea of macrophages having separate phenotypes is helpful in defining their action, it is also somewhat

misleading and over simplified. *In vivo*, macrophages are constantly encountering various external signals leading to a very fluid existence in terms of phenotypes (38). Even within the same site and population, macrophages can express different and constantly changing phenotypes, termed macrophage plasticity (39). Macrophage polarization is a result of a combination of external signals macrophages receive from their environment. Common signals that have been investigated include the Th1 cytokine interferon- γ (IFN- γ) and various PAMPs, which are associated with M1 polarization and the Th2 cytokines IL-4/IL-13, which are associated with M2 polarization. Here, we will review some recent work illuminating the role of the complement system in regulating macrophage activation and polarization.

COMPLEMENT ANAPHYLATOXINS REGULATE MACROPHAGE ACTIVATION

Complement anaphylatoxins, C3a and C5a, are pro-inflammatory and trigger monocyte and macrophage activation through various signaling mechanisms. For example, upon LPS stimulation in human monocytes, C3a induces NLRP3 inflammasome activation through an increase in ATP release mediated by extracellular signal-regulated kinase 1/2 (ERK1/2) (40). Samstad et al. found that C5a produced during complement activation by cholesterol crystals, influenced inflammation through NLRP3 inflammasome activation and IL-1 β and TNF α release, and increased the production of reactive oxygen species (ROS) (41). C5a was also correlated with IL-6 induction and development of inflammatory T-helper 17 cells (42), as well as affecting IL-17 and IL-23 production (43). Interestingly, sublethal MAC (C5b-9) deposition rather than C3a or C5a was demonstrated to trigger inflammasome activation in murine dendritic cells following LPS stimulation (44). In the CNS, C5a provides a chemotactic and activation signal for microglia and astrocytes [reviewed in Ref. (45)]. It can also synergize with damage signals such as amyloid beta (A β) to trigger enhanced inflammatory cytokine production (46). Thus, activation of complement by extracellular A β plaques can exacerbate inflammation and may play a substantial role in the pathogenesis of AD. Indeed, treatment with a C5a receptor antagonist was shown to decrease deposition of fibrillar A β and inflammatory glia and improve cognitive performance in mice models of AD (47). Many other diseases are associated with anaphylatoxin signaling, including allergic, infectious, autoimmune diseases, and cancer (48). Clearly, the pro-inflammatory signaling provided by the anaphylatoxins contributes to both beneficial (pathogen clearing) and detrimental (inflammatory disease-related) inflammation.

COMPLEMENT OPSONINS REGULATE MACROPHAGE ACTIVATION

C3b-MEDIATED OPSONIZATION

One of the major effector functions of the complement system is the tagging, or opsonization, of pathogens and/or cellular debris for clearance by phagocytes. Complement component C3 is the most abundant complement component in blood at about 1.2 mg/ml, and as such, permissible surfaces become readily coated in C3b and iC3b following cleavage of C3. CR3 is a major phagocytic receptor expressed on macrophages that is involved in clearance of iC3b opsonized particles (Figure 1). In contrast to other phagocytic receptors such as Fc γ receptors, engulfment of iC3b

coated particles via CR3 has traditionally been considered anti-inflammatory. For example, CR3-dependent engulfment does not activate the arachidonic acid cascade (49) or the release of toxic oxygen products (50). In early experiments assessing macrophage heterogeneity, Stein and colleagues demonstrated increased secretion of TNF α from macrophages elicited into the peritoneal cavity by inflammatory mediators (e.g., thioglycollate) when compared to resident peritoneal macrophages. However, independent of the macrophage phenotype, all macrophages failed to secrete significant levels of TNF α following ligation of CR3 whereas ligation of Fc γ receptors led to TNF α release by all macrophage subsets (51). In studies with the macrophage intracellular pathogen *Mycobacterium avium*, C3-independent phagocytosis of *M. avium* resulted in enhanced TNF α production (52, 53). More recent reports assessing pathogenesis of *Francisella tularensis* support a role for CR3 in inducing immune suppression and facilitating infection with this intracellular pathogen (54). While CR3-dependent immune suppression is detrimental in the course of *F. tularensis* infection or other infectious disease processes, it is beneficial in the context of clearance of apoptotic cells and/or cellular debris.

COMPLEMENT-DEPENDENT ENGULFMENT OF APOPTOTIC CELLS

Complement components readily coat the surface of apoptotic cells and facilitate ingestion by macrophages (55, 56). Ingestion of apoptotic cells is a silent process accompanied by the production of anti-inflammatory cytokines TGF β and IL-10 (57). Complement mediated opsonization of apoptotic cells is largely dependent on the classical complement pathway, and deficiencies in early components of the classical pathway (C1q, C4, and C2) result in inefficient disposal of apoptotic cells and subsequent autoimmunity (58–60). While C1q deficiency results in development of lupus in virtually all cases, absence of C3, C2, and C4 results in lupus at lower frequency indicating a role for C1q beyond classical complement pathway activation in regulation of the immune response (61). Recent reports have indicated that C1q regulates the monocyte/macrophage/dendritic cell phenotype leading to development of a phagocyte that is specialized to resolve inflammation. Specifically, the C1q-stimulated phagocyte is pro-efferocytic and anti-inflammatory (Figure 2). As such, complement opsonins are mediating activity beyond the immediate stimulation of enhanced phagocytosis; they are inducing a macrophage phenotype, or polarizing macrophages toward a pro-resolving phenotype. This would be consistent with observation that C1q deficiency results in autoimmunity and chronic inflammation.

Korb and Ahearn were the first to describe a role for C1q in the clearance of apoptotic cells, and suggested that C1q-dependent engulfment of apoptotic cells was important in prevention of autoimmunity in lupus (62). There has been wide support for this hypothesis, and Bhatia et al. demonstrated that removal of apoptotic cells by C1q was also important in prevention of the inflammatory disease atherosclerosis (63). However, the receptors/signal transduction pathway leading to C1q-dependent efferocytosis has not been clearly delineated [reviewed in Ref. (56)]. C1q binds to apoptotic cells and serves as a bridging molecule linking the apoptotic cell to the phagocyte via calreticulin (cC1qR) and its binding partner, the phagocytic receptor LRP (CD91)

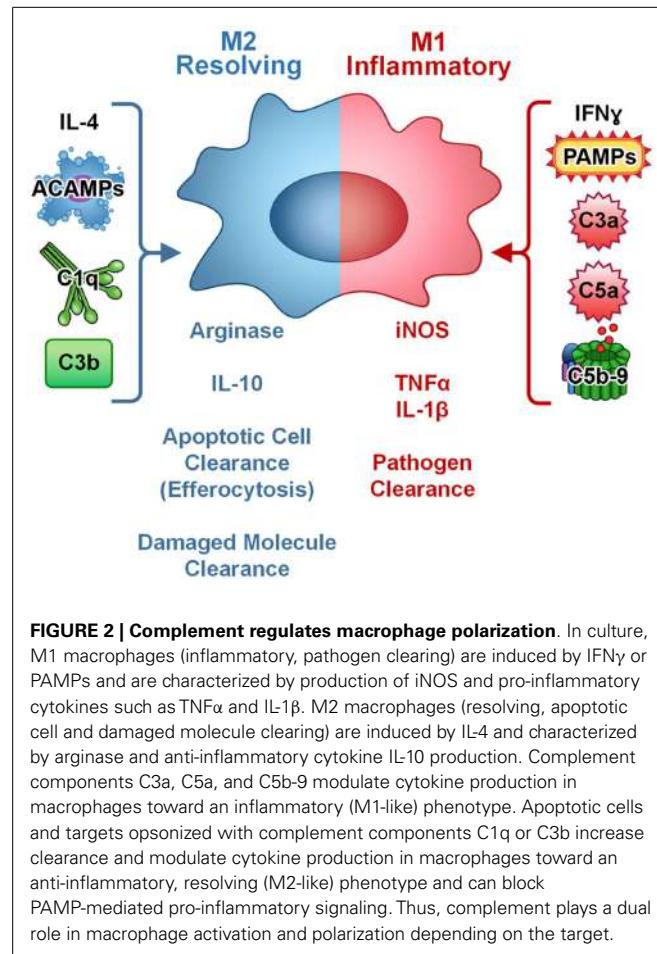


FIGURE 2 | Complement regulates macrophage polarization. In culture, M1 macrophages (inflammatory, pathogen clearing) are induced by IFN γ or PAMPs and are characterized by production of iNOS and pro-inflammatory cytokines such as TNF α and IL-1 β . M2 macrophages (resolving, apoptotic cell and damaged molecule clearing) are induced by IL-4 and characterized by arginase and anti-inflammatory cytokine IL-10 production. Complement components C3a, C5a, and C5b-9 modulate cytokine production in macrophages toward an inflammatory (M1-like) phenotype. Apoptotic cells and targets opsonized with complement components C1q or C3b increase clearance and modulate cytokine production in macrophages toward an anti-inflammatory, resolving (M2-like) phenotype and can block PAMP-mediated pro-inflammatory signaling. Thus, complement plays a dual role in macrophage activation and polarization depending on the target.

(6). However, macrophages deficient in LRP still respond to C1q with enhanced phagocytosis and efferocytosis indicating that there are multiple mechanisms of C1q-dependent engulfment (64). We demonstrated that mouse bone marrow derived macrophages and peritoneal macrophages stimulated with C1q upregulated expression of engulfment machinery including Mer tyrosine kinase and the MerTK ligand Gas6, leading to development of a macrophage that is primed for efferocytosis (65). More recently, we showed that this pathway is shared with a C1q homolog, adiponectin (66) and not with MBL, a C1q-related collectin (65).

C1q AND ADIPONECTIN MEDIATE EFFEROCYTOSIS VIA A SHARED PATHWAY

Adiponectin is referred to as an adipokine; it is produced by adipocytes and is secreted into circulation where it modulates biological responses via several receptors including adiponectin receptor 1 (AdipoR1), adiponectin receptor 2 (AdipoR2), or T cadherin (T-cad) (67, 68). More recent studies have suggested there is an additional receptor on macrophages that mediates adiponectin signaling; however, this receptor has not been identified (69). Adiponectin signaling leads to activation of 5' adenosine monophosphate-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor alpha (PPAR α), and adiponectin-dependent regulation of metabolism is mediated via

these key signaling nodes (70). Much information has been gathered regarding the role of AMPK in the regulation of metabolic activity; however, recent studies suggest that AMPK also influences the immune response, including macrophage cytokine expression and phagocytosis. For example, silencing of AMPK inhibits LPS- and fatty acid-mediated inflammation in macrophages (71). In addition, AMPK activation is associated with enhanced phagocytosis and efferocytosis, as well as macrophage polarization (72, 73). Similarly, C1q stimulates enhanced phagocytosis and diminution of pro-inflammatory cytokine production from myeloid cells, and we recently demonstrated that C1q and adiponectin-dependent Mer expression and efferocytosis require activation of AMPK (66). These studies have begun to define the mechanism by which C1q and related opsonins modulate macrophage activation.

