

# 10 YEARS OF INFLAMMATION PHARMACOLOGY

EDITED BY: Paola Patrignani and Dieter Steinheilber  
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# 10 YEARS OF INFLAMMATION PHARMACOLOGY

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## The past and future of inflammatory pharmacology research: a hot topic in health and disease

Inflammation is a physiological response to a traumatic injury, bacterial, or viral infection. However, if not appropriately controlled, it contributes to a long list of diseases, including asthma, atherosclerosis, multiple sclerosis, arthritis, and cancer. Different are the types of inflammatory responses. Acute inflammation is an immediate body response to the cellular damage induced by pathogens, noxious stimuli, or physical injury – it is a short-term response resulting in healing via time-dependent changes of leukocyte functions. First, a leukocytes infiltration happens within the damaged region with the purpose of eliminating the stimulus and repairing the tissue. Chronic inflammation, by contrast, is a prolonged and dysregulated response where the active inflammation contributes both to tissue destruction and to the development of many chronic human conditions and diseases. In the context of exaggerated inflammation, which occurs as a consequence of severe burns or trauma, the body response called sepsis can be associated with fatal outcome.

Increased knowledge of the cellular and molecular mechanisms taking part in the different types of inflammation is a central requirement to develop more effective and safer treatments. This is a necessary step to prevent potential severe consequences, i.e., organ failure associated with tissue fibrosis.

The mission of Inflammation Pharmacology (section of *Frontiers in Pharmacology*) is to publish scientifically sound studies that advance our knowledge on different aspects of inflammation and contribute to the development of more effective and safer anti-inflammatory agents. Within the present eBook are collected the top articles published in the Inflammation Pharmacology section in the last 10 years.

Some articles explored the roles played by different lipid mediators generated from arachidonic acid, including leukotrienes and prostanoids [such as prostacyclin and prostaglandin(PG)F<sub>2</sub> $\alpha$ ], in inflammatory conditions. Moreover, the protectin (PD) family of specialized pro-resolving mediators biosynthesized from the two omega-3 polyunsaturated fatty acids docosahexaenoic acid (DHA) and n-3 docosapentaenoic acid (n-3 DPA) were described for their biological effects, the G-coupled protein receptors pharmacology, biosynthesis, and medicinal chemistry. Some other articles focused on the development of novel strategies to counteract inflammation or to induce its resolution. The current concepts and controversies on classification, pathogenesis, and clinical management of cutaneous adverse events induced by biologic agents used in the treatment of rheumatologic conditions were discussed in another article.

The whole-exome and whole-genome sequencing data identifying new and old loci associated with atherosclerosis will lead to discovering new molecular targets for blocking atherosclerosis even in its early stages. This critical issue was reviewed in another paper. Numerous information on an individual clinical condition is held in their platelet-derived microparticles (MPs); the assessment of their number and size together with their content can represent the signature to acquire diagnostic information and to monitor the efficacy of therapeutic agents.

Some other articles discussed the role of fibroblasts in the development of fibrosis and potential therapies under investigation. It was enlightened the role of the activation and transdifferentiation of hepatic stellate cells (HSCs) into contractile, matrix-producing myofibroblasts (MFBs) as central events in hepatic fibrogenesis, and summarized the current strategies for targeted delivery of drugs to pro-fibrogenic liver cells, including the development of therapeutics specifically targeting HSCs.

### **Future directions in the field of inflammation**

Several lines of experimental and clinical data support that reducing inflammation may mitigate the risk of cardiovascular disease. This hypothesis was recently proven by the efficacy of the anti-inflammatory therapy targeting the IL-1 $\beta$  innate immunity pathway with the monoclonal antibody canakinumab to significantly lowering the rate of recurrent cardiovascular events than placebo, independent of lipid-level lowering effects, in patients with prior myocardial infarction. Interestingly, in the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) study, the authors noted a dramatic reduction in the number of incident cases of lung cancer (Ridker PM et al. *New Engl J Med* 2017;377:1119–1131; *Lancet* 2017;390:1833–1841). These results are intriguing and should push further research to accumulate evidence on the benefit of anti-inflammatory therapies in cancer. Another field of great interest is the therapeutic use of immune checkpoint blockade in cancer treatment. The checkpoints help keep immune responses from being too strong and sometimes can keep T cells from killing cancer cells. When these checkpoints are blocked, T cells can kill cancer cells better. Examples of checkpoint proteins found on T cells

or cancer cells include PD-1/PD-L1 and CTLA-4/B7-1/B7-2. Further research should be performed to clarify the cellular players contributing to cancer immune escape. Interestingly, it has been recently shown that platelets may directly dampen T cell function both in vitro and in vivo. Furthermore, it has become clear that besides the classical signaling cascades which rely on proteins, miRNA and other RNA species have been found to regulate inflammation and might also be an attractive target for development of new drugs.

Platelet-related transforming growth factor (TGF) $\beta$  activation contributed dominantly to this immunosuppressive effect via the cell surface TGF $\beta$ -docking receptor glycoprotein A repetitions predominant (GARP) (Rachidi S, et al. *Sci Immunol* 2017; 2: eaai7911). This opens the way to the treatment of antiplatelet agents to prevent the immune escape (Patrignani & Patrono, *Platelets*. 2018;29:779-785). Several pieces of evidence are consistent with the hypothesis that activated platelets contribute to colorectal tumorigenesis and metastatization through direct cell-cell interactions and the release of different lipid and protein mediators, and microvesicles (Patrignani & Patrono *JACC* 2016; 68: 967-76). Antiplatelet agents, notably low-dose aspirin and possibly P2Y12 antagonists, may slow down and/or prevent the development and progression of cancer by targeting the platelet contribution to various processes (Patrignani & Patrono *JACC* 2016; 68: 967-76; Ballerini et al. *Front. Pharmacol.*, 02 February 2018) However, further basic research and clinical studies are necessary to further characterize the mechanism of action and dose-response of aspirin.

There is an urgent need for the development of safer anti-inflammatory agents for the chronic treatment of patients with arthritis. Novel strategies for the tissue-specific delivery of old or new anti-inflammatory agents should be implemented. Furthermore, the development and validation of genetic and biochemical markers to identify susceptible individuals at risk of developing inflammatory-related diseases is an urgent clinical need. A relevant field that *Frontiers Inflammation Pharmacology* aims to promote is the elucidation of the mechanisms involved in the development of the pneumonia, called COVID-19, from SARS-CoV-2 and possible development of appropriate anti-inflammatory therapeutic agents, in this setting.

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# Table of Contents

- 07 PG F<sub>2α</sub> Receptor: A Promising Therapeutic Target for Cardiovascular Disease**  
Jian Zhang, Yanjun Gong and Ying Yu
- 14 5-Lipoxygenase: Underappreciated Role of a Pro-inflammatory Enzyme in Tumorigenesis**  
Dieter Steinhilber, Astrid Stefanie Fischer, Julia Metzner, Svenja Dorothea Steinbrink, Jessica Roos, Martin Ruthardt and Thorsten Jürgen Maier
- 21 Prostacyclin: An Inflammatory Paradox**  
Jeremiah Stitham, Charles Midgett, Kathleen A. Martin and John Hwa
- 29 MicroRNA Involved in Inflammation: Control of Eicosanoid Pathway**  
Meike J. Ochs, Dieter Steinhilber and Beatrix Suess
- 36 Annexin A1 N-Terminal Derived Peptide Ac2-26 Exerts Chemokinetic Effects on Human Neutrophils**  
Jesmond Dalli, Trinidad Montero-Melendez, Simon McArthur and Mauro Perretti
- 43 Targeting Peripheral Opioid Receptors to Promote Analgesic and Anti-inflammatory Actions**  
Katerina S. Iwaszkiewicz, Jennifer J. Schneider and Susan Hua
- 50 Genetic Architecture of Human Fibrotic Diseases: Disease Risk and Disease Progression**  
Agnès Gardet, Timothy S. Zheng and Joanne L. Viney
- 62 Fibroblasts in Fibrosis: Novel Roles and Mediators**  
Ryan T. Kendall and Carol A. Feghali-Bostwick
- 75 The Epithelium in Idiopathic Pulmonary Fibrosis: Breaking the Barrier**  
Ana Camelo, Rebecca Dunmore, Matthew A. Sleeman and Deborah L. Clarke
- 86 NLRP3 Inflammasome and its Inhibitors: A Review**  
Bo-Zong Shao, Zhe-Qi Xu, Bin-Ze Han, Ding-Feng Su and Chong Liu
- 95 Adrenergic Regulation of Innate Immunity: A Review**  
Angela Scanzano and Marco Cosentino
- 113 Pharmacological Intervention in Hepatic Stellate Cell Activation and Hepatic Fibrosis**  
Hans-Theo Schon, Matthias Bartneck, Erawan Borkham-Kamphorst, Jacob Nattermann, Twan Lammers, Frank Tacke and Ralf Weiskirchen
- 135 The P2X7 Receptor-Interleukin-1 Liaison**  
Anna Lisa Giuliani, Alba C. Sarti, Simonetta Falzoni and Francesco Di Virgilio
- 145 IL-1 Inhibition May Have an Important Role in Treating Refractory Kawasaki Disease**  
Perrine Dusser and Isabelle Koné-Paut
- 152 Atherosclerosis is an Inflammatory Disease Which Lacks a Common Anti-inflammatory Therapy: How Human Genetics Can Help to This Issue. A Narrative Review**  
Cristiano Fava and Martina Montagnana

- 161 Molecular Mechanisms of T Cells Activation by Dendritic Cells in Autoimmune Diseases**  
Yu Tai, Qingtong Wang, Heinrich Korner, Lingling Zhang and Wei Wei
- 171 The Role of Nrf2 in Liver Disease: Novel Molecular Mechanisms and Therapeutic Approaches**  
Dongwei Xu, Min Xu, Seogsong Jeong, Yihan Qian, Hailong Wu, Qiang Xia and Xiaoni Kong
- 178 The Protectin Family of Specialized Pro-resolving Mediators: Potent Immunoresolvents Enabling Innovative Approaches to Target Obesity and Diabetes**  
Trond Vidar Hansen, Anders Vik and Charles N. Serhan
- 195 Paradoxical Skin Reactions to Biologics in Patients With Rheumatologic Disorders**  
Simone Garcovich, Clara De Simone, Giovanni Genovese, Emilio Berti, Massimo Cugno and Angelo Valerio Marzano
- 203 Platelet-Derived Microparticles From Obese Individuals: Characterization of Number, Size, Proteomics, and Crosstalk With Cancer and Endothelial Cells**  
Rosalia Grande, Melania Dovizio, Simone Marcone, Paulina B. Szklanna, Annalisa Bruno, H. Alexander Ebhardt, Hilary Cassidy, Fionnuala Ní Áinle, Anna Caprodossi, Paola Lanuti, Marco Marchisio, Geltrude Mингrone, Patricia B. Maguire and Paola Patrignani



# PG F<sub>2α</sub> receptor: a promising therapeutic target for cardiovascular disease

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Prostaglandins (PGs), a group of key lipid mediators, are involved in numerous physiological and pathological processes including inflammation and cardiovascular homeostasis. Each PG acts on its specific and distinct cell surface G protein-coupled receptors (GPCRs) or peroxisome proliferator-activated receptors (PPARs). Prostaglandin F<sub>2α</sub> receptor (FP) is required for female reproductive function such as luteolysis and parturition. It has recently been implicated in blood pressure regulation, atherosclerosis and other inflammation-related disorders. The emerging role of FP in cardiovascular diseases is highlighted and potential therapeutic translation is discussed in the current review.