C1q AND MBL INHIBIT PRO-INFLAMMATORY AND PROMOTE ANTI-INFLAMMATORY CYTOKINE PRODUCTION

In line with these observations, Fraser and colleagues demonstrated that both C1q-stimulated human monocytes and C1q-stimulated mouse microglia produce increased anti-inflammatory IL-10 and decreased pro-inflammatory cytokines following stimulation with the TLR4 ligand LPS (21, 22). Similar activity was shown for MBL indicating that this is a distinct mechanism for macrophage activation, independent of AMPK-mediated Mer expression since MBL failed to upregulate Mer-dependent efferocytosis. C1q/MBL-dependent activation of NF κ B p50/p50 homodimers were suggested to contribute to the anti-inflammatory phenotype via competitive inhibition of pro-inflammatory NF κ B p50/p65 heterodimer activation and/or via transcriptional activation of IL-10 (74). The same group demonstrated that C1q promoted M2 polarization and limited inflammasome activation in human monocyte derived macrophages (75). Interferon-alpha (IFN- α) is a pro-inflammatory cytokine that contributes to dendritic cell activation, a breakdown in peripheral tolerance and autoimmunity (76). C1q modulates IFN- α production from human phagocytes in response to a variety of stimuli (75, 77, 78). Santer and colleagues demonstrated that C1q deficiency in human lupus patients resulted in elevated IFN- α levels in serum and cerebrospinal fluid, and that C1q suppressed immune-complex stimulated IFN- α production from human monocytes (78–80). The anti-inflammatory effects of C1q are not limited to apoptotic cells. C1q has also been shown to enhance the uptake of atherogenic forms of lipoproteins such as oxidized or acetylated LDL (oxLDL, AcLDL) (81). During clearance of oxLDL by macrophages, C1q also modulates cytokine production toward an anti-inflammatory, resolving phenotype and dampens transcriptional activity by p50/p65 NF κ B heterodimers, which may be important in limiting inflammation in the early atherosclerotic lesion (82). Combined, these data further support the hypothesis that C1q programs macrophages toward an anti-inflammatory, pro-efferocytic/phagocytic phenotype. Future studies should delineate the relative activity of C1q-dependent constitutive efferocytosis/phagocytosis versus programed polarization in the contribution toward protection from autoimmune and inflammatory disease.

CONCLUDING REMARKS

The complement system has traditionally been considered an arm of the innate immune response required for promotion of inflammation and pathogen clearance. While these functions of complement are essential to host defense, more recent advances demonstrate a novel role for components of the complement system in resolution of inflammation and protection from autoimmune and inflammatory diseases including SLE, neurodegenerative disease, and atherosclerosis. In particular, complement component C1q directs macrophage polarization leading to generation of pro-resolving macrophages that promote clearance of apoptotic cells with diminished pro-inflammatory cytokine production and increased anti-inflammatory cytokine production. C1q is synthesized by macrophages in response to tissue injury and is likely to be an important signal in resolution of inflammation independent of other complement components. Moreover, C1q-related molecules such as the complement component MBL, and the adipokine adiponectin, also downregulate macrophage-mediated inflammatory responses and upregulate efferocytosis. Identification of the molecular mechanisms by which these molecules govern macrophage activation, as well as their relative contribution to disease resolution, should reveal pathways to target for development of novel therapeutics in autoimmune and inflammatory disease.

AUTHOR CONTRIBUTIONS

Suzanne Slater Bohlson outlined the review, invited co-authors, wrote the section on complement opsonins, extensively edited all sections, and prepared the manuscript for publication. Sean David O’Conner drafted the section on macrophage polarization. Holly Jo Hulsebus drafted the section on complement anaphylatoxins. Minh-Minh Ho designed and created the figures and figure legends. Deborah Ann Fraser wrote the section on complement and extensively edited the entire manuscript. All authors reviewed the final document prior to submission.

ACKNOWLEDGMENTS

This work was supported by NIH NIAID R56AI099010-02 to Suzanne Slater Bohlson.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 26 June 2014; accepted: 07 August 2014; published online: 21 August 2014.

Citation: Bohlson SS, O'Conner SD, Hulsebus HJ, Ho M-M and Fraser DA (2014) Complement, C1q, and C1q-related molecules regulate macrophage polarization. *Front. Immunol.* **5**:402. doi: 10.3389/fimmu.2014.00402

This article was submitted to Inflammation, a section of the journal *Frontiers in Immunology*.

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Purinergic and calcium signaling in macrophage function and plasticity

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In addition to a fundamental role in cellular bioenergetics, the purine nucleotide adenosine triphosphate (ATP) plays a crucial role in the extracellular space as a signaling molecule. ATP and its metabolites serve as ligands for a family of receptors that are collectively referred to as purinergic receptors. These receptors were first described and characterized in the nervous system but it soon became evident that they are expressed ubiquitously. In the immune system, purinergic signals regulate the migration and activation of immune cells and they may also orchestrate the resolution of inflammation (1, 2). The intracellular signal transduction initiated by purinergic receptors is strongly coupled to Ca^{2+} -signaling, and co-ordination of these pathways plays a critical role in innate immunity. In this review, we provide an overview of purinergic and Ca^{2+} -signaling in the context of macrophage phenotypic polarization and discuss the implications on macrophage function in physiological and pathological conditions.

Keywords: macrophages, calcium, purinergic receptors, inflammation, inflammasome activation

PURINERGIC RECEPTORS IN MACROPHAGES

Purinergic receptors are divided into P1 and P2 receptors. The adenosine receptors are referred to as P1 receptors. P2 receptors are the receptors for adenosine triphosphate (ATP) and can be further subdivided into metabotropic P2Y receptors, which are G-protein-coupled receptors and ionotropic P2X receptors, which are cation-selective ion channels. Macrophages express a wide variety of P2X and P2Y receptors; analysis of mouse macrophages using a variety of techniques indicates the presence of P2X₄, P2X₇, P2Y₁, P2Y₂, P2Y₄, and P2Y₆ receptors (3). Additionally, the expression of P2Y₁₂ receptor has been reported in microglia (4) and macrophages (5). Historically, macrophages were also thought to express an additional ATP-sensitive large conductance channel termed P2Z receptor (6). However, this receptor was later shown to be P2X₇ by Surprenant et al. (7).

RELEASE OF ATP AND OTHER NUCLEOTIDES INTO THE EXTRACELLULAR SPACE

Cytosolic ATP can be secreted through the release of ATP-loaded vesicles or through the activation of large conductance channels (8). A variety of inflammation-related biological processes result in ATP release from cells, and macrophages respond to this extracellular ATP rapidly. Elegant studies by Ravichandran and colleagues have shown that cells undergoing apoptosis release ATP as a find-me signal that attracts phagocytes (9). In the case of dying cells, the release of ATP and other nucleotides is accomplished through Pannexin 1, a hemi-channel that is activated through caspase-dependent cleavage (10). Interestingly, monocytes stimulated with pathogen-associated ligands or danger molecules, such as uric acid can secrete ATP, which may execute an autocrine signal that results in the activation of inflammasomes and secretion of IL-1 β and IL-18 (11). Moreover, the activation of the complement cascade has also been shown to elicit ATP efflux from

macrophages and subsequent autocrine activation of the NLRP3 inflammasome (12).

THE FUNCTION OF P2Y RECEPTORS IN MACROPHAGES

In 1989, Dubyak and colleagues showed that treatment of macrophages with extracellular ATP elicits elevations in intracellular Ca^{2+} in a wide variety of myeloid cells but not in lymphocytes. These Ca^{2+} -elevations correlated with the hydrolysis of inositol phospholipids suggesting that the ATP receptors were Gq-coupled (13). Over the course of the last two decades, it has become clear that the P2Y receptors on macrophages are Gq and Gi/o-coupled and that they perform a critical function in ATP-responsive chemotaxis. For instance, the chemotaxis of cultured microglia in response to extracellular ATP was shown to be dependent on Gi/o-coupled P2Y receptors by Honda et al. (14). This receptor was later identified as P2Y₁₂. Microglia deficient in P2Y₁₂ fail to polarize and migrate toward an ATP source *in vitro* and is unable to extend their processes toward sites of brain damage in mice (4). On a related note, microglial phagocytosis is triggered by UDP that is released by damaged neurons and is dependent on P2Y₆ receptors (15). In monocytes and macrophages, P2Y₂ plays a crucial chemotactic role in locating apoptotic cells releasing ATP (9). This study used a murine air-pouch model to demonstrate that cell supernatants from apoptotic cells were able to recruit monocytes and macrophages *in vivo* and that this recruitment was diminished in mice lacking the P2Y₂ receptor. In models of lung inflammation, P2Y₂ plays a prominent role in the chemotaxis of dendritic cells and eosinophils. Moreover, mice deficient in the P2Y₂ receptor show reduced airway inflammation in lung inflammation models where ATP has been shown to accumulate in the airways (16). Macrophages navigating in a gradient of C5a secrete ATP and use a purinergic feedback loop that involves P2Y₂, P2Y₁₂, and P1 receptors to migrate (5). However, ATP-triggered

Table 1 | Purinergic receptors and TRP channels in macrophages.

	Activation	Downstream signaling	Cellular function	Disease model phenotype after targeting
A ₁	Adenosine	Gi-coupled		
A _{2A}	Adenosine	Gs-coupled	Augment M2 polarization (63)	Extensive tissue damage and prolonged inflammation (67) Agonists induce alleviation of neural inflammation (70)
A _{2B}	Adenosine	Gs/Gq-coupled (?)	Augment M2 polarization (63)	Gene deletion exacerbates lung inflammation (71) Increased mortality in a sepsis model (72)
A ₃	Adenosine	Gi-coupled	Downregulation of inflammatory cytokines (65, 66)	Reduced arthritis using agonists (67)
P2X ₄	?	?	?	?
P2X ₇	1 mM ATP	Non-selective cation flux	Activation of NLRP3 and caspase-1 Lysosomal secretion of cathepsins (27)	Resistance to contact allergen sensitivity (23) Reduced GVHD (24) Resistant to pulmonary inflammation (25)
P2Y ₂	ATP (9)	?	Dendritic cell chemotaxis (9)	Reduced airway inflammation (16)
P2Y ₄	?	?	?	?
P2Y ₆	UDP (15)	Gq-coupled?	Microglial phagocytosis (15)	
P2Y ₁₂	ATP (4)	Gi/o-coupled (14)	Microglial chemotaxis (4)	
TRPC1	?	Ca ²⁺ -influx (42)	Unconventional secretion (42)	
TRPV2	?	Ca ²⁺ -influx (39)	Initiation of phagocytosis (39)	
TRPM2	ROS	Ca ²⁺ -influx (38)	Chemokine secretion (38)	Reduced neutrophil infiltration and intestinal inflammation (38)
TRPML1	?	Lysosomal Ca ²⁺ -release (42)	Focal exocytosis during phagocytosis (42)	Decreased bacterial clearance

?, Unknown.

chemotactic differences in M1 and M2 macrophages have not been explored and the functional contribution of various P2Y receptors in macrophage phenotypes remains uncharacterized (**Table 1**).