**Keywords:** prostaglandin F2alpha, hypertension, atherosclerosis, FP receptor

## INTRODUCTION

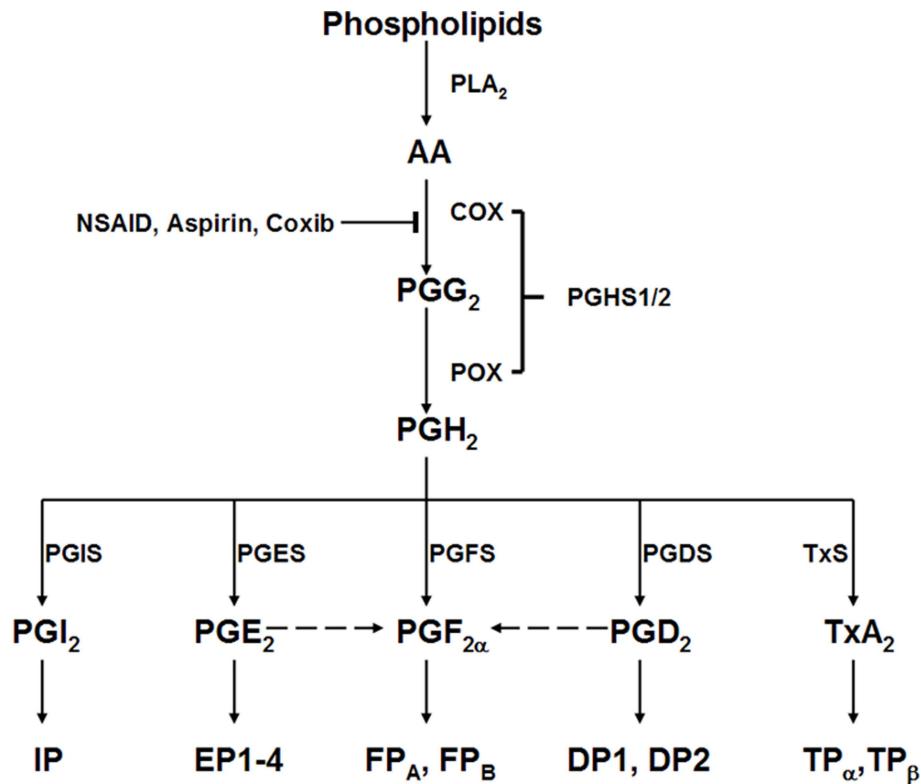
Prostanoids, including prostaglandin (PG) E<sub>2</sub>, PGD<sub>2</sub>, prostacyclin (PGI<sub>2</sub>), thromboxane A<sub>2</sub> (TXA<sub>2</sub>), and PGF<sub>2α</sub>, are generated through PGH synthase (PGHS) – known commonly as cyclooxygenase (COX), in response to a wide variety of stimuli acting as paracrine or autocrine manner. Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, ibuprofen, inhibit COX isforms to achieve antipyretic, analgesic, and anti-inflammatory actions through blocking PGs biosynthesis (Funk, 2001). Accumulating evidences demonstrate COX-derived PGs play crucial role in mediating an array of cellular processes such as cell proliferation, differentiation, and apoptosis and in regulating female reproductive function and parturition, platelet aggregation, and vascular homeostasis (Smith et al., 2000; Yu et al., 2006; Funk and FitzGerald, 2007; Yu and Funk, 2007). In addition, PGs also are involved in pathogenesis of inflammation, cancer, and cardiovascular disorders (FitzGerald and Loll, 2001; Smyth et al., 2009). The biological functions of PGs could be modulated at multiple levels such as COX, PG synthases, and downstream receptors (Narumiya and FitzGerald, 2001). Elucidating the physiological roles of COX-derived PGs in cellular and whole body homeostasis and the mechanism underlying their action will no doubt offer opportunity for developing novel therapeutics for inflammatory disease, cancer, and hypertension. Here, we summarized the recent works focusing on PGF<sub>2α</sub>/FP receptor response in cardiovascular system and reviewed the recent development of potential therapeutic target of FP receptor.

## PGF<sub>2α</sub> AND FP RECEPTOR

Prostanoids are formed through COXs on arachidonic acid via a two-step enzymatic process. First the arachidonic acid is bioconverted to PGG<sub>2</sub> through COX catalytic activity and then PGH<sub>2</sub>

through peroxidase activity (POX) of PGHS enzymes. Subsequently the PGH<sub>2</sub> is subject to metabolize to active prostanoids through individual PG synthases (Figure 1). Diversity in expression of downstream synthases results in the generation of one or two dominant PGs by individual cells. In general, PGF<sub>2α</sub> is formed by reduction of PGH<sub>2</sub> by PG endoperoxide synthase or reductase. It also can be also formed from other PGs (Figure 1) such as PGE<sub>2</sub> through 9-keto reductases and PGD<sub>2</sub> through 11-keto reductases (Watanabe et al., 1985), although relatively rare. Endogenous primary PGF<sub>2α</sub> is rapidly degraded enzymatically, half-life is less than 1 min in peripheral circulation, and its relatively stable metabolite is 15-keto-dihydro-PGF<sub>2α</sub> (Basu et al., 1992).

PGF<sub>2α</sub> exists in almost all the tissues (Basu, 2007) with more abundant in the female reproductive system (Hao and Breyer, 2008); its cellular and physiological effects are mediated by a G protein-coupled receptor—the F prostanoid receptor (the FP; Narumiya et al., 1999). Two splice forms of FP (FP<sub>A</sub> and FP<sub>B</sub>) exist in human. Initially, the FP receptor was characterized as coupling to Gq protein which lead to inositol triphosphate (IP<sub>3</sub>)/diacylglycerol (DAG) generation and mobilization of intracellular calcium (Abramovitz et al., 1994; Sugimoto et al., 1994; Watanabe et al., 1994), which is linked to the proliferation of cells (Watanabe et al., 1994). Stimulation of FP also led to activation of the small G protein Rho, resulting in phosphorylation of the p125 focal adhesion kinase, cytoskeleton rearrangement and cell morphology change (Pierce et al., 1999), and phospholipase C-mediated phosphorylation of the epidermal growth factor receptor (EGFR) and mitogen-activated protein kinase (MAPK) signaling pathways in endometrial adenocarcinoma cells (Sales et al., 2004). Recently, the coupling of Gi of FP receptor has been reported, which is response for water reabsorption in renal collecting ducts in rabbit (Hebert et al., 2005).



**FIGURE 1 | Prostanoid biosynthesis and response pathway.** AA, arachidonic acid; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; PGHS1/2, prostaglandin G/H synthase 1 or 2, which contains both cyclooxygenases (COX) and peroxidase (POX) activities; PGIS, prostaglandin I<sub>2</sub> synthase; PGES, prostaglandin E<sub>2</sub> synthase; PGFS,

prostaglandin F synthase; PGDS, prostaglandin D<sub>2</sub> synthase; TxS, thromboxane A<sub>2</sub> synthase; IP, prostaglandin I<sub>2</sub> receptor; EP, prostaglandin E<sub>2</sub> receptor; FP, prostaglandin F<sub>2α</sub> receptor; DP, prostaglandin D<sub>2</sub> receptor; TP, thromboxane A<sub>2</sub> receptor.

Expression of FP receptor and its corresponding function are summarized in **Table 1**. FP is highly expressed in the genitourinary tract (Sugimoto et al., 1997; Saito et al., 2003). Gene manipulation studies showed that, parturition is disrupted in mice lacking either cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>; Bonventre et al., 1997), that mobilizes arachidonic acid release for COX metabolism, COX-2, the more regulated form of that enzyme (Dinchuk et al., 1995; Morham et al., 1995) or the FP receptor (Sugimoto et al., 1997). Likewise, the onset of parturition is delayed in COX-1 knock out (KO) mice (Langenbach et al., 1995) but not COX-1 knockdown (KD; Yu et al., 2005). This results in high neonatal mortality that can be rescued by PGF<sub>2α</sub> replacement (Gross et al., 1998). In the eye, the FP is expressed in the vasculature, the iris sphincter and in the anterior circular muscles, all relevant to the increased uveoscleral outflow of aqueous humor provoked by PGF<sub>2α</sub> (Mukhopadhyay et al., 2001). FP agonists are approved for local application in the treatment of glaucoma (Ishida et al., 2006). Recently, abundant FP expression has also been detected in the distal convoluted tubules (DCT) and cortical collecting ducts (CCD) of the kidney (Saito et al., 2003), implicating its role in water and electrolyte homeostasis (Hebert et al., 2005). FP is observed in lung tissue and lung fibroblasts, which facilitates bleomycin-induced pulmonary fibrosis independently of transforming growth factor β (TGFβ; Oga et al., 2009). No FP receptor seems been detected in immune system organs such as spleen and thymus (Tilley et al., 2001).

## FP IN CARDIOVASCULAR DISEASES

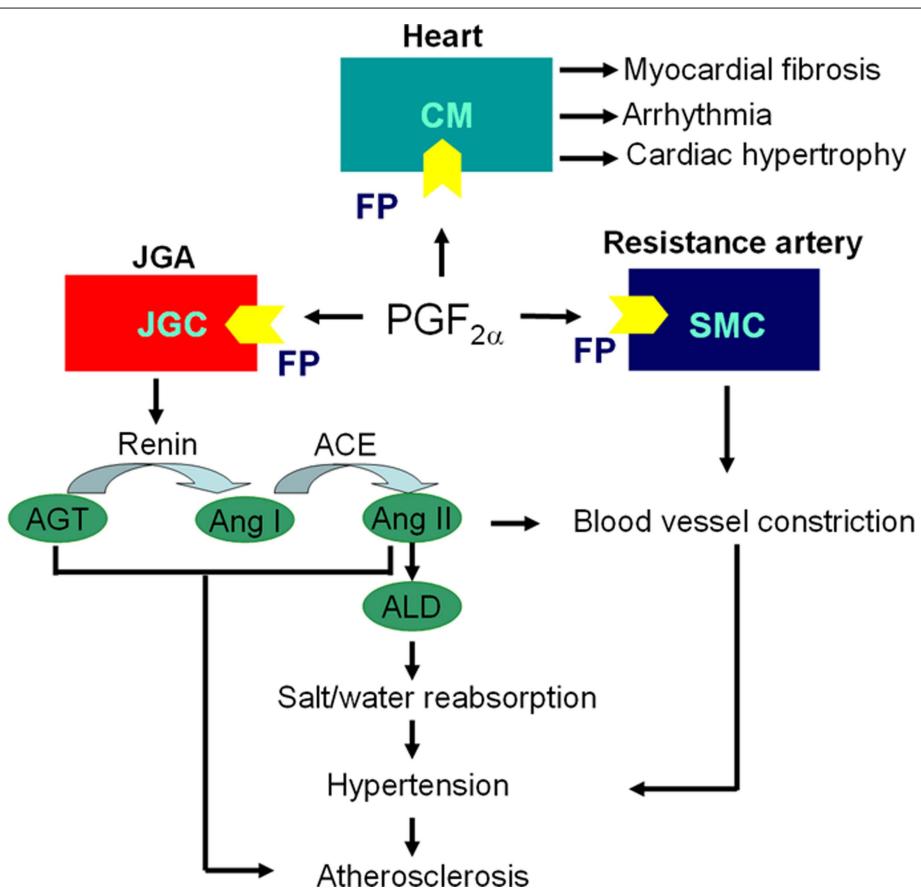
In the heart, PGF<sub>2α</sub> derives mainly from cardiac fibroblasts and its formation is increased in endocardium by ischemia (Rabinowitz et al., 1992), where it depresses contractile recovery through a mechanism associated with altered cellular energy metabolism and increased calcium accumulation (Karmazyn et al., 1993). Through FP receptor, PGF<sub>2α</sub> promotes expression of c-fos, atrial natriuretic factor (ANF), and alpha-skeletal actin in cardiomyocytes and induces cardiac myocyte hypertrophy *in vitro* and cardiac growth in rat (Lai et al., 1996), but does not affect myocyte proliferation in culture (Adams et al., 1996). Mechanistic studies showed PGF<sub>2α</sub> inhibits expression Ca<sup>2+</sup>-ATPase (SERCA2) via induction of Early Growth Response Protein 1 (Egr-1) in cultured neonatal cardiac myocytes (Hara et al., 2008). We have recently found that selective deletion of cardiomyocyte COX-2 releases a restraint on expression of fibroblast COX-2, thereby augmenting PGF<sub>2α</sub> formation. This, in turn, coincides with an increase in myocardial fibrosis and a predisposition to arrhythmogenesis (Wang et al., 2009). COX-2 derived PGF<sub>2α</sub> can further promote fibroblast PGF<sub>2α</sub> formation in a feed forward manner (Yoshida et al., 2002) and progressively promote fibrosis (Almirza et al., 2008). PGF<sub>2α</sub> promotes arrhythmias in cultured neonatal rat cardiac myocytes (Kunapuli et al., 1997; Li et al., 1997) and FP deletion protects against inflammatory tachycardia in mice *in vivo* (Takayama et al., 2005). Thus, PGF<sub>2α</sub>/FP response is involved in

multiple aspects of ischemia heart disease (**Figure 2**), blockage of the FP may facilitate recovery from cardiac ischemia-reperfusion induced injury.