THE FUNCTION OF P2X RECEPTORS IN MACROPHAGES

In comparison to P2Y receptors, the P2X receptors have a significantly lower affinity for ATP but their ability to respond to ATP is influenced by the ionic conditions. In the case of P2X₇, replacement of Na⁺ with K⁺ greatly increases the responsiveness to ATP suggesting a physiological role in damaged tissues with altered ionic conditions (17). Activation of P2X₇ by high concentrations of ATP mediates caspase-1-dependent cell death accompanied by the release of proinflammatory cytokines, such as IL-1 β and IL-18. This process is greatly potentiated in macrophages activated by LPS (18). The processing of IL-1 β and IL-18 by caspase-1 is followed by their unconventional secretion with or without accompanying pyroptosis, a caspase-1 mediated pathway of inflammatory cell death. These mechanisms appear to be greatly potentiated by the influx of extracellular Ca²⁺ through the P2X₇ channels (19). Precisely how Ca²⁺ modulates the machinery mediating the secretion of IL-1 β is not clear. Activation of P2X₇ also induces membrane blebbing and activation of Rho-effector kinases but whether the influx of Ca²⁺ is essential for these processes is also not clear. Comitantly, P2X₇ is thought to regulate inflammasome-dependent activation of caspase-1 by mediating K⁺ efflux (20) and through

the potentiation of an NF κ B-driven transcriptional program (21). In a related process, P2X₇ has been shown to control the secretion of MHC class II-containing exosomes in NLRP3-dependent but caspase-1-independent manner (22).

Due to the crucial role played by P2X₇ in the regulation of the NLRP3 inflammasome, it has been implicated as a molecular target in a large variety of inflammatory diseases (**Table 1**). Mice deficient in P2X₇ are not sensitized to contact allergens and fail to release IL-1 β in response to LPS and ATP (23). This study suggests that the ligation of P2X₇ by ATP is crucial for initiating skin inflammation. Similarly, P2X₇ expression on antigen presenting cells appears to have a major impact on graft-versus-host disease (GVHD) (24). How P2X₇ affects antigen processing and presentation is not clear yet. The P2X₇-deficient mice have also been shown to be highly resistant to pulmonary inflammation induced by exposure to cigarette smoke (25). In the case of intestinal inflammation, mast cells expressing P2X₇ have been shown to play a central role in initiating the inflammatory cascade (26). In this case, it seems likely that the influx of Ca²⁺ through P2X₇ potentiates the degranulation of mast cells. A similar role for P2X₇-mediated potentiation of lysosomal secretion of cathepsins has been reported in a mouse model of arthritis (27). Recently, it was discovered that monocytes from patients afflicted with Behcets disease, a severe auto-inflammatory disorder, have increased expression of P2X₇ (28). Recent studies have also implicated P2X₇ in the modulation of adaptive immunity through the control of antigen presentation on MHC

class I molecules (29). In addition to P2X₇, macrophages also express P2X₁ channels but the functional significance is not yet clear (30). Although M1 macrophages are more efficient at ATP-induced secretion of IL-1 β (31), no significant differences in the expression levels of P2X₇ have been noted. It has been suggested that P2X₇ activation is decoupled from IL-1 β regulation in M2 macrophages (31).

CALCIUM CHANNELS IN MACROPHAGES

As non-excitable cells, macrophages rely on Ca²⁺-permeable channels that are not gated by voltage. In addition to P2X channels, macrophages express the store-operated Orai channels and some members of the transient receptor potential (TRP) channel superfamily. The regulation and function of these ion channels remains a mystery and is an emerging topic of significance to inflammation (**Table 1**).

ORAI CHANNELS IN MACROPHAGES

Historically, the elevations of intracellular Ca²⁺ in macrophages were first observed in response to platelet-activating factor (PAF) (32). In accord with the classic store-operated Ca²⁺-entry, PAF first elicited the mobilization of intracellular Ca²⁺ stores through a Gq-coupled pathway. The emptying of the ER stores was then followed by the opening of the Ca²⁺-permeable channels in the plasma membrane, the so-called CRAC channels (33). For almost two decades, the identity of CRAC channels remained a mystery but we now know their molecular identities as Orai channels (34). Recent discoveries have unraveled the regulatory mechanisms of Orai channels but their functional role in macrophage biology remains undefined. Some observations have linked store-operated Ca²⁺ response to the production of reactive oxygen species (ROS) in macrophages but definitive work and mechanistic insights have not been forthcoming (35). The Ca²⁺-influx necessary for the engulfment of apoptotic cells by macrophages is thought to be mediated by Orai channels and genetic studies in *Caenorhabditis elegans* support this notion (36) but how these channels are activated when macrophages encounter apoptotic cells is not clear and the precise role of Ca²⁺ in the engulfment process has not been clarified. Since P2Y receptors can be Gq-linked, the subsequent depletion of Ca²⁺ stores through IP₃ receptors should result in activation of Orai channels. Whether this actually occurs and whether Ca²⁺-influx through Orai channels is critical for the cellular outputs of P2Y receptor stimulation is not yet clear.

TRP CHANNELS IN MACROPHAGES

The 28 members of TRP channel superfamily are subdivided into TRPC (seven members), TRPV (six members), TRPM (eight members), TRPML (three members), TRPP (three members), and TRPA (one member) families (37). TRP channels are cation-selective channels that are weakly voltage-sensitive and diversely gated by temperature, mechanical force, electrophiles, ligands, and internal cues, such as membrane composition and pH. Recent reports have highlighted the potent functional impact of these channels in macrophages. In monocytes lacking TRPM2, the Ca²⁺-influx in response to ROS is diminished and the cells are unable to produce chemokines necessary for the recruitment of other cells (38). In a mouse model of intestinal inflammation,

ulceration and neutrophil infiltration were significantly attenuated in mice lacking TRPM2 (38).

Transient receptor potential channels have also been shown to play a major role in phagocytosis. Macrophages lacking TRPV2 are deficient in the triggering of phagocytosis when they encounter zymosan and IgG opsonized particles (39). Whether this function of TRPV2 is coupled to the influx of Ca²⁺ or other cations is not entirely clear but abnormalities in cytoskeletal rearrangements during phagocytosis were observed and the cells were also found to be deficient in chemotaxis. Mice lacking TRPV2 respond poorly when challenged with *Listeria monocytogenes*. They show increased mortality and greater bacterial load in their organs (39). During phagocytosis, macrophages replenish their membranes through a process termed focal exocytosis. This process was thought to be independent of Ca²⁺ (40), but for the phagocytosis of large particles, the process requires the activity of TRPML1 (41). Xu and colleagues have shown that TRPML1 is a lysosomal channel that is essential for the phagocytosis of large particles. Through the combined use of electrophysiology and live-cell imaging, authors show convincingly that TRPML1 mediates the release of lysosomal Ca²⁺ at the site of membrane uptake during large particle phagocytosis.

Recently, TRPC1 has been shown to play a role in restraining the unconventional secretion of IL-1 β (42). Secretion of IL-1 β is greatly potentiated after the degradation of TRPC1 by caspase-11 and macrophages lacking TRPC1 show increased secretion of IL-1 β in response to inflammatory stimuli. The precise mechanism through which TRPC1 regulates this unconventional secretion machinery is not yet clear.

COUPLING OF PURINERGIC AND CALCIUM SIGNALING IN MACROPHAGES

Extracellular ATP induces Ca²⁺ elevations in myeloid cells through the activation of G_q-coupled P2Y receptors and Ca²⁺-permeable P2X channels. P2Y receptors have a higher affinity for ATP and can elicit Ca²⁺ mobilization from the intracellular stores at low micromolar concentrations of extracellular ATP (13). In contrast, the P2X channels open at millimolar concentrations of ATP and mediate the influx of extracellular Ca²⁺ and other cations (43). Notably, the mobilization of Ca²⁺ stored in the endoplasmic reticulum is not sufficient for the activation of caspase-1 and secretion of IL-1 β . When cells are stimulated with ATP in extracellular medium that is depleted of Ca²⁺, IL-1 β secretion is nearly abolished (19). These observations indicate that activation of caspase-1 requires a sustained and more intense rise in intracellular Ca²⁺, which can be mediated by the activation of P2X₇ channels but not P2Y receptors.

An alternative explanation for differential requirements of Ca²⁺ stores and Ca²⁺-entry in the activation of caspase-1 involves the efflux of K⁺ through the activated P2X₇ channels. In this model, a concomitant efflux of K⁺ is necessary for the activation of caspase-1 and the rise in intracellular Ca²⁺ without K⁺ efflux is insufficient (44). In any case, although the activation of P2Y receptors by low concentrations of ATP is insufficient to activate caspase-1, the resulting Ca²⁺ oscillations have been shown to promote the transcription of proinflammatory cytokines such as IL-6 (45). The relative contributions of P2X and P2Y receptors in nucleotide-induced Ca²⁺-signaling have not been adequately

defined but the use of knockout mice has provided useful insights into this complex aspect of inflammation (46). In myeloid cells, Ca²⁺-dependent activation of PKC plays a pivotal role in the NFκB pathway (47) and the cellular outputs at the site of inflammation are thus likely to be shaped by the purinergic microenvironment. Even in the absence of purinergic signals, Ca²⁺ stores can be mobilized by Toll-like receptors through the activation of tyrosine kinases and phospholipase C (48), but the presence of ATP in the microenvironment likely functions as a potent amplifying mechanism for inflammatory processes.

In addition to the regulation of proinflammatory gene expression and cytokine secretion, Ca²⁺-signaling plays a major role in phagosome maturation. This link is exploited by the internalized mycobacterium for immunoevasive block of phagosome maturation (49). Although the role of Ca²⁺ in phagosome maturation is incompletely defined, it is clear that the lysosomal synaptotagmin VII, a Ca²⁺-sensitive protein, is essential for the fusion of lysosomes with phagosomes (50, 51). Ca²⁺-influx is also essential for the engulfment of apoptotic cells and a subsequent anti-inflammatory response (36). Macrophage phenotypic polarization results in significant differences in the execution of phagocytosis and phagosome maturation but the associated differences in the role of purinergic and Ca²⁺-signals between differentially polarized macrophages remain undefined (52). Ca²⁺-influx has also been shown to be essential to maintain the leading-edge structure in migrating macrophages. In this context, Ca²⁺-influx may be necessary for the activity of PKC α , which is preferentially localized at the leading edge (53).

CHEMOTACTIC AND PHAGOCYTIC RESPONSES OF MACROPHAGES TO EXTRACELLULAR ATP

Macrophages and other cells of myeloid lineage respond at three basic levels to extracellular ATP gradients. First, they migrate toward increasing ATP concentrations; second, they use the ATP gradients emanating from dying cells as a “find-me” signal to locate and phagocytose the cell corpse; and third, at high concentrations of ATP, macrophages respond by robust secretion of proinflammatory cytokines.

Chemotactic responses to ATP were first convincingly demonstrated using cultured microglial cells. Extracellular ATP at micro-molar concentrations induced pronounced membrane ruffling, chemokinesis, and chemotaxis (14). This aspect of purinergic response was not confined to ATP gradients emanating from a distant site, indicating ATP acted on cells in an autocrine manner. Indeed, migrating human neutrophils release ATP from their leading edges to amplify and steer their migration using an autocrine feedback loop that involves multiple types of purinergic receptors (54). In macrophages, the chemotactic response to C5a also utilizes an “autocrine purinergic loop” that involves the release of ATP at the leading edge and activation of multiple purinergic receptors (5). In asthmatic airway inflammation, ATP-induced chemotaxis appears to play a critical role in eosinophil and dendritic cell infiltration (55).