Vascular endothelial cells secrete surprisingly large amounts of PGF<sub>2α</sub> in response to shear stress *in vitro* (Di Francesco et al., 2009). The relevance of this phenomenon is poorly understood

**Table 1 | FP expression and its physiological/pathological function.**

Tissue/cell distribution	Physiological/pathological process	References
Ovary	Luteolysis, parturition	Sugimoto et al. (1997), Gross et al. (1998), Saito et al. (2003)
Myometrium	Uterine contraction	Brodt-Eppley and Myatt (1999), Fischer et al. (2008)
Ocular vasculature; iris sphincter; ocular circular muscles	Aqueous humor homeostasis	Mukhopadhyay et al. (2001)
Renal distal convoluted tubule, cortical collecting duct	Water and electrolyte reabsorption	Saito et al. (2003), Hebert et al. (2005), Hao and Breyer (2008)
Juxtaglomerular apparatus	Renin secretion; blood pressure regulation	Yu et al. (2009)
Lung fibroblast	Pulmonary fibrosis	Oga et al. (2009)
Cardiac fibroblast; cardiomyocyte	Myocardial fibrosis; arrhythmias; myocyte hypertrophy	Lai et al. (1996), Kunapuli et al. (1997), Li et al. (1997), Yoshida et al. (2002), Takayama et al. (2005), Almirza et al. (2008), Wang et al. (2009)
Vascular smooth muscle cell (VSMC)	VSMC hypertrophy; vasoconstriction	Whittle et al. (1985b), Rice et al. (2008), Yu et al. (2009)



**FIGURE 2 | Scheme of PGF<sub>2α</sub>/FP pathway involved in pathogenesis of cardiovascular disease.** Cardiac fibroblasts derived PGF<sub>2α</sub> induces cardiac hypertrophy, fibrosis and arrhythmia through FP receptor in adjacent cardiomyocytes (CMs); PGF<sub>2α</sub> stimulates renin release from juxtaglomerular granular cells (JGCs) by FP receptor in an autocrine fashion, and activate renin–angiotensin–aldosterone system (RAAS) to elevate blood pressure

through enhancing salt/water reabsorption in kidney and constricting blood vessels directly via Angiotensin II (Ang II); PGF<sub>2α</sub> promotes resistance artery constriction through FP in smooth muscle cells (SMCs), which eventually increases blood pressure and contributes to atherosclerosis; Activated RAAS also accelerates atherosclerosis. JGA, juxtaglomerular apparatus; AGT, angiotensinogen; ACE, angiotensin-converting enzyme; ALD, aldosterone.

but in sufficient quantities. PGF<sub>2α</sub> may act as an incidental ligand at the TxA<sub>2</sub> receptor-the TP (Wong et al., 2009). Furthermore, the expression of FP receptors in the medial layer of resistance vessels was observed (Yu et al., 2009), which is involved in vasoconstriction (Whittle et al., 1985a). Thus it might prove relevant to the regulation of systemic blood pressure (BP) as PGF<sub>2α</sub> direct infusion causes dose-dependent elevation of BP in anesthetized mice (Yu et al., 2009). Moreover, PGF<sub>2α</sub> increases reactive oxygen species (ROS) and induces vascular smooth muscle cells (VSMCs) hypertrophy through translocation of mammalian target of rapamycin (mTOR) from nucleus to cytoplasm and activation of phosphatidylinositol 3-kinase (PI3K) pathway (Rice et al., 2008). In mice, FP deletion reduces significantly BP in mice, both when they are placed on a regular chow diet and after manipulation of dietary fat or sodium intake. This coincides with decreased activation of renin–angiotensin–aldosterone system (RAAS; Yu et al., 2009). FP receptor expression is marked in afferent arterioles of the juxtaglomerular apparatus (JGA) and renin-containing granular cells are decreased in the FP deficient mice (FP<sup>-/-</sup>). Indeed, activation of the FP appears to regulate juxtaglomerular (JG) cell differentiation and consequent renin expression, explaining depressed activation of the RAAS in FP<sup>-/-</sup> mice. Although FP expression was not detected in the aorta or even when it was complicated by atherosclerotic lesions, FP deletion attenuates atherogenesis in hyperlipidemic mice [low-density lipoprotein (LDL) receptor knockout, Ldlr<sup>-/-</sup>]. Perhaps restraint of atherogenesis in Ldlr/FP double knockout (Ldlr<sup>-/-</sup>/FP<sup>-/-</sup>) mice merely results from disruption of renal RAAS activation with a consequent impact on systemic BP (Figure 2). Taken together, antagonism of the FP receptor may afford a strategy for the control of hypertension and its attendant vascular diseases such as atherosclerosis (Yu et al., 2009).

### PGF<sub>2α</sub> IN HUMAN INFLAMMATORY DISEASE

In human studies, PGF<sub>2α</sub> is one of the more abundant PGs formed at sites of inflammation (Scher and Pillinger, 2009), and is subject to inhibition by NSAIDs such as low dose aspirin (Helmersson et al., 2005b). Similar to PGE<sub>2</sub>, PGF<sub>2α</sub> is present in joint fluid collected from rheumatoid arthritis, psoriatic arthritis, osteoarthritis patients (Trang et al., 1977; Basu et al., 2001), and the levels of these PGs could also be effectively retarded by NSAIDs treatment. In addition, the synovial cells from rheumatoid arthritis patient are able to secrete PGF<sub>2α</sub> *in vitro* (Seppala, 1987). Along with 8-Iso-PGF<sub>2α</sub>-oxidative stress marker, PGF<sub>2α</sub> was elevated during the first hour in acute myocardial infarction (AMI) patient treated with percutaneous coronary intervention (PCI; Berg et al., 2005) and 24 h after post-surgery in elective PCI patients probably due to aspirin treatment before operation (Berg et al., 2004).

Atherosclerosis is a chronic vascular inflammation diseases characterized by the thickening of the arterial wall (Rader and Daugherty, 2008). Vascular endothelial dysfunction is believed as initial step during atherogenesis, high plasma LDL, free oxygen radicals caused by cigarette smoking, hypertension, and diabetes mellitus, and other genetic defects could cause endothelial dysfunction leading to atherosclerosis (Ross, 1999). As the major metabolite of PGF<sub>2α</sub>, 15-keto-dihydro-PGF<sub>2α</sub> is elevated in the conditions

associated with those increased cardiovascular risk, such as smoking (Helmersson et al., 2005a), obese (Sinaiko et al., 2005), rheumatic disease (Basu et al., 2001), type I (Basu et al., 2005) and type II (Helmersson et al., 2004) diabetes mellitus; increased PGF<sub>2α</sub> was found in urine from population with hypercholesterolemia and smoking – the conditions associated with oxidative stress (Yin et al., 2007). Moreover, plasma PGF<sub>2α</sub> level in the elder man is positively related with common carotid artery intima-media thickness (CCA-IMT) (Wohlin et al., 2007) – a valid index of atherosclerosis. Moreover, a polymorphism in COX-1 gene (rs10306135) identified recently is associated with significantly decreased PGF<sub>2α</sub> and further lower susceptibility for cardiovascular disease (Helmersson et al., 2009). Hence, PGF<sub>2α</sub> maybe involved in initiation and progression of chronic cardiovascular diseases, such as atherosclerosis and hypertension.

### PHARMACOLOGY OF FP MODULATION

Given the accumulating evidence pleading for the involvement of PGF<sub>2α</sub>/FP receptor response pathway in regulating ocular uveoscleral outflow and normal parturition as well as pathogenesis of hypertension and atherosclerosis, the exploration of novel compounds able to specifically stimulate or inhibit FP receptor will constitute promising therapeutic avenues.

Human FP receptors are expressed in the human ocular trabecular meshwork (Anthony et al., 1998) and topical exogenous PGF<sub>2α</sub> and FP agonists reduce intraocular pressure (IOP) in monkeys and humans without causing inflammation (Weinreb et al., 2002). Thus, FP agonists, latanoprost, bimatoprost, and travoprost, are used in the treatment of glaucoma and ocular hypertension (Ishida et al., 2006), although the precise mechanism by which they work is poorly understood. More directly relevant has been the suggestion that FP antagonism may delay luteolysis and uterine contraction during parturition (Bernal, 2001), with the potential to delay preterm birth (Olson, 2005). Until recently, AL-8810, reported 10 years ago, is the first described FP antagonist, albeit that it is a partial agonist (Griffin et al., 1999) with which there is much experience in model systems (Sharif et al., 2000; Hirst et al., 2005). Theratechnologies compound THG 113 tested as FP receptor blocker, inhibits the contractile activity of smooth muscle cells from mouse (Peri et al., 2002), sheep (Hirst et al., 2005), and human myometrium (Friel et al., 2005) in response to exogenous PGF<sub>2α</sub> *in vitro* probably through activating Ca<sup>2+</sup>-activated K<sup>+</sup> channel (BKCa; Doheny et al., 2007), and delays lipopolysaccharide (LPS)-induced preterm birth in mice (Peri et al., 2002), and lowers uterine electromyographic activity and delays RU486 (a progesterone receptor blocker)-induced preterm birth in sheep (Hirst et al., 2005). More recently, AS604872, another patented FP antagonist, was shown to be effective to delay preterm parturition in rodents (Chollet et al., 2007; Cirillo et al., 2007). Thus, FP receptor could be a potential target for the pharmacological management of preterm labor. Given that renin is elevated in pregnancy-induced hypertension with decreased PGI<sub>2</sub> biosynthesis (Fitzgerald et al., 1987), FP antagonist seems more suitable theoretically for management of pregnancy-induced hypertension with broad gestational benefits. However, further clinical investigation is required regarding therapeutic efficacy of FP antagonist in clinic.

## CONCLUSION

In summary, PGF<sub>2α</sub>, an early focus of prostaglandin research has been quite neglected outside the field of reproductive biology in recent decades. However, emerging evidence, particularly from mice lacking its FP receptor, hint at its importance in BP regulation and atherosclerosis. PGI<sub>2</sub> is a potent renin secretagogue, antagonism or deletion of its receptor (the IP) protects against high-renin hypertension in renoprival models of in rodents (Fujino et al., 2004), while accelerates atherogenesis (Kobayashi et al., 2004). Thus, blockade of the FP may represent a novel therapeutic strategy in syndromes of renin dependent hypertension with a more cardioprotective profile than suppressing synthesis or disrupting activation of the PGI<sub>2</sub> receptor (IP).

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Given the use of FP agonists in the treatment of glaucoma, the synthesis of antagonists seems readily tractable. Such pharmacological probes will facilitate our determination of whether FP antagonists might have utility in a wide variety of cardiovascular disorders in the future.

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# 5-Lipoxygenase: underappreciated role of a pro-inflammatory enzyme in tumorigenesis

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Leukotrienes constitute a group of bioactive lipids generated by the 5-lipoxygenase (5-LO) pathway. An increasing body of evidence supports an acute role for 5-LO products already during the earliest stages of pancreatic, prostate, and colorectal carcinogenesis. Several pieces of experimental data form the basis for this hypothesis and suggest a correlation between 5-LO expression and tumor cell viability. First, several independent studies documented an overexpression of 5-LO in primary tumor cells as well as in established cancer cell lines. Second, addition of 5-LO products to cultured tumor cells also led to increased cell proliferation and activation of anti-apoptotic signaling pathways. 5-LO antisense technology approaches demonstrated impaired tumor cell growth due to reduction of 5-LO expression. Lastly, pharmacological inhibition of 5-LO potently suppressed tumor cell growth by inducing cell cycle arrest and triggering cell death via the intrinsic apoptotic pathway. However, the documented strong cytotoxic off-target effects of 5-LO inhibitors, in combination with the relatively high concentrations of 5-LO products needed to achieve mitogenic effects in cell culture assays, raise concern over the assignment of the cause, and question the relationship between 5-LO products and tumorigenesis.

**Keywords:** leukotriene, apoptosis, cell proliferation, mitogenic effects, cytotoxicity

## INTRODUCTION

Cancer still ranks the second leading cause of death worldwide despite the emergence of a variety of novel therapeutic options over the past decade. Malignancy of cells reflects an up-regulation of various oncogenic signal cascades that elevate tumor cell proliferation, suppress apoptosis, trigger angiogenesis, and promote metastasis. Lipid mediators, such as leukotrienes (LTs) and prostaglandins constitute a recently discovered class of tumor promoters acting by increasing tumor cell viability and triggering metastasis inducing events. Pharmacological modulation of these biosynthetic pathways is steadily increasing in importance. The present article summarizes several of these experimental findings, which implicate an emerging role of 5-lipoxygenase (5-LO)-derived LTs in carcinogenesis and critically examines the potential shortcomings of previously conducted research. This can provide direction for future investigations on 5-LO in tumorigenesis.

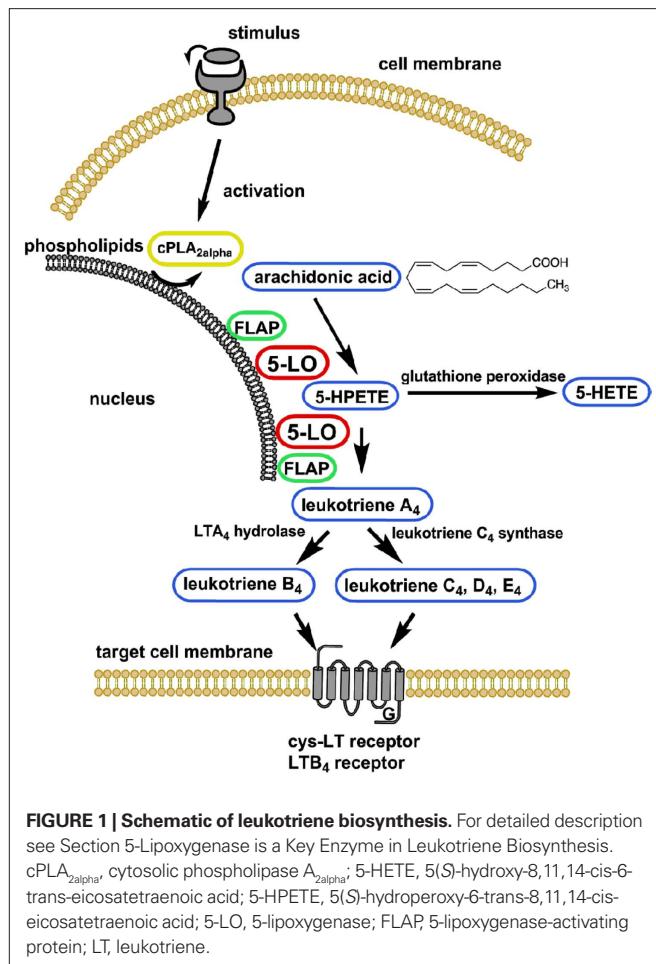
## 5-LIPOXYGENASE IS A KEY ENZYME IN LEUKOTRIENE BIOSYNTHESIS

Leukotrienes constitute a group of bioactive lipids that are generated by the 5-LO pathway. 5-LO expression is typically restricted to certain types of leukocytes, such as granulocytes (neutrophils,

eosinophils, and basophils) and monocytes/macrophages, which constitute the major source of LTs due to high 5-LO expression and enzymatic activity. 5-LO expression can also be detected in dendritic cells, mast cells, and B-lymphocytes. LTs primarily mediate inflammatory and allergic reactions (Funk, 2001; Tong et al., 2002; Ohd et al., 2003; Titos et al., 2003; Yoshimura et al., 2003; Zhi et al., 2003; Hayashi et al., 2006) by enhancing chemotaxis of migrating neutrophils and triggering vascular permeability and edema formation in inflamed tissues. Furthermore, LTs are potent bronchoconstrictory agents, crucially involved in asthma pathogenesis, and enhance plaque formation in foam cells of human atherosclerotic tissue. An increasing body of evidence also suggests a potential role of 5-LO products in early stage pancreatic, prostate, and colorectal carcinogenesis (Qiao et al., 1995; Bortuzzo et al., 1996; Avis et al., 2001; Ding et al., 2003; Tong et al., 2005).

5-Lipoxygenase catalyzes the first two steps in LT formation. Biosynthesis begins with dioxygenation of arachidonic acid (AA) released by cytosolic phospholipase A<sub>2</sub>α (cPLA<sub>2</sub>α) from cellular phospholipids, which results in two chemically unstable intermediates, 5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HPETE) and LTA<sub>4</sub> (Figure 1). A non-heme coordinated iron atom, located in the catalytical site of 5-LO, is involved in both chemical reactions. Depending on the cellular enzymes present, LTA<sub>4</sub> can be either converted to LTB<sub>4</sub> by LTA<sub>4</sub>-hydrolase or conjugated with glutathione by LTC<sub>4</sub>-synthase to generate LTC<sub>4</sub>. Further processing of LTC<sub>4</sub> produces LTD<sub>4</sub> and LTE<sub>4</sub>. 5-HPETE can be reduced by glutathione peroxidases to form the corresponding 5(S)-hydroxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HETE; Radmark et al., 2007).

**Abbreviations:** AA, arachidonic acid; cPLA<sub>2</sub>α, cytosolic phospholipase A<sub>2</sub>α; FLAP, 5-lipoxygenase-activating protein; 5-HETE, 5(S)-hydroxy-8,11,14-cis-6-trans-eicosatetraenoic acid; 5-HPETE, 5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid; 5-LO, 5-lipoxygenase; LT, leukotriene; NDGA, nordihydroguaiaretic acid; PPAR, peroxisome proliferator-activated receptors; RTK, receptor tyrosine kinase; VEGF, vascular endothelial growth factor.



**FIGURE 1 | Schematic of leukotriene biosynthesis.** For detailed description see Section 5-Lipoxygenase is a Key Enzyme in Leukotriene Biosynthesis. cPLA<sub>2alpha</sub>, cytosolic phospholipase A<sub>2alpha</sub>; 5-HETE, 5(S)-hydroxy-8,11,14-cis-6-trans-eicosatetraenoic acid; 5-HPETE, 5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid; 5-LO, 5-lipoxygenase; FLAP, 5-lipoxygenase-activating protein; LT, leukotriene.

Notably, 5-LO is of importance not only for the biosynthesis of LTs but also of related bioactive eicosanoids, such as 5-oxo-ETE formed by the enzymatic oxidation of 5-HETE (Powell et al., 1992). Furthermore, the lipoxins are formed by the cooperative action of 5- and 15-LO on AA (Serhan et al., 1984) and are involved in programmed resolution of acute inflammation (Levy et al., 2001).

In resting cells, 5-LO resides in either the nucleus or the cytosol, depending on the cell type. Upon activation, 5-LO translocates to the nuclear membrane, where the 5-LO activating protein (FLAP) is thought to facilitate the transfer of phospholipid-derived AA to 5-LO and to enhance the efficiency of conversion of 5-HPETE to LTA<sub>4</sub>, thereby triggering 5-LO product formation (Abramovitz et al., 1993; Mancini et al., 1993). The LTs produced then exert their biological effects by binding to specific G-protein-coupled transmembrane receptors at the cell surface denoted BLT1/2 for LTB<sub>4</sub> and CysLT1/2 activated by the cysteinyl LTs (Funk, 2001).

Several pharmacological strategies exist to suppress 5-LO product formation. Non-redox and redox type inhibitors, including CJ-13,610, Rev-5901, and AA-861 compete with fatty acids for binding to the active site cleft(s). Iron-ligand inhibitors such as zileuton and BWA4C suppress enzyme activity through chelation of the central iron atom and/or by stabilizing the ferrous oxidized state. FLAP inhibitors such as MK-886 act indirectly by interfering with the availability of AA (Ford-Hutchinson et al., 1994).

## STUDIES THAT PROVIDE EVIDENCE FOR A ROLE OF 5-LO IN TUMOR CELL PROLIFERATION

Increasing evidence in literature implicates 5-LO in the growth of several tumor types, including pancreatic, colorectal, prostate, and breast cancer. Numerous studies demonstrated overexpression of 5-LO in tissue samples of primary tumor cells as well as in established cancer cell lines (Chen et al., 2006). Addition of 5-LO products to cultured tumor cells led to increased cell proliferation and activation of anti-apoptotic signaling pathways (Ding et al., 2003; Tong et al., 2005). 5-LO antisense technology approaches impaired tumor cell growth by reducing 5-LO expression (Sveinbjornsson et al., 2008). Finally, pharmacological inhibition of 5-LO has been shown to potently suppress tumor cell growth by inducing cell cycle arrest and triggering cell death via the intrinsic apoptotic pathway (Ghosh and Myers, 1998; Ding et al., 1999). Based on these findings, anti-LT drugs were considered a promising and novel pharmacological strategy for cancer prevention and therapy.

## TUMOR-ASSOCIATED OVEREXPRESSION OF 5-LO, LT RECEPTORS AND OTHER ENZYMES INVOLVED IN LT BIOSYNTHESIS

The first evidence of a potential role for 5-LO in cancer growth was based on expression studies reported by Hong et al. (1999), who reported that 5-LO and FLAP were universally expressed in numerous epithelial cancer cell lines. These findings were in agreement with observations by Hennig et al. (2002), who demonstrated increased expression of 5-LO in a set of human pancreatic cancer cells and a decreased expression in normal pancreatic ductal cells. Subsequently, Gupta et al. (2001) demonstrated 5-LO overexpression in samples taken from prostate carcinoma patients, where the mean level of 5-LO mRNA was sixfold higher in malignant tissues compared to healthy tissues. Overexpression was further documented in malignant pleural mesothelial cells (Romano et al., 2001), in bladder carcinomas (Yoshimura et al., 2003), esophageal tumors (Zhi et al., 2003), and for breast cancer (Jiang et al., 2003).

In support of this hypothesis, an elevated expression of the LTB<sub>4</sub> receptor was detected in human pancreatic cancer tissue (Hennig et al., 2002). Assessing colorectal cancer samples from 84 patients, Ohd et al. (2003) were able to show a correlation between expression of CysLT receptors, 5-LO, an increased viability of the tumor cells and declined prognosis for patient survival. Notably, overexpression not only applies to 5-LO but also to other 5-LO binding enzymes involved in LT biosynthesis. Hong et al. (1999) found universal overexpression of FLAP in a series of epithelial cancer cell lines. Overexpression of FLAP associated with higher tumor aggressiveness and a poor prognosis for survival was demonstrated by analyzing breast cancer tissue samples of patients (Jiang et al., 2006). LTA<sub>4</sub> hydrolase overexpression and activity was found to be an early event in esophageal and oral adenocarcinogenesis (Chen et al., 2004; Sun et al., 2006), whereas a crucial role of LTC<sub>4</sub> synthase and cPLA<sub>2alpha</sub> is evident particularly in pathogenesis of leukemia (Stenke et al., 1998; Sjolinder et al., 2000; Runarsson et al., 2007). Recently, simultaneous overexpression of various enzymes and receptors involved in LT biosynthesis and action, including, 5-LO, FLAP, LTC<sub>4</sub> synthase, LTA<sub>4</sub> hydrolase, BLT, and CysLT receptors, was detected in the majority of human primary neuroblastoma tumors as well as in respective cell lines. Whereas 5-LO is well-recognized as pro-carcinogenic, the related enzyme 15-LO-2 is down-regulated

in malignant tissues, considered to function as a tumor suppressor and to inhibit carcinogenesis (Shappell et al., 1999; Hsi et al., 2002). The role of 15-LO-1 is still discussed controversially in the literature (Pidgeon et al., 2007). Depending on the tumor cell type, overexpression of one enzyme of the LT synthesizing machinery in tumor cells does not inevitably account for an overexpression of the other enzymes. Thus, granulocytes from acute myeloid leukemia patients showed suppressed LTB<sub>4</sub> formation accompanied by elevated LTC<sub>4</sub> hydrolase expression and LTC<sub>4</sub> synthesis compared to leukocytes from healthy patients (Stenke et al., 1998).

### CYTOTOXIC EFFECTS BY 5-LO INHIBITORS

Numerous studies have demonstrated cytotoxic and anti-proliferative effects of 5-LO inhibitors in cultured tumor cells as an important basis for the involvement of 5-LO in tumorigenesis. Tsukada et al. were amongst the first to describe the potent anti-proliferative effects of the 5-LO inhibitor AA-861 in a human leukemic cell line. This was accompanied by a sharp reduction in cellular DNA, RNA, and protein synthesis. It was concluded from this result that LTs potentially play an essential role in cancer cell viability (Tsukada et al., 1986). Similar growth-inhibitory effects were observed with the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA) in stomach cancer cells (Shimakura and Boland, 1992).

These data agree well with studies reporting that the 5-LO inhibitor AA-861 was capable of abolishing the AA stimulated increase of prostate cancer cell growth (Ghosh and Myers, 1997) and that inhibition of 5-LO by MK-886 (FLAP inhibitor) triggers severe apoptosis in human prostate cancer cells (Ghosh and Myers, 1998). Subsequent studies described that AA-861 is capable of suppressing the growth of esophageal cancer cells *in vitro* (Hoque et al., 2005) and the proliferation of MCF-7 breast cancer cells (Hammamieh et al., 2007). AA-861 also has been shown to have similar effects in colorectal cancer cells (Ihara et al., 2007). Strong cytotoxic effects in various cancer cell lines were also observed with the 5-LO inhibitor and LTD<sub>4</sub> receptor antagonist Rev-5901 (Ding et al., 1999; Tong et al., 2002; Titos et al., 2003; Hayashi et al., 2006; Melstrom et al., 2008; Sveinbjornsson et al., 2008). Recent findings with the LTB<sub>4</sub> receptor antagonist, LY293111,

which demonstrated potent anti-pancreatic cancer effects by inducing tumor cell apoptosis (Ding et al., 2005) and triggering S-phase cell cycle arrest (Tong et al., 2007), further supported the hypothesis that 5-LO and its downstream products, play crucial roles in tumorigenesis. Finally, a chemopreventive activity of Rev-5901 against colorectal adenocarcinoma xenografts was recently demonstrated in an animal tumor model (Melstrom et al., 2008). Taken together, the diverse set of 5-LO inhibitors was shown to suppress the growth of several types of tumor cells by cytotoxic mechanisms, primarily through activation of the intrinsic pathway of apoptosis. However, in addition to the pro-apoptotic activity of the drugs, it should be noted that additional anti-proliferative effects were observed, such as a decrease in DNA synthesis and induction of cell cycle arrest, which could harbor great significance. A summary of the described drug effects can be found in **Table 1**.