The analysis of dendritic cell responses to ATP clearly demonstrates that the chemotactic response to ATP is mechanistically dissociated from other cellular effects of ATP such as secretion of proinflammatory cytokines (16). However, the ATP-induced

chemotactic response is intricately connected to the role of purinergic signaling in the location and phagocytosis of apoptotic cells. In the central nervous system, damaged neurons release UDP, which triggers the phagocytic response in neighboring microglial cells (15) and similar mechanisms are likely at work in other tissue-resident macrophages. A definitive role for extracellular ATP as a find-me signal for phagocytes was demonstrated by Elliott et al. (9). The release of ATP and UTP is dependent on the activation of caspases and Pannexin 1 during the early stages of apoptosis and the concentration gradient generated by such release is highly efficient at recruiting monocytes and macrophages. Whether different macrophage phenotypes migrate differently in response to ATP gradients has not been explored. Phagocytosis of dying cells that release ATP serves an anti-inflammatory role, and phagocytic capacity is drastically inhibited in Mox macrophages that accumulate at the sites of oxidative tissue damage (56). In this context, it would be interesting to know whether alternatively activated M2 or M2-like macrophages show any significant specialization in locating dying cells using the ATP gradients.

EXTRACELLULAR ATP AS A TRIGGER FOR PROINFLAMMATORY CYTOKINE SECRETION AND PYROPTOSIS

Sustained exposure to relatively high concentrations of ATP has been shown to be a critical signal for the secretion of proinflammatory cytokines, such as IL-1 β and IL-18. The unconventional secretion of these cytokines is accomplished through proteolytic processing by caspase-1. The activation of caspase-1 is regulated by large multimeric complexes called inflammasomes and the activation of one such inflammasome, the NLRP3 inflammasome, is highly sensitive to the presence of extracellular ATP. Early evidence for ATP-induced maturation of IL-1 β came from studies involving apoptosis of peritoneal exudate cells when exposed to high concentrations of extracellular ATP. This form of apoptosis, which we now refer to as pyroptosis, was accompanied by proteolytic processing and release of IL-1 β (57), and was especially pronounced in LPS-stimulated mouse peritoneal macrophages where exposure to millimolar concentrations of ATP resulted in rapid processing and release of IL-1 β . It was further demonstrated that exposure to high concentrations of extracellular K⁺ prevented the processing and release of IL-1 β (58), suggesting that depletion of intracellular K⁺ was essential for ATP-induced IL-1 β processing. Subsequent studies of this phenomenon were greatly facilitated by the isolation of the human monocytic cell line THP-1, which was shown to be highly sensitive to purinergic stimulation of IL-1 β processing. At least in human monocytes, the purinergic activation of IL-1 β processing and secretion is also accompanied by release of proteolytically activated caspase-1 and a commitment to cell death (59).

A study conducted by Dixit and colleagues tested the role of channel-mediated ATP release by characterizing Pannexin 1-deficient mice in the context of inflammasome activation (60). Authors show that the activation of caspase-1 and secretion of IL-1 β in response to a wide variety of stimuli including ATP is normal in Pannexin-1-deficient macrophages. In contrast, Pannexin 1-deficient thymocytes failed to recruit macrophages after undergoing apoptosis. Overall, these studies indicate that ATP released through Pannexin 1 is sufficient to reach extracellular

concentrations that are of functional relevance to chemotaxis. However, the activation of caspase-1 typically requires high concentrations of ATP that are unlikely to be reached when cytosolic ATP is released through Pannexin 1. The role of vesicular release of ATP in the autocrine activation of inflammasome has not yet been ruled out and that may hold the key to reconcile these studies. A key component of vesicular ATP release is the vesicular nucleotide transporter VNUT (also known as SLC17A9), which is responsible for the accumulation of ATP into secretory vesicles (61). The human monocytic cell line THP-1 has been shown to express VNUT, which mediates the rapid secretion of ATP in response to LPS treatment (62). The function of vesicular ATP secretion in mouse macrophages and its physiological significance have not been reported yet. It is also not clear whether M1 and M2 macrophages exhibit mechanistic and functional differences in ATP release mechanisms.

The mechanisms through which activated caspase-1 and IL-1 β are secreted have remained unclear but there is evidence for the involvement of Ca²⁺-influx elicited by ATP (19). Interestingly, although high concentrations of ATP are required for caspase-1 activation, low concentrations of ATP (10 μ M) or UTP (10 μ M) are sufficient to induce oscillations in intracellular Ca²⁺ and increased transcription of the proinflammatory cytokine IL-6 (45). An autocrine role for ATP-induced activation of inflammasome has also been suggested in the case of primary human monocytes that are stimulated by danger-associated uric acid. According to this model, the activation of inflammasome is dependent upon the initial release of ATP, which then acts on purinergic receptors in an autocrine manner. Whether such secretion results in local ATP concentrations that are high enough to activate the inflammasomes is not clear (11). In addition to the regulation of IL-1 β and IL-18 secretion, ATP has also been shown to regulate the secretion of lysosomal cathepsins (27). These proteases are involved in the degradation of extracellular matrix and can result in auto-inflammatory tissue damage. Recently, ATP was shown to potentiate the release of IFN β in LPS-stimulated macrophages (30).

With respect to macrophage phenotypes, a recent study provided evidence that inflammatory M1 macrophages are more sensitive to ATP and more efficient at ATP-induced IL-1 β release when compared to M2 macrophages (31). While the physiological significance of these findings remains to be elucidated, this study provides the initial exploratory foray into these outstanding questions pertaining to how macrophage polarization fine-tunes the sensitivity to the purinergic microenvironment.

THE ATP METABOLITE ADENOSINE REGULATES THE RESOLUTION OF INFLAMMATION

Many cells express membrane-bound ectonucleotidases that convert the extracellular ATP and ADP to adenosine. A common pathway involves the conversion of ATP and ADP to AMP by CD39 and subsequent conversion of AMP to adenosine by CD73. These enzymatic biochemical conversions have potent implications for the termination of inflammatory response due to the reduction in ATP levels. More significantly, adenosine serves as a ligand for G-protein coupled adenosine receptors or P1 receptors on myeloid cells (Table 1). The four P1 adenosine receptors

(A₁, A_{2A}, A_{2B}, and A₃) transmit a “calm down” signal that may orchestrate the resolution of inflammation, a process conceptually different from anti-inflammatory signals that restrain the initiation of inflammatory process by preventing the recruitment and activation of immunocytes. Pertinently, extracellular adenosine has been shown to augment the polarization of macrophages toward the M2 phenotype (63).

Early studies showed that adenosine inhibited the secretion of TNF α , IL-6, and IL-8 by LPS-activated human monocytes (64). Subsequently, it was shown that the adenosine receptor A₃ plays a major role in downregulating the synthesis of proinflammatory cytokines in monocytes and macrophages in response to extracellular adenosine (65, 66). Synthetic agonists of A₃ adenosine receptor have been shown to have potent therapeutic effects in various mouse models of rheumatoid arthritis (67). A similar role for the A_{2A} adenosine receptor has also been reported. Mice deficient in the A_{2A} receptor show extensive tissue damage and prolonged inflammation to sub-threshold doses of inflammatory stimuli in three different disease models (68). In the context of infectious diseases and polymicrobial sepsis, A_{2A} receptors are required for the control of IL-10 production by alternatively activated macrophages (69). The A_{2A} receptors also play an anti-inflammatory role in neuroinflammation. The ligation of A_{2A} receptors on the activated microglia has been shown to retract their processes and initiate the resolution of inflammation in the brain (70).

In the case of endotoxin-induced lung injury, the pharmacological inhibition or genetic deletion of A_{2B} receptors greatly exacerbates lung injury. In contrast, the A_{2B} receptor agonist attenuates endotoxin-induced lung inflammation (71). The role of A_{2B} receptors in dampening endotoxin-induced inflammation is evident in mouse models of polymicrobial sepsis. Deletion of A_{2B} receptors greatly increases the mortality of mice from cecal ligation and puncture-induced sepsis (72). The A_{2B}-deficient mice showed increased levels of proinflammatory cytokines and chemokines in the serum, coincident with augmented activation of NF κ B and p38 in the spleen.

In summary, the adenosine receptors provide crucial information to monocytes and macrophages and calibrate their response to the complex mix of purinergic stimuli in the inflammatory microenvironment (73). In this context, the ratio of ATP and adenosine may provide the crucial cues necessary for the polarization of macrophages toward the M2 phenotype induced by IL-4. The role of A_{2A} and A_{2B} receptors to augment this process of M2 polarization has been illuminated (63) but surprisingly, a recent study shows that adenosine may control this process independent of IL-4 (74). Similarly, a recent study provides evidence suggesting a role for adenosine in IL-10-induced STAT3 activation in alternatively activated phenotype termed M2c (75). Manipulation of ATP and adenosine levels in the tissue microenvironment is thus likely to emerge as a potent mechanism to guide the plasticity of macrophages and holds clinical potential for therapeutic intervention. This concept may find traction in a large variety of diseases where inflammation plays a major pathological role. A recent pre-clinical study exemplifies the application of this strategy in the treatment of osteolysis; the authors show that activation of A_{2A} receptors prevents wear-induced osteolysis (76).

EXTRACELLULAR ATP PLAYS A PROMINENT ROLE IN INFLAMMATORY DISEASES

Some of the earliest evidence indicating a role for extracellular ATP in monocyte and macrophage function came from the findings that the cells of the myeloid lineage exhibit rapid elevations in intracellular Ca^{2+} when treated with micromolar amounts of ATP (13). The *in vivo* significance of this finding was further highlighted by the studies of Bertics and colleagues who showed that LPS-induced activation of macrophages was greatly enhanced by extracellular ATP (77) and that mice treated with the adenine nucleotide analog 2-methylthio-ATP were protected from endotoxic shock (78). Subsequent studies indicated a potent role for purinergic signals in controlling the inflammatory gene expression in response to LPS stimulation (79). The extensive contribution of purinergic signaling in inflammatory processes has now been established in a wide variety of pathologies (2), some of which are outlined below.

The tissue-resident macrophages of the central nervous system, the microglia, have been shown to be especially sensitive to extracellular ATP. *In vitro* studies with cultured microglia revealed that extracellular ATP and ADP stimulate chemotaxis and morphological changes (14). Local trauma in the brain, which results in cell death, is thought to increase the extracellular levels of ATP significantly and elegant studies using multiphoton imaging have demonstrated that microglia respond rapidly to local injury through dynamic changes in their morphology. The convergence of microglial processes at the injury site could also be stimulated by local injection of ATP and this response was demonstrated to be highly sensitive to the presence of ATP-hydrolyzing enzymes and blockers of purinergic receptors (80).

In the lungs of asthmatic patients, allergic challenges cause rapid accumulation of ATP and this has been modeled successfully in mice using experimentally induced asthma (55). Interestingly, hydrolysis of ATP in the airways through the application of apyrase greatly reduces eosinophil infiltration, production of Th2 cytokines, and bronchial hyper-reactivity. Corollary experiments show that exogenous ATP potentiates airway inflammation (55) and similar findings have demonstrated a role for purinergic signals in cigarette smoke-induced inflammation and emphysema (25). The significance of purinergic signals in allergic reactions is also evident in the skin. The accumulation of ATP in response to contact allergens is critical for the production of inflammatory cytokines by the myeloid cells and the subsequent sensitization process (23). Accumulation of ATP has also been observed in the ascites of patients and mice undergoing GVHD. In mice, the severity of GVHD is greatly reduced by neutralizing the ATP or by blocking purinergic signaling (24). Furthermore, recent studies have also implicated extracellular ATP in the development of intestinal inflammation in patients with Crohn's disease. In corresponding mouse models, blocking purinergic signaling greatly reduces the activation of intestinal mast cells and thereby blocks the subsequent rise in proinflammatory cytokines and leukotrienes (26).

In summary, studies in human beings and mice provide conclusive evidence that purinergic signals play a major role in inflammation and tissue injury. Purinergic receptors are expressed ubiquitously and a comprehensive understanding of how purinergic

signals influence the physiology and pathology is still in rudimentary stages. Purinergic control of macrophage function promises to play a central role in these processes and understanding the effects of purinergic signals on macrophage function provides an immediate window toward therapeutic intervention.

CONCLUDING REMARKS

Our understanding of the fundamental role played by purinergic and Ca^{2+} signaling in macrophage activity is increasing rapidly but the signaling mechanisms that drive specific cellular outputs still remain largely enigmatic. The close coupling of purinergic stimulation and Ca^{2+} influx suggests that the purinergic receptors, Orai channels, and TRP channels function in a co-ordinated network that responds rapidly to the changes in the inflammatory microenvironment. By virtue of being excellent drug targets, purinergic G-protein coupled receptors and ion channels offer an enticing pharmacological path to shape the plasticity of macrophage function in various diseases. To make this a reality, we will need to develop experimental models where the influence of the purinergic microenvironment and the resulting Ca^{2+} -dynamics in macrophages can be interrogated *in situ*. All indications are that we have only just scratched the surface in this exciting area of innate immunity.

ACKNOWLEDGMENTS

This work was supported by NIH-P01HL120840.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 15 August 2014; accepted: 29 October 2014; published online: 27 November 2014.

Citation: Desai BN and Leitinger N (2014) Purinergic and calcium signaling in macrophage function and plasticity. Front. Immunol. 5:580. doi: 10.3389/fimmu.2014.00580

This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology.

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Nitric oxide synthase: non-canonical expression patterns

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Science can move ahead by questioning established or canonical views and, so it may be with the enzymes, nitric oxide synthases (NOS). Nitric oxide (NO) is generated by NOS isoforms that are often described by their tissue-specific expression patterns. NOS1 (nNOS) is abundant in neural tissue, NOS2 is upregulated in activated macrophages and known as inducible NOS (iNOS), and NOS3 (eNOS) is abundant in endothelium where it regulates vascular tone. These isoforms are described as constitutive or inducible, but in this perspective we question the broad application of these labels. Are there instances where "constitutive" NOS (NOS1 and NOS3) are inducibly expressed; conversely, are there instances where NOS2 is constitutively expressed? NOS1 and NOS3 inducibility may be linked to post-translational regulation, making their actual patterns activity much more difficult to detect. Constitutive NOS2 expression has been observed in several tissues, especially the human pulmonary epithelium where it may regulate airway tone. These data suggest that expression of the three NOS enzymes may include non-established patterns. Such information should be useful in designing strategies to modulate these important enzymes in different disease states.

Keywords: NOS1, NOS2, NOS3, iNOS, eNOS, nNOS, nitric oxide, nitric oxide synthase

INTRODUCTION

Nitric oxide synthases (NOS) are enzymes that catalyze the conversion of L-arginine to L-citrulline and nitric oxide (NO), a free radical involved in homeostatic and immunological functions. There are three NOS isoforms and each isoform is associated with a set of characteristics and expression pattern. These expression patterns have been used to define the isoform's nomenclature. NOS1 is often called nNOS because of its expression in neurons and the brain. NOS2 is referred to as iNOS, because its expression can be induced by cellular activation. NOS3 is often referred to as eNOS because of its association with the endothelium. The purpose of this Perspective is to examine the concept of inducible and constitutive NOS expression, and suggest that although the current paradigm is supported in many instances, the constitutive versus inducible dichotomy has been applied too broadly and may restrict our understanding of these enzymes' functions in health and disease. A complete examination of potential NO-mediated physiological functions and NOS-expressing cells throughout an organism's tissues is beyond the scope of this work. Our focus will be on immunologically relevant cells (e.g., lymphocytes, macrophages, and the epithelium), but we will also include some non-typical NOS-expressing cells (osteoclasts and cancer). Moreover, to avoid any confusion associated with the tissue-origin nomenclature, we will identify each NOS isoform by its numeric descriptor (e.g., NOS1, NOS2, NOS3).

BASIC NOS BIOCHEMISTRY

All three NOS enzymes are catalytically active when dimerized and require two substrates, L-arginine, molecular oxygen, in combination with several co-factors including nicotinamide-adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide

(FAD), flavin mononucleotide (FMN), and (6R)5,6,7,8-tetrahydro-L-biopterin (BH4) to generate NO (1). Two NOS isoforms, NOS1 and NOS3, are commonly associated with constitutive expression. NOS1 and NOS3 activity is calcium dependent and requires interaction between the NOS enzyme and calmodulin-bound calcium to facilitate the catalysis of L-arginine and production of NO. In addition to the required co-factors and enzyme substrates, NOS1 and NOS3 are regulated through a variety of post-translational mechanisms including phosphorylation, myristylation, and palmitoylation, and modification of subcellular localization (2, 3). NOS1 and NOS3 are commonly associated with the "low" levels of NO production that mediate intracellular signaling processes (NOS1) and vascular homeostasis (NOS3). In addition to NO production, NOS3 can function in an "uncoupled" manner and produce ROS when the available stores of BH4 are removed or oxidized, L-arginine depleted, or the NOS3 inhibitor asymmetric dimethyl-L-arginine overexpressed (1, 4). NOS1 and NOS3 are most commonly found in non-immunological cells (e.g., neurons, muscle, endothelium), and, because their NO output is relatively low, these isoforms are considered to be less immunologically important than their inducible, immunologically relevant counterpart, NOS2.

Inducible expression of NOS has long been associated with immunological functions. Immune cells use NO, often in conjunction with reactive oxygen intermediates (ROI), to kill pathogens and cancer cells (5, 6). NO acts non-specifically on a variety of targets and can kill targets at micromolar concentrations (7). This lack of specificity can cause collateral damage to normal cells and tissues and consequently, NO production is tightly regulated. NOS2 is minimally expressed or is not abundant intracellularly in macrophages unless immune-related stimulation and gene

transcription occurs (hence its label as the “inducible” NOS isoform). Once transcribed, NOS2 has a high-affinity binding site for calmodulin and can function in a calcium-independent manner suggesting that any time it is expressed it is likely to be active. While the factors inducing and regulating NOS2 have been extensively studied in rodent models, NOS2 has been more difficult to study in primates. There has been controversy regarding its importance in human immune responses (8), or even whether NOS2 is expressed in human macrophages (9–11). There are several reasons why NOS2 expression has been difficult to identify in primate macrophages, including the different signals required for induction, inappropriate culture conditions, or intrinsic differences in NOS expression, but it is increasingly clear that NOS2 is expressed by human macrophages and has implications for human disease (12). A variety of immune cells other than macrophages [ranging from memory T cells (13, 14) to chondrocytes (15)] also express NOS2 in response to stimulation, suggesting that NOS2 expression is more flexible and extensive than previously reported.

IS NOS EXPRESSION “TRUE TO FORM?”

While NOS1, NOS2, and NOS3 have been associated with particular cell types and expression patterns, questions remain about whether these associations have been applied too strictly. Although the concept of constitutive (NOS1, NOS3) vs. inducible (NOS2) expression appears to be convenient, is it biologically plausible that NOS1 or NOS3 expression can be inducible under some circumstances, and conversely, can NOS2 be constitutively expressed in other circumstances? This issue has important clinical and therapeutic implications that need to be considered when designing new immunomodulatory therapies that rely on NOS expression to fight cancer or infectious diseases, or exploring current therapies for unanticipated effects. The answers are complicated by inconsistent data from experiments using different cell lines, animal models or clinical samples, and experimental techniques, but there are likely to be some generally applicable concepts and examples

that we can use as guidelines. The remainder of this review will be focused on identifying the evidence for inducible NOS1 and NOS3, and constitutive NOS2 expression (summarized in Table 1).

INDUCIBLE EXPRESSION OF “CONSTITUTIVE” NOS ISOFORMS

There are a few instances of NOS1 expression that are clearly associated with upregulation in response to external stimuli. Classically, homeostatic NOS1 expression has been associated with neuronal signaling, although inflammatory stimuli can increase neuronal NOS1 expression, potentially leading to NO-mediated damage (30, 31). The relative contribution of NOS1 to pathology in this context is often confounded by co-induction of NOS2 expression. NOS1 splice variants are expressed in skeletal, cardiac, and smooth muscle cells and can generate NO that increases blood vessel dilatation and improved blood flow to nearby muscle tissue (32). The paucity of data on NOS1 expression in monocyte-derived macrophages has suggested, perhaps incorrectly, that NOS1 has little expression or importance for tissue macrophages. That said, NOS1 expression has been identified in human bronchoalveolar lavage cells (16), lung cancer (33), and alveolar and epithelioid macrophages from humans with tuberculosis (17). Although these observations do not necessarily indicate that NOS1 is upregulated in these cells, they demonstrate non-canonical NOS1 expression, and suggest that NOS1 may be immunologically important in unanticipated ways. Significantly, recent data indicate that NOS1 activity may be regulated post-transcriptionally, with important consequences for macrophage activation and function. In unprimed murine bone marrow-derived macrophages, immune complexes can stimulate calcium-dependent NOS1 and NOS3 activity that leads to increased phagocytosis by these cells (18) indicating the upregulated activity of the “constitutive” NOS isoforms that may have unappreciated roles in immunity. There may be other systems and cell types where post-transcriptional

Table 1 | Examples of non-canonical NOS expression in non-cancerous cells and tissues.

Isoform	Cell type and reference	Species	Pathology	Expression pattern
NOS1	Bronchial epithelial cells (16)	Human	No	Ca ²⁺ flux-dependent induction
NOS1	Alveolar macrophages (17)	Human	Tuberculosis	Induced-immune stimulation?
NOS1	Epithelioid macrophages (17)	human	Tuberculosis	Induced-immune stimulation?
NOS1	BMD ^a macrophage (18)	mouse	N/A ^b	Ca ²⁺ flux-dependent induction
NOS2	Colonic epithelium (19, 20)	Human	No	Constitutive expression
NOS2	Lung epithelium (21–23)	Human, macaque	No	Constitutive expression
NOS2	Brain, spinal tissue (24, 25)	Rat	No	Constitutive expression
NOS3	Alveolar macrophages (17, 26)	Human, macaque	Tuberculosis	Induced-immune stimulation?
NOS3	Epithelioid macrophages (17)	Human, macaque	Tuberculosis	Induced-immune stimulation?
NOS3	RAW264.7 macrophages ^c (27)	Mouse	N/A	Ca ²⁺ flux-dependent induction
NOS3	BMD ^a macrophages (28)	Mouse	N/A	LPS-stimulated activity
NOS3	Osteoclasts (29)	human	No	Ca ²⁺ flux-dependent induction

^aBMD, macrophages differentiated from bone marrow-derived monocytes.

^bN/A, not applicable.

^cRAW264.7 macrophages are a murine macrophage-like cell line.

regulation of NOS1 expression through Ca^{2+} -dependent or other modulatory mechanisms can confer inducible-like characteristics to this “constitutively” expressed isoform. However, identifying these mechanisms will require a deeper understanding of cellular dynamics and responses *in vivo*, and this cannot be obtained using immunohistochemistry or studying isolated cells or cell lines.