### MITOGENIC EFFECTS OF 5-LO PRODUCTS

Several AA metabolites synthesized via the 5-LO pathways have been shown to promote tumor cell viability and to exert protective effects toward the 5-LO inhibitor induced cytotoxicity. The precise molecular mechanisms through which these molecules act on cancer cells remain incompletely understood. Direct proliferative and anti-apoptotic stimuli as well as an enhanced tumor angiogenesis may contribute. 5-HETE and LTB<sub>4</sub> increased cell proliferation and viability of pancreatic cancer cells by activating the mitogenic and anti-apoptotic MAPK and Akt kinase signaling pathways (Ding et al., 2003; Tong et al., 2005). A susceptibility toward 5-HETE also was described for several other tumor cell types, including breast cancer (Avis et al., 2001), and cancer of the lung (Avis et al., 1996). The effects of 5-HETE may, at least partially, derive from 5-oxo-EET, which is formed by the cellular oxidation of 5-HETE (Powell et al., 1992). 5-oxo-EET acts by binding to the G-protein-coupled OXE surface receptors (Grant et al., 2009). Some tumor cell types, including colorectal cancer cells, displayed specific mitogenic effects in response to LTB<sub>4</sub>, but not to other 5-LO products (Qiao et al., 1995; Bortuzzo et al., 1996). A few additional studies report proliferative and anti-apoptotic effects for CysLTs. Accordingly, LTD<sub>4</sub>

**Table 1 |** Cytotoxic and anti-proliferative effects by 5-LO inhibitors in cell culture assays.

Inhibitor	Concentration ( $\mu$ M)	Cell type	Effects	Literature
AA-861	20	Human leukemia cell lines	Potent anti-proliferative effects	Tsukada et al. (1986)
AA-861	60	Human prostate cancer cells	Inhibition of arachidonic acid stimulated cell growth	Ghosh et al. (1997)
MK-886	10	Human prostate cancer cells	Triggered cell death via activation of the apoptotic pathway	Ghosh et al. (1998)
Rev-5901	15	Human pancreatic cancer cell lines	Inhibition of cell proliferation, reversal by 5-HETE and 12-HETE	Ding et al. (1999)
LY293111	1	pancreatic cancer cells	Inhibition of pancreatic cancer growth, induction of tumor cell apoptosis	Ding et al. (2005)
AA-861	60	Esophageal cancer cells	Suppression of cell growth by induction of apoptosis	Hoque et al. (2005)
AA-861	30	Human bladder cancer cell lines	Strong growth suppression	Hayashi et al. (2006)
MK-591, MK-886	20	MCF-7 breast cancer cell line	Inhibition of cell proliferation	Hammamieh et al. (2007)

increased the proliferation and survival of intestinal epithelial cells (Paruchuri et al., 2005) and potentially enhanced tumor growth by up-regulating the transcriptional activity of the oncogenic protein beta-catenin (Mezhybovska et al., 2006). Romano et al. (2001) were able to show that 5(S)-HETE and LTA<sub>4</sub> but not LTB<sub>4</sub>, potently up-regulated vascular endothelial growth factor (VEGF) transcription and expression in a human malignant mesothelioma model, which may contribute to the reported pro-angiogenic and anti-apoptotic effects of 5-LO products. Recent findings uncovered a novel function of LTB<sub>4</sub> in driving oncogenic ras-induced metastasis by acting on BLT-2 receptors (Kim et al., 2009). Furthermore, LTB<sub>4</sub>-induced breast cancer cell survival was linked to BLT-2 mediated generation of reactive oxygen species. Collectively, the body of research supports a model in which 5-LO products modulate proliferative and anti-apoptotic events through multiple signaling pathways. **Table 2** provides a summary of the described mitogenic effects.

### 5-LO KNOCK-DOWN STUDIES IN CULTURED CELLS AND IN 5-LO KNOCK-OUT ANIMALS

Several studies found reduced cell proliferation rates due to down-regulation of 5-LO expression by antisense approaches. Using 5-LO antisense oligonucleotides, Romano et al. (2001) were amongst the first to provide direct evidence for 5-LO participation in the growth of malignant pleural mesothelial cells. Moreover, reduced expression of the 5-*oxo*-ETE receptor by siRNA approaches significantly impaired the viability of prostate cancer cells, suggesting a tumorigenic function of the 5-LO product 5-*oxo*-ETE (Sundaram and Ghosh, 2006). Finally, silencing of LT receptors was capable of suppressing growth of colorectal cancer cells (Thara et al., 2007) and neuroblastoma cells (Sveinbjornsson et al., 2008). Comparatively few studies have investigated the role of 5-LO in mouse models. Chen et al. (2009) demonstrated that 5-LO-deficient knock-out (KO) mice show significantly impaired induction of chronic myeloid leukemia through specific suppression of leukemic stem cell proliferation. A summary of experimental findings accounting for a role of 5-LO in tumorigenesis can be found in **Figure 2**.

### STUDIES THAT QUESTION THE ROLE OF 5-LO IN TUMORIGENESIS

#### CYTOTOXIC OFF-TARGET EFFECTS OF 5-LO INHIBITORS

The increasing number of publications that report a crucial role of 5-LO, and its products in tumorigenesis have been accompanied by additional studies that question the correlation between 5-LO and cancer. There is little disagreement that 5-LO inhibitors exert strong

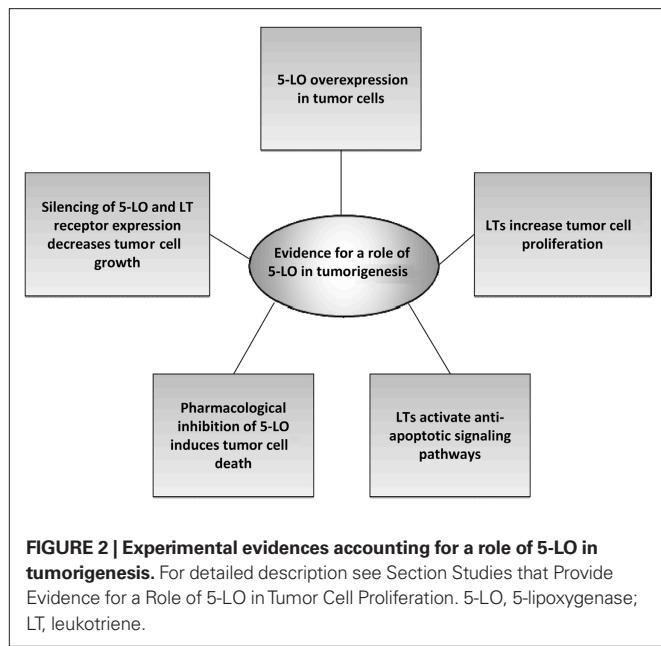
cytotoxic activities against 5-LO overexpressing tumor types and cultured tumor cells, which represents a significant basis for concluding that 5-LO products directly stimulate tumor cell proliferation. However, we recently demonstrated that the common 5-LO inhibitors AA-861, Rev-5901, BWA4C, and CJ-13,610 can reduce the viability of pancreatic cancer cells, cervix carcinoma cells, and leukemic cells independently of suppression of 5-LO product formation (Fischer et al., 2010). The hypothesis of 5-LO-independent cytotoxicity and anti-proliferation was substantiated using several experimental approaches. First, the various 5-LO inhibitors were shown to possess highly different abilities to reduce cell viability, to induce cytotoxic effects and to suppress the proliferation of cultured 5-LO-positive Capan-2 pancreas carcinoma cells. While the commonly used inhibitors AA-861, MK-886, and Rev-5901 produced strong cytotoxicity, other, more selective and more potent 5-LO inhibitors, including CJ-13,610, BWA4C, failed in this respect. Notably, zileuton, the only commercialized 5-LO inhibitor, failed to induce an anti-proliferative or cytotoxic response in all types of tumor cells employed. Additionally, the IC<sub>50</sub> values of cytotoxicity for AA-861, MK-886, and Rev-5901 exceeded the respective IC<sub>50</sub> values for inhibition of 5-LO enzyme activity by more than 20-fold (Rev-5901) and up to 5,000-fold (AA-861). Lastly, well-established 5-LO-negative tumor cell lines exhibited a higher susceptibility toward the 5-LO inhibitors than their morphologically related 5-LO-p counterparts. These observations are in line with a report by Datta et al. in which MK-886 induced severe apoptosis independently of FLAP (Datta et al., 1999; Fischer et al., 2010). Sabirsh et al. (2005) recently described 5-LO-independent effects of various LT synthesis inhibitors on Ca<sup>2+</sup> signaling in 5-LO-deficient HeLa carcinoma cells. Also the apoptotic effects of licoferone, a dual COX/5-LO inhibitor, were found to occur independently of the ability of the drug to affect the AA cascade (Tavolari et al., 2008).

Recently, the 5-LO inhibitor zileuton was shown to suppress prostaglandin E<sub>2</sub> biosynthesis in macrophages with an IC<sub>50</sub> value of 1.94 μM (Rossi et al., 2010), a value which is close to the IC<sub>50</sub> of the drug for suppression of LT production in cell-based assays (Carter et al., 1991). Suppression of the tumor-promoting mediator PGE<sub>2</sub> by zileuton was also observed in human whole blood at clinically achievable concentrations and in rats at standard doses. This raises concerns over the relationship between the drugs chemopreventive effects and suppression of 5-LO.

In contrast to this, some non-tumor cell types showed obvious susceptibility toward 5-LO products, such as freshly isolated murine neuronal stem cells, which produced considerable basal amounts of LTB<sub>4</sub> (~7 ng per 10<sup>6</sup> cells) and whose growth was suppressed

**Table 2 | Mitogenic effects of 5-LO products in cell culture assays.**

5-LO product	Cell type	Effect	Literature
LTB <sub>4</sub>	Colorectal cancer cells	Increase of cell proliferation	Qiao et al. (1995), Bortuzzo et al. (1996)
5-HETE, LTA <sub>4</sub>	Human malignant pleural mesothelial cells	Angiogenic and anti-apoptotic effects, in combination with potent up-regulation of vascular endothelial growth factor	Romano et al. (2001)
5-HETE, LTB <sub>4</sub>	Human pancreatic cancer cells	Stimulation of cell viability and proliferation via MAPK pathway	Ding et al. (2003), Tong et al. (2005)
LTD <sub>4</sub>	Intestinal epithelial cells	Increase of cell survival and cell proliferation possibly mediated via activation of wnt-signaling	Paruchuri et al. (2005), Mezhybovska et al. (2006)



by AA-861 at concentrations less than 1  $\mu\text{M}$  (Wada et al., 2006). Furthermore, zileuton, the only drug devoid of anti-proliferative and cytotoxic off-target effects (Fischer et al., 2010), was capable of inhibiting the proliferation of RAW 264.7 macrophages below 1  $\mu\text{M}$ . The macrophages considerably synthesized LTB<sub>4</sub> and addition of physiologically relevant concentrations of LTB<sub>4</sub> reversed the growth-inhibitory effects of zileuton (Nieves and Moreno, 2006). In sum, in many cases, the cytotoxic and chemopreventive effects 5-LO inhibitors in cell culture assays and in animal tumor models may derive from molecular mechanisms other than suppression of LT biosynthesis and warrant reassessment.

### MITOGENIC EFFECTS OF 5-LO PRODUCTS

Several studies reported accelerated proliferation of tumor cells in the presence of exogenously added 5-LO products and attenuated anti-proliferative effects by 5-LO inhibitors (see Mitogenic Effects of 5-LO Products, page 3, 2nd paragraph). The extreme concentrations frequently required to observe these effects, however, represent one caveat to these experiments; the final concentrations of 5-LO products used often exceeded those present in the medium of untreated cells by up to 10,000-fold (Ghosh and Myers, 1998; Hoque et al., 2005; Sveinbjornsson et al., 2008). Experiments using physiologically relevant concentrations will be one step forward to test the mitogenicity and the pleiotropic effects of 5-LO products. Experiments are also needed to exclude that high concentrations of these 5-LO products induce non-specific anti-apoptotic responses that may counteract diverse stimuli or drugs inducing the intrinsic pathway of apoptosis.

### LACKING EVIDENCE FOR A CLINICAL EFFICACY OF 5-LO INHIBITORS IN CANCER THERAPY

Few clinical trials have assessed the efficacy of anti-LT drugs in clinical cancer therapy. A randomized double-blind phase II study with the LTB<sub>4</sub> antagonist LY293111 in patients suffering from advanced

adenocarcinoma of the pancreas did not reveal any therapeutic benefit (Saif et al., 2009). This is a surprising result as a significant body of literature has demonstrated a remarkable susceptibility of pancreatic cancer cells toward anti-LT drugs in cell culture studies (see Cytotoxic Effects by 5-LO Inhibitors) and therefore suggests a certain lack of correlation between the effects of 5-LO inhibitors in cell culture assays and in patients. Notably, the mean  $C_{\max}$  plasma concentrations of the drug achieved in patients after a dosage of 600 mg BID were found to be 4.4  $\mu\text{M}$  (Schwartz et al., 2005) and should lead to almost complete suppression of LTB<sub>4</sub> signal transduction (Marder et al., 1995). Consequently, pleiotropic effects of the drug including modulation of PPAR (peroxisome proliferator-activated receptor) signal transduction are currently discussed (Adrian et al., 2008).