There is considerable evidence indicating NOS3 expression is inducible under the right conditions. Forstermann et al. found that expression of NOS3 could be modulated by a range of stimuli, and that there appeared to be a species-specific difference in NOS3 regulation (34). More recently, reports have identified that NOS3 expression can be induced in human and macaque macrophages (17, 26), but the significance of the presence and inducibility of this isoform in macrophages remains to be elucidated. The macrophage-like murine cell line RAW264.7 is known for its ability to produce significant quantities of NO via an iNOS-dependent mechanism following interferon gamma and LPS stimulation, but it also constitutively expresses calcium-sensitive NOS3 and produces low levels of NO in a calcium-dependent manner (27), reminiscent of NOS1-mediated NO production (18). The quantity of NO produced at steady state was approximately 20-fold less than that produced by NOS2 following stimulation, suggesting that its function was not directly bactericidal. A later study using murine bone marrow-derived macrophages identified NOS3-generated NO as an important factor in capacitating macrophage activation by enabling increased NK- κ B activity, NOS2 expression, and NO production (28). Interestingly, mice lacking NOS3 produced less NOS2 protein and, subsequently, less NO following immune stimulation than control mice (28). In addition, it was observed that NOS2 induction led to diminished NOS3 expression, suggesting that there was an inverse feedback loop regulating NOS2- and NOS3-mediated NO production. NOS3 expression has also been observed in macrophages from non-human primates (26) and humans (17, 26) in the context of *Mycobacterium tuberculosis* infection, suggesting that NOS3 may be important in primate pulmonary immune responses. As previously mentioned, dysregulated NOS3 can produce ROS instead of NO and it cannot be ruled out that macrophage NOS3 does not generate non-traditional products instead of NO in these situations, particularly in environments rich in L-arginine-utilizing enzymes (e.g., lung and tuberculous granuloma (26, 35)). There is also evidence that NOS3 may be important in bone remodeling and can be regulated by controlling access to Ca^{2+} - and NOS2-mediated NO production (29). Unstimulated osteoclasts (macrophage-like cells responsible for bone remodeling) constitutively express both NOS2 and NOS3, with bone resorption associated with Ca^{2+} -dependent NOS3-mediated NO production and inhibition of osteoclast function mediated by NOS2 (36). As with NOS1, it may be difficult to identify upregulated NOS3-mediated NO production in instances where this increase is attributable to post-translational events. There is also some evidence that NOS1 and NOS3 activity can be upregulated post-translationally by stimuli-specific release of Ca^{2+} . This type of activation has important consequences in the regulation of many physiological processes, ranging from macrophage activation to bone homeostasis.

CONSTITUTIVE EXPRESSION OF THE “INDUCIBLE” NOS2 ISOFORM

Nitric oxide synthase 2 has become the paradigm of an inducible immunoresponsive gene, particularly in rodent systems. The high-affinity calmodulin-binding domain of NOS2 enables it to function in conditions where Ca^{2+} is unavailable, suggesting that dimerized NOS2 is always active and capable of generating NO when the appropriate co-factors are present (1, 37). The ease at which NOS2 expression is induced varies across and there are significant differences in species-specific expression patterns (9, 38, 39) and even differences between individuals in genetically diverse populations (40). In mice, which are often viewed as the paradigm for inducible NOS2 expression, some strains have macrophages that readily express NOS2 when stimulated, whereas other strains have more restrained NOS2 expression (41). NOS2 expression in primate systems appears to have different requirements for its induction that can result in NO concentrations that differ by several orders of magnitude (39). That said, although NOS2 expression is generally inducible, in some circumstances, NOS2 can be constitutively expressed. Some of the best-described examples of constitutive NOS2 expression occur in the human colonic epithelium (19, 20) and pseudostratified columnar epithelia in the human (21–23) and non-human primate lung (Figure 1). In the lung, constitutive NOS2 expression by these cells is robust and likely to be responsible for the majority of exhaled NO in human breath (22). NOS2 expression from these cells is thought to help regulate ciliary beat (16) and airway tone or reactivity (22). Rat epithelium can also express NOS1 (21), suggesting that there are likely to be species-specific differences in epithelial NOS expression. It should also be noted that neither the lung nor the colonic epithelia are sterile environments, and there remains the possibility that NOS2 expression occurs in response to stimulation by the normal microbiota associated with these tissues. Neural tissue

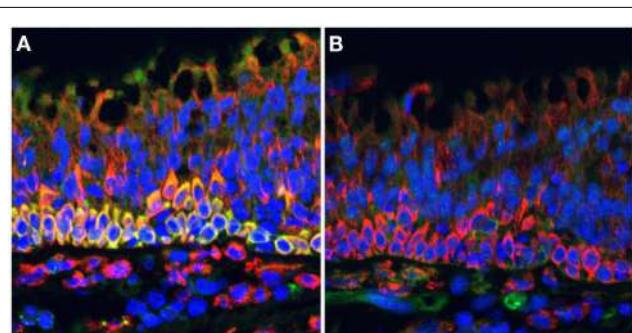


FIGURE 1 | NOS2 is strongly expressed by ciliated pseudostratified columnar epithelial cells in the cynomolgus macaque lung.

Formalin-fixed paraffin-embedded lung tissue sections were stained for (A) NOS2 (green) or (B) NOS3 (green) in combination with CD163 (red), a hemoglobin scavenger receptor expressed on macrophages and epithelial cells, and imaged by widefield epifluorescence microscopy. Intense NOS2 expression can be observed in the basal cells underlying the ciliated cells, with less intense staining in the ciliated cells. NOS3 staining is associated with cells in the lamina propria but not ciliated epithelial cells. This staining is characteristic of ciliated epithelia of both uninfected and *Mycobacterium tuberculosis*-infected macaques (pictured). DAPI-stained nuclei are indicated in blue.

is much less likely to be associated with bacteria, and there is evidence that NOS2 in rodents is constitutively expressed at low levels in brain and spinal tissue (24, 25). This can be upregulated above basal levels by inflammatory stimuli where it may be associated with disease in models of pathological conditions including Alzheimer's disease and arthritis-associated arthralgia (30, 31).

In addition to constitutive expression in normal tissues, constitutive NOS2 expression has been identified in tumors, including melanoma (42), prostate cancer (43), colorectal cancer (44), breast cancer (45), bladder cancer (46), head and neck cancer (47), and esophageal adenocarcinoma (48). In these pathologies, NOS2 is often associated with poor prognosis, potentially related to increased angiogenesis, metastatic ability, aggressive growth, resistance to apoptosis, and chemotherapy (49, 50). The mechanistic basis for why tumor progression is sometimes associated with NOS2 expression is not fully understood, but could include additional mutation by NOS-mediated DNA strand breakage, and immunosuppression of T-cell responses through both NO-dependent and NO-independent mechanisms (49). Research in this area is not without controversy and there is evidence that NOS2-generated NO has protective effects in cancer, possibly reflecting differences in a tumor's inflammatory state, the type of infiltrating immune cells, tumor location, tumor type, and the stage of disease, as well as differences in whether there are high or low levels of NO in the tumor microenvironment (49, 50). Although poorly understood at present, a better understanding of how NOS2 expression influences the tumor environment may lead to the development of novel interventional strategies and improved clinical treatment (50, 51).

CONCLUDING STATEMENT

A better understanding of the properties and expression patterns of the different NOS isoforms has shed light on the diverse range of physiological roles that these enzymes fulfill. We now know that there are instances where functions of these enzymes diverge from the dichotomous constitutive or inducible expression patterns they are often associated with. We should take this opportunity to study the full range of possible NOS function. Recognizing the possibility that NOS enzymes may act in non-canonical ways can only increase our understanding of how tissues respond to disease and give us new opportunities for developing innovative therapeutic strategies.

AUTHOR CONTRIBUTIONS

Joshua T. Mattila and Anita C. Thomas contributed to drafting the manuscript.

ACKNOWLEDGMENTS

We gratefully acknowledge JoAnne L. Flynn (University of Pittsburgh, Pittsburgh, PA, USA) for permission to image data on cynomolgus macaque lung. Joshua T. Mattila is supported in part by NIH RO1A103785 (JoAnne L. Flynn) and grants from the Bill and Melinda Gates Foundation (JoAnne L. Flynn). Anita C. Thomas is supported by funding from the British Heart Foundation.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 01 August 2014; accepted: 19 September 2014; published online: 09 October 2014.

*Citation: Mattila JT and Thomas AC (2014) Nitric oxide synthase: non-canonical expression patterns. *Front. Immunol.* 5:478. doi: 10.3389/fimmu.2014.00478*
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology.

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Metabolic reprogramming in macrophage polarization

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Studying the metabolism of immune cells in recent years has emphasized the tight link existing between the metabolic state and the phenotype of these cells. Macrophages in particular are a good example of this phenomenon. Whether the macrophage obtains its energy through glycolysis or through oxidative metabolism can give rise to different phenotypes. Classically activated or M1 macrophages are key players of the first line of defense against bacterial infections and are known to obtain energy through glycolysis. Alternatively activated or M2 macrophages on the other hand are involved in tissue repair and wound healing and use oxidative metabolism to fuel their longer-term functions. Metabolic intermediates, however, are not just a source of energy but can be directly implicated in a particular macrophage phenotype. In M1 macrophages, the Krebs cycle intermediate succinate regulates HIF1 α , which is responsible for driving the sustained production of the pro-inflammatory cytokine IL1 β . In M2 macrophages, the sedoheptulose kinase carbohydrate kinase-like protein is critical for regulating the pentose phosphate pathway. The potential to target these events and impact on disease is an exciting prospect.

Keywords: metabolism, macrophage, HIF, glycolysis, PGC-1 β

INTRODUCTION

Early insights into the metabolic status of macrophages date back to the pioneering work carried out by G. C Hard more than 40 years ago. Hard showed that activated murine peritoneal macrophages had lower levels of oxygen consumption than resting ones as well as higher levels of glycolysis (1). This study provided the first evidence of a significant metabolic change in the macrophage as a consequence of activation. Studies by Newsholme and colleagues in the 1980s provided further evidence supporting this idea, as they were able to show that enzymes involved in glucose metabolism have higher enzymatic activities in macrophages, resulting in high rates of glucose and glutamine consumption (2).

Shortly afterward, in the early 1990s, a role for IL4 in macrophage activation was described, as well as the concept of alternative activation (3, 4). At this stage, a distinction was made between classically activated macrophages, also known as M1, and alternatively activated macrophages, also referred to as M2. M1 macrophages are activated by bacterial-derived products such as lipopolysaccharide (LPS), as well as by signals associated with infection such as IFN γ . This type of activation results in a highly inflammatory macrophage with high phagocytic and bactericidal potential. M2 macrophages on the other hand can be activated by parasitic products as well as signals associated with parasitic infections, such as the cytokines IL4 and IL13. This gives rise to a macrophage with anti-parasitic and tissue repair functions (5).