### CONCLUSION AND FUTURE DIRECTION

A considerable number of studies has provided evidence for a role of 5-LO in tumorigenesis. The well-recognized overexpression of 5-LO in various types of malignant cells, the reduction of tumor cell viability by 5-LO gene silencing approaches, as well as experiments involving 5-LO KO mice, together constitute a substantial rationale for this hypothesis. However, considering that the cytotoxic activity of 5-LO inhibitors is substance-specific and may, in many cases, not derive from inhibition of 5-LO activity, the traditional hypothesis that 5-LO products are the exclusive players in 5-LO-triggered tumorigenesis may warrant reconsideration. Experiments on the role of 5-LO product formation in proliferation of cultured tumor cells using high concentrations ( $>1 \mu\text{M}$ ) of certain 5-LO inhibitors may be misleading and the use of these agents as pharmacological tools should be critically considered. Also, possible indirect tumorigenic effects of 5-LO products (e.g., promotion of angiogenesis) with relevance toward the observed situation *in vivo* but not for cell culture assays should be taken into account. Notably, non-enzymatic functions, including an interaction with cytoskeleton proteins or with the adaptor protein Grb-2, involved in receptor tyrosine kinase (RTK)-dependent growth factor signaling, have been reported for 5-LO (Lepley and Fitzpatrick, 1994). Because of the crucial role of oncogenic RTK signaling in cancer progression, a disrupted growth factor signaling may contribute to the reduction in tumor cell viability by 5-LO gene silencing approaches. Thus, experiments that assess Grb-2-dependent growth factor signaling after 5-LO gene silencing may be instructive. Taken together, a broad body of evidence from the literature suggests a crucial, albeit poorly defined, role of 5-LO in tumorigenesis of several cancer types. Elucidation of the molecular mechanisms underlying these effects may include direct proliferative actions of 5-LO products on tumor cells as well as indirect and so far neglected effects of 5-LO and thereby draw novel connections between pathways that are currently regarded as unrelated.

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# Prostacyclin: an inflammatory paradox

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Prostacyclin ( $\text{PGI}_2$ ) is a member of the prostaglandin family of bioactive lipids. Its best-characterized role is in the cardiovascular system, where it is released by vascular endothelial cells, serving as a potent vasodilator and inhibitor of platelet aggregation. In recent years, prostacyclin ( $\text{PGI}_2$ ) has also been shown to promote differentiation and inhibit proliferation in vascular smooth muscle cells. In addition to these well-described homeostatic roles within the cardiovascular system, prostacyclin ( $\text{PGI}_2$ ) also plays an important role as an inflammatory mediator. In this review, we focus on the contribution of prostacyclin ( $\text{PGI}_2$ ) as both a pathophysiological mediator and therapeutic agent in three major inflammatory-mediated disease processes, namely rheumatoid arthritis, where it promotes disease progression ("pro-inflammatory"), along with pulmonary vascular disease and atherosclerosis, where it inhibits disease progression ("anti-inflammatory"). The emerging role of prostacyclin ( $\text{PGI}_2$ ) in this context provides new opportunities for understanding the complex molecular basis for inflammatory-related diseases, and insights into the development of current and future anti-inflammatory treatments.

**Keywords:** prostacyclin, IP receptor, inflammation, atherosclerosis, rheumatoid arthritis, pulmonary fibrosis

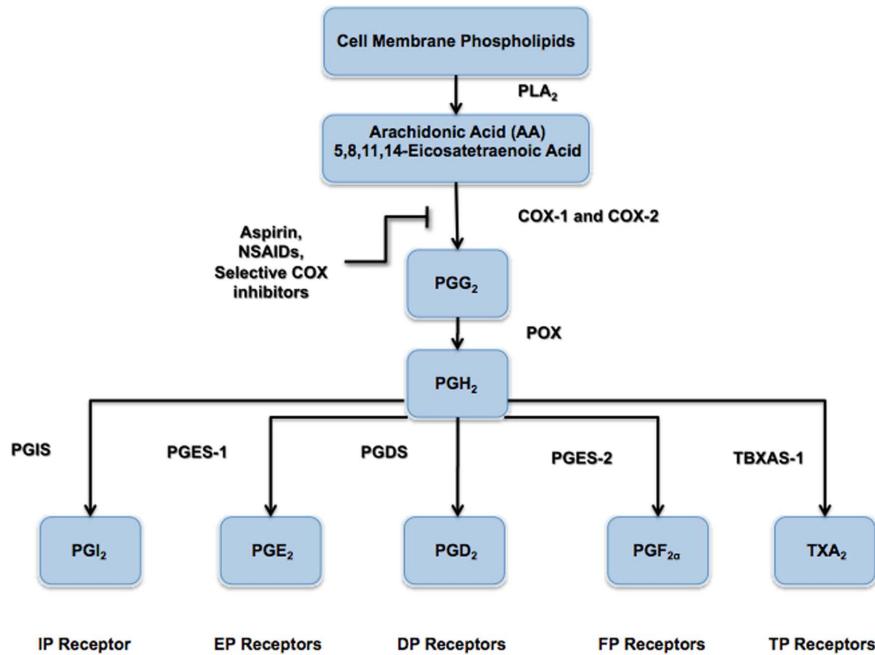
## PHARMACOLOGY OF PROSTACYCLIN AND ITS RECEPTOR

Prostacyclin ( $\text{PGI}_2$ ) is a member of the prostaglandin family of bioactive lipids, and is a derivative of the 20-carbon, omega-6 fatty acid, arachidonic acid (AA or 5,8,11,14-eicosatetraenoic acid). Both cyclooxygenase enzymes (COX-1 and COX-2) convert AA into the prostaglandin precursor PGH<sub>2</sub>, which is subsequently synthesized into prostacyclin ( $\text{PGI}_2$ ) via prostacyclin synthase (PGIS; **Figure 1**). However, the majority of  $\text{PGI}_2$  produced *in vivo*, particularly within the systemic and pulmonary vasculature (Moncada et al., 1977; Catella-Lawson et al., 1999; McAdam et al., 1999), and other regions like the synovium (Brodie et al., 1980; Crofford et al., 1994), appears to be derived from COX-2.  $\text{PGI}_2$  is unstable at physiological pH and, thus, has a very short half-life *in vivo* (<2 min), rapidly forming the inactive hydration product 6-keto-prostaglandin F1 $\alpha$  (6-keto-PGF<sub>1 $\alpha$</sub> ; Lewis and Dollery, 1983; Smyth and FitzGerald, 2002). The actions of  $\text{PGI}_2$  are mediated through a seven-transmembrane-spanning G-protein coupled receptor (GPCR), referred to as the IP receptor (International Union of Pharmacology nomenclature). The IP receptor is a Class A rhodopsin-like GPCR that couples predominately to the Gs subunit of the heterotrimeric G-protein and mediates intracellular signaling via adenylyl cyclase (AC) activation and cyclic AMP (cAMP) production (Boie et al., 1994). Animal studies have also shown that  $\text{PGI}_2$  may also signal through alternate Gq- and Gi-related pathways (Lawler et al., 2001), as well as nuclear receptor-mediated pathways, such as the peroxisome proliferator activated receptor gamma (PPAR $\delta$ ) pathway (Lim and Dey, 2002). Stitham et al. (2003) have elucidated the putative binding pocket for the human IP receptor, which has been reported to also accommodate type E prostanoids (i.e., PGE<sub>1</sub> and PGE<sub>2</sub>) in addition to its native ligand  $\text{PGI}_2$  and its analogs (Boie et al., 1994; Nakagawa et al., 1994). The physiological effects of  $\text{PGI}_2$  are vast with much remaining to be uncovered. Within the vasculature,  $\text{PGI}_2$  serves as a potent vasodilator and is the major inhibitory prostanoid in platelet aggregation (Smyth et al., 2009), and has also been shown

to inhibit vascular smooth muscle cell (VSMC) proliferation and de-differentiation (Fetalvero et al., 2006, 2007). Within the lungs,  $\text{PGI}_2$  reduces pulmonary blood pressure as well as bronchial hyper-responsiveness (Idzko et al., 2007). Within the kidneys,  $\text{PGI}_2$  serves to regulate renal blood flow and glomerular filtration rate, as well as mediates the release of renin (Komhoff et al., 1998). In the nervous system,  $\text{PGI}_2$  has been shown to elicit nociceptive pain response (Murata et al., 1997).

## PROSTACYCLIN AS AN INFLAMMATORY MEDIATOR

As described, prostacyclin ( $\text{PGI}_2$ ) is best known for its regulatory role within the cardiovascular system, where it promotes VSMC relaxation (vasodilatation) and inhibits platelet aggregation (anti-thrombotic). However, it is also an important inflammatory mediator. The seminal work by Vane (1971) demonstrating the inhibition of prostaglandin biosynthesis as the mechanism of action for aspirin (acetylsalicylic acid) and other aspirin-like drugs first highlighted the importance of the prostaglandin family of molecules, and set the stage for the development of many pharmacologic agents, such as traditional, non-selective non-steroidal anti-inflammatory drugs (tNSAIDs) and the newer selective COX-2 inhibitors. Further work by Davies et al. (1984) pinpointed particular prostaglandins, principally prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and prostacyclin ( $\text{PGI}_2$ ), in the mediation of vascular permeability associated with the hyperemia and edema seen with acute inflammation. Murata et al. (1997) demonstrated the involvement of prostacyclin ( $\text{PGI}_2$ )-mediated inflammatory swelling *in vivo*, using prostacyclin receptor deficient (IP $-/-$ ) mice. In these critical experiments, it was shown that mice lacking the prostacyclin receptor had a reduced inflammatory response, as measured by percent change in vascular permeability using a carrageenan-induced paw-edema model (Murata et al., 1997). Limb edema was decreased by approximately 50% in IP-deficient mice, similar to levels seen in mice pre-treated with the non-steroidal anti-inflammatory agent indomethacin. Moreover, a significant



**FIGURE 1 | Prostanoid biosynthesis pathway.** The enzyme phospholipase A2 (PLA2) hydrolyzes arachidonic acid (AA) from the phospholipids of the extracellular membrane. Arachidonic acid is modified by the cyclooxygenase (COX) enzymes (COX-1 and COX-2) to form the intermediate precursor prostaglandin G<sub>2</sub> (PGG<sub>2</sub>) via the addition of two oxygen (O<sub>2</sub>) molecules. Prostaglandin H<sub>2</sub> (PGH<sub>2</sub>) is subsequently formed by the actions of peroxidase

enzyme, which releases a single oxygen (O<sub>2</sub>) molecule. As shown, all prostanoids are derived from the parent compound PGH<sub>2</sub> and are formed via their respective synthase enzymes, namely prostaglandin I<sub>2</sub> synthase (PGIS), prostaglandin E<sub>2</sub> synthase (PGES-1), prostaglandin D<sub>2</sub> synthase (PGDS), prostaglandin F<sub>2α</sub> synthase (PGES-2), and thromboxane A<sub>2</sub> synthase (TBXAS-1).

reduction in lung exudate volume, using a carrageenan-induced pleurisy model, was also observed (although data not shown) for IP-deficient mice as well (Murata et al., 1997). In contrast, a study by Takahashi et al. (2002) demonstrated the IP-deficient mice showed higher skin and airway immune responses (relating to increased capillary permeability in these tissues) in antigen-sensitized inflammation, suggesting a protective role for PGI<sub>2</sub> in allergic inflammation. These studies (among others) have highlighted prostacyclin as a major endogenous mediator of inflammation – both pro-inflammatory and anti-inflammatory, depending upon the tissue and pathological model being studied (Figure 2).

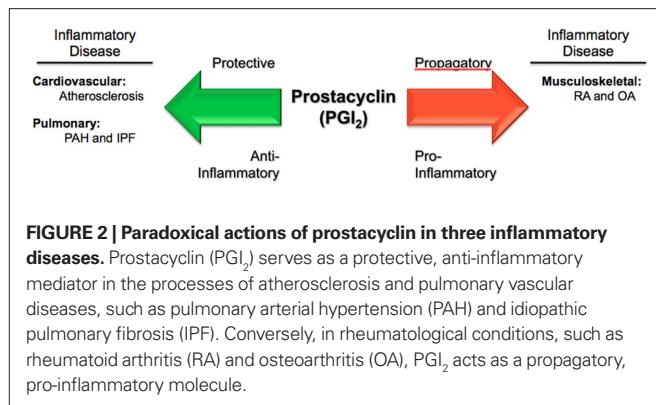
## ROLE OF PROSTACYCLIN IN ARTHRITIS

While the majority of focus has centered around the role of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) in rheumatoid arthritis (RA), some studies have shown that the predominate prostaglandin detected within the synovial fluid of patients with RA is in fact prostacyclin (PGI<sub>2</sub>; Brodie et al., 1980). Moreover, using both collagen-induced arthritis and collagen-antibody-induced arthritis models, Honda et al. (2006) showed that prostacyclin receptor knockout (IP<sup>-/-</sup>) mice exhibited significantly reduced clinical and histological arthritic scores versus control mice, in both arthritis models, placing further emphasis on receptor-mediated prostacyclin activity in the pathogenesis of RA. Using a K/BxN serum-transfer arthritis model, Chen et al. (2008) administered serum from arthritic K/BxN mice to induce an IgG-mediated autoantibody-induced inflammatory arthritis to recipient mice lacking either prostaglandin E synthase-1 (mPGES-1<sup>-/-</sup>)

or the prostacyclin receptor (IP<sup>-/-</sup>), in order to determine the relative importance of PGE<sub>2</sub> and PGI<sub>2</sub>, respectively. Findings revealed that mice deficient in prostaglandin E synthase-1 mPGES-1 (and therefore unable to produce PGE<sub>2</sub>), developed arthritis in normal fashion, whereas mice lacking the receptor for PGI<sub>2</sub> demonstrated a significant decrease (31% versus wild-type) in clinical arthritis. Furthermore, using COX-1 and COX-2 knockout animals, it was shown that mice lacking the COX-1 isoform were resistant to the development of arthritic disease, while those lacking the COX-2 isoform remained vulnerable (Wang et al., 2008).

These results convey two important points: (1) a substantial proportion of the prostanoid contribution to joint inflammation (at least in the K/BxN serum-transfer arthritis model) can be accounted for by PGI<sub>2</sub> and its interaction with the IP receptor, and (2) selective COX-1 inhibition through genetic knockout prevented the development of disease, suggesting that COX-1-derived PGI<sub>2</sub> is the major inflammatory mediator within this arthritis model (Wang et al., 2008). More importantly, such studies lend weight to the involvement of prostacyclin in *chronic* inflammatory disease processes, as well as being an *acute* mediator, and also call into question the paradigm regarding COX-1- and COX-2-derived prostaglandin functions *in vivo* (i.e., regulatory “housekeeping” versus inflammatory induction).

Interestingly, from the perspective of clinical therapies, there does not seem to be a difference in efficacy according to COX selectivity. As Chen et al. (2008) showed, meta-analysis from a systematic review of 145 randomized controlled trials, examining the clinical



effectiveness of a variety of COX-2 inhibitors (including etodolac, meloxicam, celecoxib, rofecoxib, etoricoxib, valdecoxib, and lumiracoxib), showed similar efficacy compared to non-selective NSAIDs (including naproxen, diclofenac, ibuprofen, loxoprofen, nabumetone, piroxicam, indomethacin, tenoxicam, and nimesulide) in the symptomatic relief of both RA and osteoarthritis (OA), but with superior gastrointestinal tolerability and protection against complicated upper gastrointestinal events (e.g., ulcers, bleeding, perforations) – the majority of evidence coming from OA populations. However, the amount of evidence for this gastro-protective effect varied considerably across individual drugs. Moreover, an increased risk of myocardial infarction (MI) was also observed among those drugs with greater volume of evidence in terms of exposure in patient-years (Chen et al., 2008), presumably as a by-product of the discriminating suppression of COX-2-derived  $\text{PGI}_2$ , which has been shown to be cardioprotective (Murata et al., 1997; Cheng et al., 2002; Egan et al., 2004). Along these same lines, as chronic inflammation has been linked to enhanced development of atherogenesis (Libby et al., 2002), individuals with RA may already be at increased risk for cardiovascular disease. In fact, a recent comparative study involving disease-duration-matched RA and diabetes mellitus (DM) patients found that RA was a substantial and independent cardiovascular risk factor, with similar severity to DM, with significantly worsened preclinical atherosclerotic markers, increased intima-to-media thickness, as well as lower flow-mediated dilatation (measure of endothelial function; Stamatelopoulos et al., 2009). Thus, the combination of chronic RA-mediated inflammation, perpetuating an increased atherosclerotic burden, along with COX inhibition therapy – either selective (COX-2 inhibitor) or non-selective (NSAIDs) – would undoubtedly increase cardiovascular risk drastically, given what we now know about  $\text{PGI}_2$ . Thus,  $\text{PGI}_2$  is emerging as an important intermediary in inflammatory conditions such as rheumatoid and OA, with the dualistic purpose of pro-inflammatory mediator on the one hand – involved in disease pathophysiology – and cardioprotective factor on the other – both of which are central in the consideration of pharmacologic therapies and adverse effects.

## ROLE OF PROSTACYCLIN IN PULMONARY FIBROSIS AND PULMONARY HYPERTENSION

While pulmonary fibrosis and pulmonary hypertension are distinct pathophysiological entities, they do share some commonalities, some of which involve  $\text{PGI}_2$ . Pulmonary arterial hypertension

(PAH) is in fact a heterogeneous group of diseases sharing similarities in pathophysiological mechanisms, clinical presentation, and therapeutic approaches (Simonneau et al., 2009). The pathogenesis of PAH is complex and incompletely understood, with both genetic and environmental factors contributing to altered vascular structure and function (Badesch et al., 2007). The main vascular changes in PAH are vasoconstriction, VSMC proliferation, endothelial loss or dysfunction, and thrombosis (Farber and Loscalzo, 2004), which implicates a disruption of vascular hemostasis and its principle mediators, particularly  $\text{PGI}_2$  and  $\text{TxA}_2$ , among others. This is evidenced by findings in patients with PAH, whereby both the production of prostacyclin synthase (PGIS) within small-to-medium pulmonary arteries, as well as urinary metabolites (6-keto-prostacyclin  $\text{F2}\alpha$ ) of prostacyclin, were shown to be decreased, while levels of thromboxane metabolites (thromboxane B2) were increased (Christman et al., 1992; Tuder et al., 1999). Interestingly, this imbalance of  $\text{PGI}_2$  and  $\text{TxA}_2$  within the pulmonary vasculature, leading to increased mean pulmonary artery pressure, mimics that of the cardio-systemic vasculature system. In fact, many of the pathophysiological mechanisms identified in PAH overlap with those involved in atherogenesis, including vascular smooth muscle and endothelial cell dysfunction, enhanced platelet activity and thrombosis, inflammation, and cellular chemotaxis (Essop, 2010). Owing to its potent vasodilatory, anti-thrombotic, and anti-proliferative effects,  $\text{PGI}_2$  has secured a central role in the treatment of PAH. Continuous intravenous epoprostenol (synthetic  $\text{PGI}_2$ ) is the best-studied advanced therapy for PAH and remains a first-line agent, particularly for those with severe disease (WHO functional class IV), as it has been shown to improve overall symptoms, exercise capacity, and hemodynamic status in controlled clinical trials (Barst et al., 2009), as well as confer a survival benefit in both idiopathic and heritable forms of PAH (IPAH and HPAH; Barst et al., 2009). There are limitations to treatment with epoprostenol based upon its pharmacology (plasma half-life = 3–5 min) and long-term use requires a permanent central venous catheter and a portable infusion pump. Analogs of  $\text{PGI}_2$  have also been used clinically, and are administered by a variety of routes, including intravenously (e.g., treprostinil and iloprost), subcutaneously (e.g., treprostinil), inhalation (e.g., iloprost), and orally (e.g., beraprost). These medications are generally more stable with longer half-lives, but have variable safety and efficacy equivalencies compared to epoprostenol, and clinical trials with these (and other) alternative agents are limited with respect to severe disease classification (Barst et al., 1996). Other vasoactive therapies for PAH include the dual endothelin receptor ( $\text{ET}_A$  and  $\text{ET}_B$ ) antagonist (e.g., bosentan) and phosphodiesterase type-5 (PDE-5) inhibitors, which have been proven effective, alone or in combination therapy, in milder forms of PAH (WHO functional class II and III; Rubin et al., 2002; Sitbon et al., 2003; Galie et al., 2005; McLaughlin et al., 2005; Pepke-Zaba et al., 2008). Again, the central role of  $\text{PGI}_2$ , as both an inflammatory and hemodynamic mediator, puts it at the forefront in understanding the pathophysiology and pharmacological treatment of pulmonary vascular diseases, particularly PAH.

Interestingly,  $\text{PGI}_2$  seems to have a similar safeguarding effect in the chronic inflammatory condition of idiopathic pulmonary fibrosis (IPF), as recent studies have shown that COX-2-derived  $\text{PGI}_2$  serves a protective role against bleomycin-induced pulmonary fibrosis – a

major animal model for IPF that mimics the progressive fibrosis and interstitial inflammation of sub-pleural lung tissue in humans. In a study by Lovgren et al. (2006), IP-deficient mice were more susceptible to bleomycin-induced pulmonary fibrosis, demonstrating increased collagen deposition and cellularity after bleomycin administration compared with the wild-type mice. These observations correlated with increases in quantitative measurements of histological lung scores and hydroxyproline levels within the lung parenchyma. Similar results were found using COX-2-deficient mice, but were not supported using knockouts for either the EP2 or EP4 receptors, which bind COX-2-derived prostaglandin E<sub>2</sub> (PGE<sub>2</sub>; Lovgren et al., 2006). Such findings put direct focus on the loss of COX-2-derived prostacyclin as a protective factor. While COX-2-derived prostacyclin is now well known for its protective effects within the cardiovascular system, such results provide compelling evidence for PGI<sub>2</sub>-mediated protection against fibrotic pathologies as well.

A more recent animal study by Zhu et al. (2010) also confirms these findings, pharmacologically, and identifies PGI<sub>2</sub> as a potential new therapeutic agent for pulmonary fibrotic disease. Using intra-peritoneal injections of iloprost, a stable PGI<sub>2</sub> analog, it was demonstrated that a single dose of iloprost (200 µg/kg; prior to bleomycin injection) could preclude pulmonary inflammation and fibrosis in mice (Zhu et al., 2010). Pre-treatment with iloprost seemed to significantly reduce both inflammatory infiltration and collagen deposition with the pulmonary interstitium, as well as improve lung mechanics (reduced static compliance and elevated tissue elastance; Zhu et al., 2010). However, the specific inflammatory cell subtype being suppressed could not be delineated in this current study. Iloprost pre-treatment decreased production of pro-inflammatory and fibrotic cytokines, such as TNF-alpha, IL-6, and TGF-beta-1, and increased release of anti-fibrotic mediators, including IFN-gamma and chemokine CXCL10/IP-10, as measured by mRNA expression levels or ELISA. Moreover, the cumulative mortality in iloprost-treated mice was 10% at day 21 versus 60% in the non-iloprost-treated cohort (Zhu et al., 2010).

In human studies, inhaled iloprost has been proven efficacious in the treatment of various forms of pulmonary hypertension, including pulmonary hypertension secondary to pulmonary fibrosis (Olschewski et al., 1999). In fact, it has been suggested that the majority of vascular resistance in fibrotic lung disease is due to persistent vasoconstriction (Olschewski et al., 1999), which may explain the effectiveness of PGI<sub>2</sub> analog therapy. Certain studies also suggest a long-term clinical benefit from continued therapy with inhaled iloprost, which was well tolerated and required no substantial dose increase over a 2-year trial (Olschewski et al., 2010). In the United States, its use has been approved for PAH New York Heart Association (NYHA) functional class III and IV; for individuals with marked limitations or inability to carry on physical activity (Gomberg-Maitland and Olschewski, 2008). Thus, PGI<sub>2</sub> seems to be an important effector in both these fibro-proliferative disorders of the lung, playing both a protective role against disease development, as well as a therapeutic role in symptom management.

## ROLE OF PROSTACYCLIN IN ATHEROSCLEROSIS

Atherosclerosis is now known as an inflammatory disease, with the same complex cellular interactions involving monocytes, macrophages, lymphocytes, extracellular matrix (ECM) components, and

connective tissue cells as seen in other chronic inflammatory and fibro-proliferative diseases (e.g., RA, pulmonary fibrosis, glomerulosclerosis; Ross, 1999). The critical role of PGI<sub>2</sub> in atherosclerosis is quickly emerging, with evidence spanning from molecular and cell biology to human clinical trials. Mounting data has demonstrated the protective effect of prostacyclin activity against the development of atherothrombotic cardiovascular disease through the inhibition of various cellular processes, including platelet activation, leukocyte adhesion, as well as VSMC modulation. As such, PGI<sub>2</sub> analogs (e.g., iloprost) are able to down-regulate lymphocyte adhesion to endothelial cells, which suggests an ability to block the early events in atherosclerosis (Della Bella et al., 2001). Furthermore, interactions within the realm of lipid metabolism has lent further support toward the atheroprotective properties of PGI<sub>2</sub> (Thiemermann, 1991). Specifically, HDL has been shown to induce COX-2 expression and PGI<sub>2</sub> production in both endothelial and VSMCs (Pomerantz et al., 1985; Vinals et al., 1997, 1999) while, conversely, PGI<sub>2</sub> has been shown to induce cholesterol ester hydrolase activity, which catalyzes the first step in the removal of cholesterol from foam cells, critical components in atherogenesis (Hajjar and Weksler, 1983; Weksler et al., 1983; Hajjar et al., 1989). The effects of PGI<sub>2</sub> on VSMCs are becoming an important target for understanding both the pathophysiology of atherothrombosis and the atheroprotective effects of prostacyclin. In mature blood vessels, VSMCs are quiescent and exhibit a differentiated, contractile phenotype. However, in response to vascular injury, these cells have been shown to re-enter the cell cycle, proliferate, migrate toward attractants, down-regulate expression of contractile proteins, and up-regulate synthesis of proteins, particularly ECM (Campbell et al., 1988). PGI<sub>2</sub> has been shown to exert both anti-proliferative (Grosser et al., 1995; Zucker et al., 1998) and anti-migratory (Blindt et al., 2002) effects on smooth muscle cells. In advanced atherosclerotic lesions (as well as restenotic lesions), expression levels of smooth-muscle-specific differentiation markers are markedly reduced (Wilcox, 1992; O'Brien et al., 1993), and as Fetalvero et al. (2006) have shown, treatment with the stable PGI<sub>2</sub> analog, iloprost, induces VSMC differentiation via a cAMP-PKA-mediated signaling pathway. In similar fashion, Kasza et al. (2009) went on to further show that, in addition to increased contractile protein expression and contractile morphology, iloprost-treated VSMCs up-regulate COX-2 expression, mediated not only by cAMP-PKA, but also novel pathways involving ERK-1/2 activation and Akt-1 inhibition. In turn, the up-regulated COX-2 expression lead to subsequent PGI<sub>2</sub> release (i.e., prostacyclin-induced prostacyclin release), which was shown to have a paracrine, positive-feedback effect on neighboring VSMCs (not exposed to iloprost), inducing similar cellular responses (Kasza et al., 2009). Thus, there appears to be a clear link between the major atheroprotective effects of prostacyclin and VSMC modulation. As such, the phenotypic change in VSMCs toward a proliferative and de-differentiated state, which is a hallmark occurrence in the progression of atherosclerosis and restenosis, necessitates a clear understanding of this regulatory process and is an extremely important area of research.