Also during this period, further research was carried out on the metabolic changes associated with macrophage activation. Bustos and Sobrino suggested for the first time that the inhibition of cytokine production in macrophages caused by glucocorticoids could be due to the inhibition of the glycolytic enzymes PFK1 and PFK2, thus directly implicating impaired metabolism with impaired function (6). A key discovery was, however, in arginine metabolism. Inés María Corraliza and colleagues were able

to show that different enzymes responsible for the metabolism of arginine would be induced in a macrophage depending on the type of activation. In an M1 macrophage, nitric oxide synthase (iNOS) is upregulated, resulting in the catabolism of arginine to citrulline and nitric oxide, the latter playing key role in the intracellular killing of pathogens. In an M2 macrophage on the other hand, arginase-1 (Arg1) is induced, which results in the production of urea, polyamines, and ornithine, which are important for the wound healing actions of this macrophage population (7, 8). The differential metabolism of arginine is as of today, one of the most reliable discriminating factors between M1 and M2 macrophages. In fact, it is the only factor identified so far that can be used to detect M2 macrophage polarization in human samples (9). This provides an example of how studying the metabolic status of macrophages has proven more useful than studying function alone as well as unveiling the potential for therapeutic targeting in disease.

GLYCOLYTIC M1 VERSUS OXIDATIVE M2 MACROPHAGES

Although studies into the metabolism of immune cells date back a few decades, it has only been in recent years that the tight link between metabolism and function has become apparent. The clear metabolic differences existing between M1 and M2 macrophages exemplify this idea. An M1 macrophage is part of the first line of defense of the innate immune system, which takes place within hours to days, as opposed to an M2 macrophage, which plays a bigger role within the resolution phase and thus has longer-term functions. Their metabolism is unsurprisingly a clear reflection of those functions.

In M1 macrophages, aerobic glycolysis is induced upon activation, which involves an increase in glucose uptake as well as the conversion of pyruvate to lactate (Figure 1). At the same time, the activities of the respiratory chain are attenuated, allowing for reactive-oxygen species (ROS) production. Further evidence

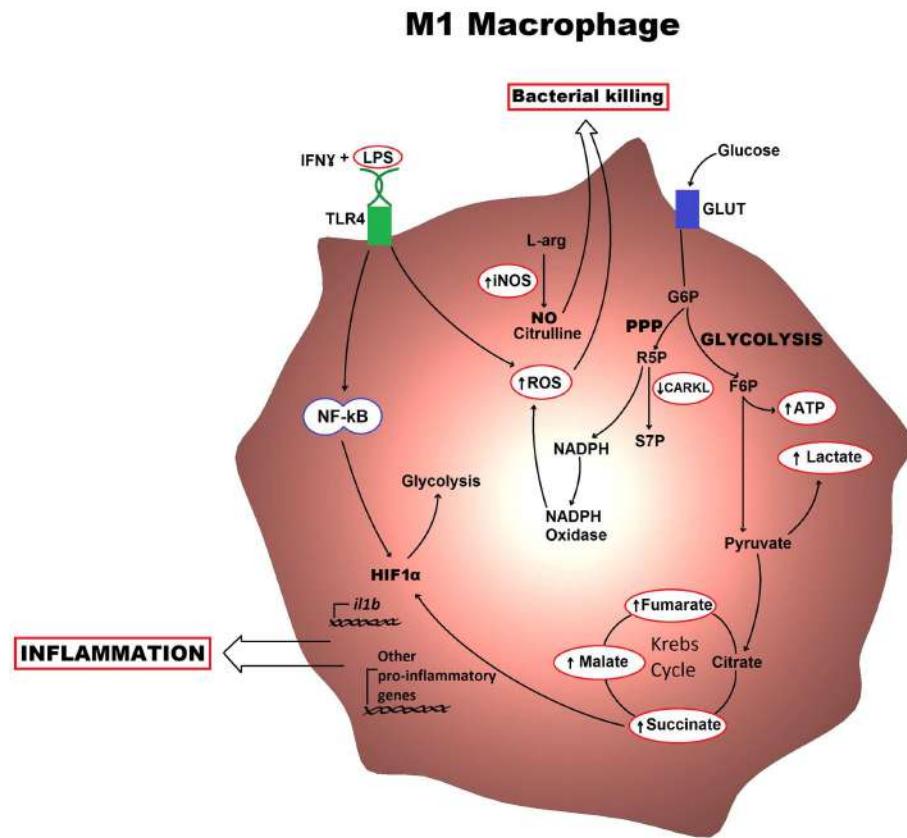


FIGURE 1 | Metabolic profile of an M1 macrophage is shown.
Classically activated macrophages induce an aerobic glycolytic program that results in lactate production and increased levels of intermediates of the Krebs cycle. The HIF1 α transcription factor also becomes activated and can drive production of pro-inflammatory cytokines. The key

functional consequences are bacterial killing, mostly through the production of ROS and NO, and inflammation, which occurs via cytokine production. G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; R5P, ribulose-5-phosphate; S7P, sedoheptulose phosphate; NO, nitric oxide; ROS, reactive-oxygen species.

for this is provided when treating macrophages with the electron transport chain inhibitors rotenone and antimycin A as this mimics the effects of toll-like receptor (TLR) agonists in driving ROS production from the mitochondria (10). Furthermore, the pentose phosphate pathway is also induced following classical activation. This pathway is key for the generation of NADPH for the NADPH oxidase, which is important for ROS production, but also for nitric oxide synthesis (11). Altogether, these metabolic events can provide the cell with rapid energy and reducing equivalents, which are required for bactericidal activity. M2 macrophages on the other hand obtain much of their energy from fatty acid oxidation and oxidative metabolism, which can be sustained for longer. Following activation, they can induce expression of constituents of the electron transport chain that will perform oxidative phosphorylation as well as driving the pyruvate into the Krebs cycle (Figure 2). The pentose phosphate pathway is also more limited in M2 macrophages. Blocking oxidative metabolism not only blocks the M2 phenotype but also drives the macrophage into an M1 state. Similarly, forcing oxidative metabolism in an M1 macrophage potentiates the M2 phenotype (12, 13). These key metabolic differences between differentially activated macrophages are widely

accepted; however, the switches responsible for orchestrating these different profiles at the molecular level remain largely unknown and how exactly the cell's metabolic status regulates polarization is not yet well understood.

Following classical activation, there is a switch in the expression of 6-phosphofructose-2-kinase/fructose-2,6-bisphosphatase (PFK2) isoforms from the liver-form (L-PFK2) to the more active ubiquitous form (u-PFK2), leading to fructose-2,6-bisphosphate accumulation, which pushes the glycolytic flux. This switching occurs at the transcriptional level with the L-PFK2 gene, *PFKB3*, being induced following activation (12). Additionally, there seems to be a requirement for downregulation of the carbohydrate kinase-like protein (CARKL) for the development of an M1 phenotype. CARKL catalyzes the production of sedoheptulose-7-phosphate, an intermediate of the pentose phosphate pathway (Figure 1). Besides expression levels of CARKL rapidly decreasing following classical activation, CARKL-expressing cells show defects in LPS-induced superoxide production. Furthermore, overexpression of CARKL results in a decrease in the production of pro-inflammatory cytokines in accordance with an M2 phenotype. Altogether, this would suggest that CARKL may help

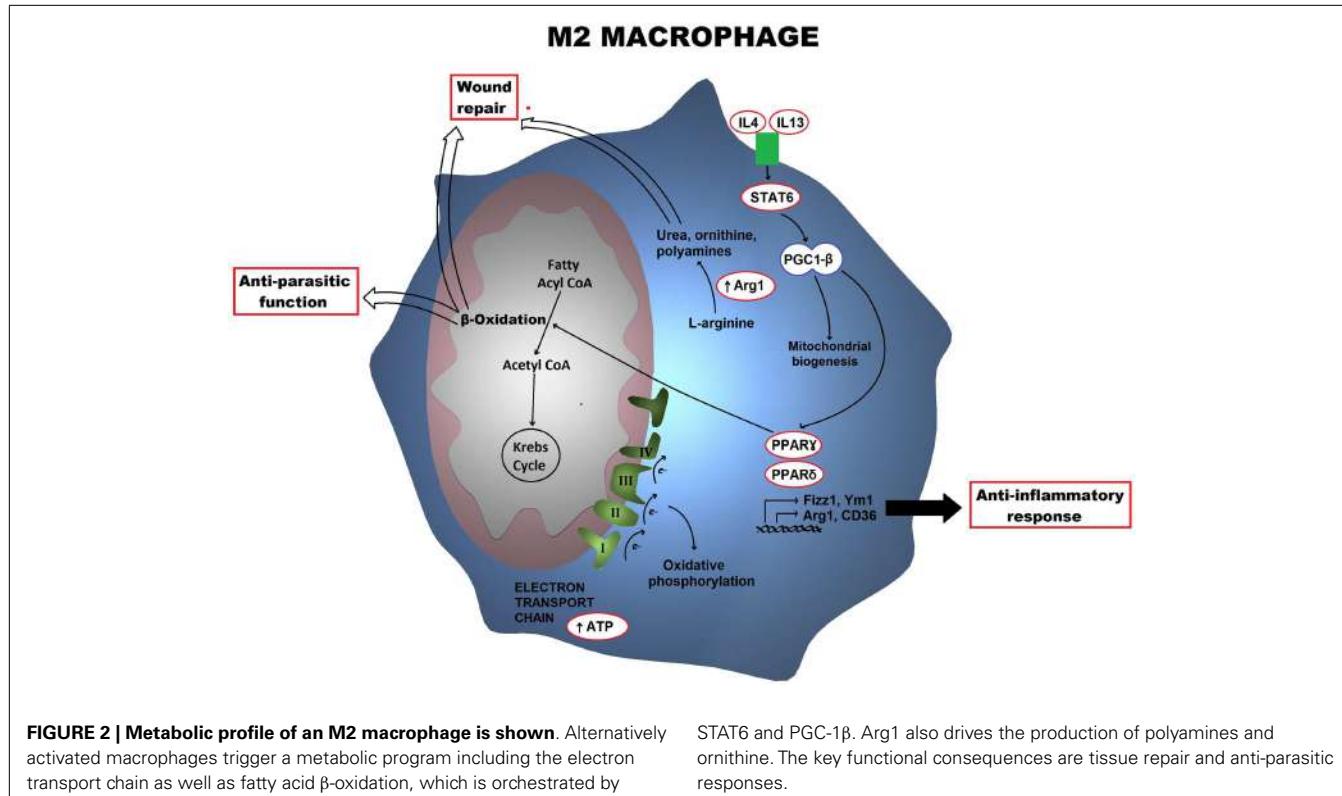


FIGURE 2 | Metabolic profile of an M2 macrophage is shown. Alternatively activated macrophages trigger a metabolic program including the electron transport chain as well as fatty acid β -oxidation, which is orchestrated by

STAT6 and PGC-1 β . Arg1 also drives the production of polyamines and ornithine. The key functional consequences are tissue repair and anti-parasitic responses.

drive the macrophage metabolism toward increased pentose phosphate pathway activity and increased redox state, thus supporting M1 polarization (14). Finally, activation of macrophages with LPS results in increased levels of Krebs cycle intermediates such as succinate and malate. Succinate, in particular, was shown to drive IL1 β production through HIF1 α , a response that could be blocked by inhibition of glycolysis using 2-deoxyglucose (15). This exemplifies how the macrophage metabolism is not simply needed for providing the energy required but can also have a direct involvement in the transcriptional regulation of the immune response.

Following alternative activation, the *PFK1* gene instead of the *PFK3* is expressed, resulting in higher levels of the liver isoform of PFK2 and lower levels of fructose-2,6-bisphosphate. The lower glycolytic levels are compensated with an increase in oxidative phosphorylation. Following macrophage activation with IL4, there is massive induction of an oxidative metabolic program, ranging from fatty acid uptake and oxidation, to oxidative phosphorylation and mitochondrial respiration. The mechanism behind this increase is somewhat better understood than that of glycolysis in M1 macrophages. Following IL4 treatment, the transcription factor STAT6, which is responsible for mediating the transcriptional responses of this cytokine, becomes activated. Active STAT6 can induce the coactivator protein peroxisome proliferator-activated receptor (PPAR) γ -coactivator-1 β (PGC-1 β). PGC-1 β can induce mitochondrial respiration as well as mitochondrial biogenesis. Furthermore, together with the transcription factors, nuclear respiratory factor 1 (NRF-1) and estrogen-related receptor- α (ERR α), it drives the production of key mitochondrial components, such as cytochrome *c* and ATP synthase (16, 17). It is therefore not

surprising that PGC-1 β is considered as the key player responsible for the metabolic switch in M2 macrophages (Figure 2). In fact, knockdown of PGC-1 β impairs not only the metabolic profile of M2 macrophages but also their functions (13). Furthermore, while PGC-1 β is the key trigger, PPARs, particularly PPAR γ and PPAR δ , have a key role in maintaining the phenotype. PPAR δ is responsible for orchestrating the effector functions of alternative activation, for instance, expression of the macrophage galactose-type C-type lectin 1 (MGL-1) as well as costimulatory molecules and other factors involve in the anti-inflammatory response. PPAR γ on the other hand, is involved in the transcription of different factors required for β -oxidation of fatty acids (18, 19).

Recently, the protein TNF-alpha-induced protein 8-like 2 (TIPE2) has also been associated with an M2 phenotype, through the induction of arginine metabolism, which as already mentioned, is the most distinguished metabolic feature of M2 macrophages. Interestingly, TIPE2 exerts such function following long-term classical activation of macrophages with LPS and not alternative activation. Thus, TIPE2 uses the switching to arginine metabolism to negatively regulate inflammation, and can therefore re-program a classically activated macrophage into its anti-inflammatory counterpart (20).

HYPOXIA-INDUCIBLE FACTOR IN MACROPHAGE POLARIZATION

Macrophages, as well as other immune cells, are usually found in inflamed sites, which are characterized by low oxygen levels. The transcription factor HIF thus plays an important role as one of the key mediators in the adaptation of macrophages to hypoxic

conditions. This heterodimeric protein is composed of two subunits, an α and a β subunit. Three isoforms of the oxygen-sensitive α subunit have been identified. The HIF1 α isoform is expressed ubiquitously, and is tightly linked to the inflammatory response and microbicidal activities. HIF2 α on the other hand, is expressed in a more limited fashion, but it is present in myeloid cells (21, 22). There is evidence in the literature suggesting a role for the two HIF α isoforms, 1 and 2, in macrophage polarization. While HIF1 α has been associated with classical macrophage activation, HIF2 α has been recently linked to an M2 phenotype. These differential roles are, however, far from clear.

HIF1 α expression can be driven by different classical activators through NF- κ B, resulting in the production of pro-inflammatory cytokines and other mediators of the M1 phenotype, such as glycolytic enzymes and glucose transporters. HIF2 α expression, on the other hand, occurs independently of NF- κ B, which would be in accordance with alternative activation. Interestingly though, both isoforms seem to be important in maintaining levels of the NF- κ B subunit p65 (23). A key mediator regulated by HIF1 α is the M1 marker iNOS. Under hypoxic conditions, nitric oxide production through iNOS is HIF1 α -dependent thus implicating HIF1 α in bacterial clearance (24). In fact, HIF1 $\alpha^{-/-}$ macrophages have impaired capacity to clear both Gram-positive and Gram-negative bacteria. Nevertheless, superoxide production during the respiratory burst, which is also required for bacterial clearance, seems to be a HIF1 α -independent event (25). This is, interestingly, not the only HIF1 α -independent event that occurs following classical activation. A critical event in the reprogramming of metabolism to glycolysis is the switch from L-PFK2 to u-PFK2, which also occurs independently of HIF1 α (12). This would suggest the presence of some other yet unidentified factor responsible for mediating the metabolic switch in M1 macrophages, either independently or in association with HIF1 α .

The potential role of HIF2 α in promoting the M2 phenotype, although promising, remains obscure. HIF2 α has been shown to regulate transcription of the M2 marker, Arg1. This finding is supported by the half-life of both proteins, as both the mRNAs for HIF2 α as well as Arg1, have relatively long half-lives. The mRNAs for HIF1 α and iNOS, however, are relatively short-lived (24). This would agree with the initial idea of how the metabolism of polarized macrophages goes hand in hand with the timing of their functions, and would support the association of HIF1 α and HIF2 α with M1 and M2 macrophages, respectively. There are, however, incongruences regarding the role of HIF2 α . For instance, HIF2 α also controls IL1 β production, which is associated with an M1 phenotype rather than M2 (15). Additionally, HIF2 α has also been associated with NF- κ B activity as mentioned above, which is also associated with an M1 phenotype (23). Studies have shown, however, that both isoforms seem to have redundant and overlapping functions, even though when one is knocked down, the other does not seem to be able to compensate (23). This highlights the fact that there are still major gaps in our understanding of the differential activities of the two isoforms.

POLARIZATION OF HUMAN MACROPHAGES

Most current knowledge of macrophage polarization comes from murine studies; however, our understanding of this topic in human macrophages remains quite poor. Furthermore, the limited studies

that have been carried out using human macrophages have identified major interspecies differences. For instance, classic murine M2 macrophage markers, such as Ym1 or Fizz1, lack human homologs and can therefore not be used as markers in human macrophages (26).

Interestingly, a recent proteomic analysis of differentially activated human macrophages suggests that the major functional differences between the two lie within metabolic pathways. The study identifies major metabolic enzymes such as glucose-6-phosphate dehydrogenase, fructose-1,6-bisphosphatase 1 (Fbp1), alpha enolase, and fructose bisphosphate aldolase A as being differentially expressed in human M1 and M2 macrophages (27). In agreement with murine studies, human M1 macrophages also upregulate glycolysis to give rise to a pro-inflammatory phenotype characterized by the production of cytokines such as IL12p40, TNF α , or IL6. However, oxidative metabolism and fatty acid oxidation do not seem to predominate in human M2 macrophages, but instead, gluconeogenesis, driven by Fbp1, seems to play a major role (27). This finding is supported by a subsequent study suggesting that fatty acid oxidation is dispensable in human M2 macrophages. D. Namgaladze and B. Brüne show that IL4-induced human M2 macrophages do not induce PGC-1 β , the key transcription factor responsible for driving the fatty acid oxidation program. In contrast with murine studies, the use of a fatty acid oxidation inhibitor does not impair the ability of human macrophages to produce high levels of CCL18 and Mrc1 and low levels of IL1 β and IL6, suggesting that they maintain the M2 phenotype (28).

Another major aspect that seems to differ considerably between murine and human macrophages is the role of iNOS and Arg1 in M1 and M2 macrophages, respectively. Attempts to demonstrate significant production of NO by human macrophages in culture have mostly failed. When successfully detected, it has only been after a period of stimulation of a few days and in much smaller amounts than that detected in murine macrophages (29). Furthermore, a recent report indicates that epigenetic modifications silence the *nos2* gene in humans, suggesting that there is no role for iNOS in M1-mediated inflammation (30). Intriguingly, macrophage-derived NO production has been reported in cases of acute inflammation such as those presented by rheumatoid arthritis patients as well as those suffering from malaria (31, 32). On the other hand, it is not just the role of iNOS that has been questioned, but also that of Arg1. Neither Cameron et al. nor Sheemann et al. could detect any arginase activity from human macrophages in culture (33, 34). However, Anika Geelhaar-Karsch and colleagues have recently shown that patients suffering from classical Whipple's disease, which is associated with elevated levels of M2 macrophages, present with higher levels of arginase activity as well as Arg1-derived products, such as urea (9). Interestingly, this could only be detected in plasma and fresh biopsies and not in macrophages in culture. Therefore, although the major differences existing between mice and humans in this regard are undisputable, the switch toward iNOS versus Arg1 may still play an important role in human diseases.

FINAL PERSPECTIVES

The metabolic aspects behind macrophage activation have long been an area of interest for many. Metabolism as a key aspect of macrophage polarization, however, is an intriguing area within

macrophage biology that has only started to develop more recently. Although we are still very much in the dark regarding our understanding of the metabolic molecular events driving macrophage polarization, the evidence discussed suggests that the role of metabolic intermediates is much more important than expected. The key question is why M1 and M2 macrophages would have such different metabolic profiles? It is possible that M1 macrophages are mainly found in hypoxic environments and therefore have to rely on glycolysis, produced via HIF1 α , for their ATP production. Glycolysis can also be rapidly induced, which is perhaps needed for the rapid activation that occurs in M1 macrophages during infection. The attenuation in the respiratory chain will also allow M1 macrophages to produce ROS, as will the NADPH produced by the pentose phosphate pathway, which is required for the NADPH oxidase. For M2 macrophages, acute activation is less of an issue, as their main function is in wound healing and anti-parasitic defense. M2 macrophages also do not generate ROS and therefore have a fully functional respiratory redox chain, allowing for oxidation of fatty acids. β -oxidation of fatty acids has, in fact, been shown to be anti-inflammatory, possibly because of a decrease in the production of prostaglandins, although this is not fully understood (35). Perhaps, the more sustained role of M2 macrophages mainly involves the metabolism of fat reserves with less ROS being a safe-ground against injury during tissue repair.

The translation of these discoveries to human diseases is an intriguing prospect, especially, since there are diseases that have been associated with one particular macrophage phenotype or another. For instance, patients presenting with chronic venous ulcers suffer from chronic inflammation as a result of failing to switch from M1 macrophages to M2 (36). On the other hand, those suffering from classical Whipple's disease, a result of chronic infection caused by *Tropheryma whipplei*, fail to clear the infection due to the lack of inflammation and excess presence of M2 macrophages (37). Interestingly, there are also reports suggesting that the distribution of M1 and M2 macrophages varies between males and females. In fact, the higher incidence of asthma in female mice was associated with higher levels of M2 macrophages when compared to male mice (38). Since females are known to present with higher incidence of not only asthma but also other autoimmune diseases, it would be interesting to speculate whether gender-associated differences in macrophage polarization might play a role.

Finally, manipulation of macrophage polarization has already proven to be somewhat successful clinically. Administration of the classical M1 macrophage activator IFN γ had beneficial effects in patients with ovarian carcinoma (39, 40). Therefore, our current understanding of the metabolic status of differentially activated macrophages holds great potential for clinical applications, although further research is required in order to capitalize clinically on the observations made to date in both murine and human systems.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 11 July 2014; paper pending published: 04 August 2014; accepted: 18 August 2014; published online: 02 September 2014.

Citation: Galván-Peña S and O'Neill LAJ (2014) Metabolic reprogramming in macrophage polarization. *Front. Immunol.* **5**:420. doi: 10.3389/fimmu.2014.00420
This article was submitted to Inflammation, a section of the journal *Frontiers in Immunology*.

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