Mouse models have provided valuable insight into the role of prostacyclin in cardiovascular homeostasis and pathogenesis. IP-deficient (IP<sup>-/-</sup>) mice display increased propensities toward thrombosis (Murata et al., 1997), intimal hyperplasia

and restenosis (Cheng et al., 2002), and reperfusion injury (Xiao et al., 2001). Moreover, in both atherogenic apolipoprotein E (apo-E)- and low-density lipoprotein receptor (LDL-R)-deficient backgrounds, mice lacking the IP receptor demonstrated greater atherosclerotic burden with higher platelet reactivity and leukocyte adhesion to endothelial cells (Egan et al., 2004; Kobayashi et al., 2004). Moreover, Egan et al. (2004) went on to further demonstrate that, in female pre-menopausal LDL-R knockout mice, the atheroprotective effect of estrogen is significantly reduced in the absence of the IP receptor (i.e., double LDL-R and IP knockout). Moreover, as long-term estrogen exposure was shown to increase COX-2 expression, as well as the formation of the PGI<sub>2</sub> metabolite 6-keto-PGF<sub>1α</sub> in mouse aortic smooth muscle cells (mASMCs), this study suggests a significant contribution of estrogen-mediated, COX-2-derived PGI<sub>2</sub> in the protection against atherogenesis (Egan et al., 2004). Such *in vivo* findings highlight the important functional presence of prostacyclin activity in the maintenance of cardiovascular homeostasis and, in turn, implicate receptor-ligand (hIP-PGI<sub>2</sub>) dysfunction in the acceleration of atherogenesis and the subsequent development of related disorders, including stroke, MI, and hypertension. In humans, the importance of PGI<sub>2</sub> in atherogenesis has also been shown. Arehart et al. (2008) demonstrated that patients harboring a dysfunctional human prostacyclin receptor variant (R212C) exhibited an enhanced atherothrombotic phenotype, with a higher incidence of triple-vessel coronary artery disease (CAD) and greater number of clinical cardiovascular events (including MI, stroke, PTCA, CABG, PVD, and unstable angina) in high-risk patients versus age- and risk-factor-matched normal-allele patients. Another study by Patrignani et al. (2008) correlated the R212C prostacyclin receptor polymorphism, as well as two other synonymous variants, with intimal hyperplasia and progressive deep venous thrombosis, respectively. Biochemical analyses have revealed potential mechanistic explanations for these R212C disease association studies. Ibrahim et al. (2010) demonstrated that co-expression of the R212C variant, together with wild-type hIP, resulted in dominant-negative inhibition of signaling through receptor homo-dimerization, with enhanced wild-type IP localization to the endoplasmic reticulum. Interestingly, a similar effect was observed with co-expression of the R212C variant and the wild-type thromboxane receptor α (TPα), which revealed R212C hetero-dimerization, with subsequent inhibition of TPα receptor activity (Ibrahim et al., 2010). Since this time, Stitham, Arehart, and colleagues have identified other functionally deficient prostacyclin receptor polymorphisms (i.e., R215C and L104R) associated with increased CAD (Ibrahim et al., 2010). While such mutations are rare, it is notable that single, heterozygous, point mutations within the prostacyclin receptor gene (*PTGIR*) are associated with clinically significant disease changes.

Similarly, mutations and polymorphisms in the prostacyclin synthase (*PGIS*) gene have been associated with essential hypertension, MI, and cerebral infarction (Iwai et al., 1999; Nakayama, 2005). The sum of these results correspond not only with the aforementioned IP knockout mouse studies, but also parallel findings from the world-wide withdrawal of the selective COX-2 inhibitors (e.g., rofecoxib and valdecoxib) whose discriminating suppression of COX-2-derived prostacyclin (PGI<sub>2</sub>) resulted in increased risk of cardiovascular events (e.g., MI and thrombotic stroke), particularly in

predisposed patients (Fitzgerald, 2004; White et al., 2004; Bresalier et al., 2005). These latter findings relating adverse cardiovascular events to unmatched suppression of COX-2-derived PGI<sub>2</sub> have been widely discussed and demonstrate that a disrupted balance between PGI<sub>2</sub> and TXA<sub>2</sub>—favoring unopposed COX-1-derived TXA<sub>2</sub> production—is responsible, at least in fair part, for pro-thrombotic and perhaps pro-atherogenic effects (McAdam et al., 1999; Vane, 2002). In a recent opinion article, Rovati et al. (2010) proposed that concomitant TP receptor antagonism, along with selective COX-2 inhibition (dual COXIB-TP antagonists), may abrogate such adverse cardiovascular events (caused by the imbalance between PGI<sub>2</sub> and TXA<sub>2</sub>) and improve the safety profile of selective COX-2 inhibitors. Other groups have proposed this concept as well, but clinical trials have yet to be pursued. As another approach, Capone et al. (2010) suggest assessment of COX-2 activity in whole blood *ex vivo*, perhaps in combination with biomarkers—such as biochemical (urinary levels of 6-keto-PGF<sub>1α</sub>) and genetic (IP receptor and other prostacyclin-related polymorphisms)—as potential surrogate endpoints to assess for prostacyclin synthesis *in vivo* as a predictor of cardiovascular risk.

## NOVEL PROSTACYCLIN-RELATED THERAPIES IN INFLAMMATION

As the role of PGI<sub>2</sub> is becoming more defined in inflammatory-related diseases, the development of novel agonists and antagonists for the IP receptor is at the forefront of research. As previously stated, the role of PGI<sub>2</sub> in arthritic diseases (RA and OA) is one of a pro-inflammatory mediator, and the use of wide-ranging inhibitors of prostaglandin synthesis (NSAIDs and selective COX-2 inhibitors) has remained a mainstay of therapy. However, targeted antagonism of PGI<sub>2</sub> activity has proven effective in reducing pain and inflammation in preclinical trials. Using a mono-iodoacetate (MIA)-induced rodent model of chronic OA, Pulichino et al. (2006) have shown that a novel arylamide compound (Keitz et al., 2004) with specific IP receptor antagonism significantly reduced joint discomfort in a dose-dependent manner, and with similar efficacy to diclofenac as well as an MF-tricyclic COX-2 inhibitor. Furthermore, in a collagen-antibody-induced model of RA, the same IP antagonist reduced mean scores for all arthritic parameters by 93% (AUC of the clinical scores) when given in prophylactic mode 1 day prior to collagen antibody injection, in IP+/- mice (Pulichino et al., 2006). Comparably, a 91% reduction in arthritic scores was observed for IP−/− mice and a 98% reduction for COX-2-treated mice. Interestingly, treatment in therapeutic mode (6 days post-injection) had no effect on clinical scores (Pulichino et al., 2006). However, as discussed earlier, targeted PGI<sub>2</sub> antagonism has the potential for increased risk of adverse cardiovascular effects, which may be even more pronounced than with broader-spectrum NSAIDs or selective COX-2 inhibitor therapies.

For the treatment of pulmonary vascular disease such as PAH and IPF, the vasoactive PGI<sub>2</sub> analog formulations (e.g., epoprostenol, treprostinil, and iloprost) are all in clinical use, but are reserved for advanced therapy for persistent disease (Barst et al., 2009). Moreover, these agents have limitations, including short half-lives, parenteral (non-oral) routes of administration, and heterogeneous therapeutic response. Thus, the quest for novel therapies for these diseases is ongoing. In preclinical studies of

both PAH and IPF, a novel, non-prostanoid prostacyclin receptor agonist (ONO-1301, Toray Industries) that also has potent inhibitory activity against thromboxane synthase (TBXAS1) has shown promise. Murakami et al. (2006) have demonstrated attenuation of bleomycin-induced pulmonary fibrosis, as well as improved survival in mice, using ONO-1301. Kataoka et al. (2005) demonstrated similar protective results in a monocrotaline-induced PAH model, whereby rats treated with ONO-1301 showed improved pulmonary hemodynamics and survival, along with a reduction of vascular remodeling (media hypertrophy) and plasma TxA<sub>2</sub> metabolites. Moreover, the pharmacokinetic profile (half-life approximately 5.6 h) of ONO-1301 appears to be better than some of the PGI<sub>2</sub> analogs in current therapeutic use (Antoniu, 2006) and, thus, if the same clinical efficacy can be proved in humans, ONO-1301 could be a new therapy for PAH and IPF. In lieu of this, several new orally available, non-prostanoid, selective IP receptor agonists have reached clinical trial status for PAH, including APD811 (Arena Pharmaceuticals), which is in Phase I and NS-304, a.k.a., Selexipag (Actelion Pharmaceuticals; Kuwano et al., 2007, 2008), which is currently in Phase III.

With the imbalance between PGI<sub>2</sub> and TxA<sub>2</sub> playing an important role in atherogenesis and atherothrombosis, the development of dual-acting compounds seems like a promising direction for the development of novel therapeutic agents. In a similar fashion to the aforementioned dual COXIB-TP antagonists, both Miyamoto and Yamada have demonstrated the potential for TRA-418 (Toray Industries), a novel compound with both PGI<sub>2</sub> (IP receptor) agonistic and TxA<sub>2</sub> (TP receptor) antagonistic activities (Miyamoto et al., 2003; Yamada et al., 2003). *In vitro* studies showed that TRA-418 inhibited platelet aggregation through impedance of both glycoprotein IIb/IIIa (GPIIb/IIIa) activation and P-selectin expression, which are key markers of platelet activation (Miyamoto et al., 2003). Moreover, the TRA-814 compound was also shown to inhibit platelet-leukocyte complex formation in a dose-dependent manner (Miyamoto et al., 2010). These results suggest that this compound may be useful as both an anti-thrombotic and anti-atherogenic agent. Similarly, Ohno et al. (2005) have developed a dual-acting benzofuran compound (Toray Industries) that

possesses similar PGI<sub>2</sub> agonism and TxA<sub>2</sub> antagonism, which maintains potent anti-platelet activity with minimal effect on blood vessel dilation.

Thus, while the current pool of PGI<sub>2</sub>-centered compounds is primarily investigational, and clinical safety and efficacy in human disease still largely unproven, their development emphasizes the centrality of PGI<sub>2</sub> activity in various inflammatory-mediated pathologies, and provides exciting new directions to steer drug development.

## CONCLUSION

The culmination of data presented in this review reinforces the notion that Bunting et al. (1983) put forth almost 30 years ago – that a dynamic balance between the prostaglandins prostacyclin (PGI<sub>2</sub>) and thromboxane A<sub>2</sub> (TxA<sub>2</sub>; in addition to many other mediators) is crucial in maintaining cardiovascular homeostasis, and has critical pathophysiological and therapeutic implications. However, we are just now realizing the potential breadth and scope of this seminal proposition with PGI<sub>2</sub> playing a central role. Our current knowledge of PGI<sub>2</sub>, as both a physiological-pathophysiological mediator and therapeutic agent, in a host of inflammatory-related diseases, is growing rapidly. As demonstrated, PGI<sub>2</sub> has been shown to play protective roles in atherogenesis – relating to CAD, MI, stroke, and other cardiovascular abnormalities. It has also been shown to be involved in certain fibro-proliferative and pulmonary vascular diseases, such as IPF and PAH, where it serves as both a protective factor and first-line pharmacotherapy. Furthermore, in the setting of RA, PGI<sub>2</sub> seems to play a pro-inflammatory role and, as evidence increases, perhaps may one day be considered a therapeutic target in RA and related disorders (Figure 2). Further study of this important prostaglandin, in both the realms of basic science and clinical medicine, is needed and will undoubtedly yield new insights into inflammatory disorders and pharmacological treatments.

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# MicroRNA involved in inflammation: control of eicosanoid pathway

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MicroRNAs (miRNAs) have emerged as important regulators in human physiological and pathological processes. Recent investigations implicated the involvement of miRNAs in the immune system development and function and demonstrated an unexpected new regulatory level. We summarize the current knowledge about miRNA control in the development of the immune system and discuss their role in the immune and inflammatory responses with a special focus on eicosanoid signaling.

**Keywords:** microRNA, immune system, eicosanoids, arachidonic acid cascade, inflammation

## INTRODUCTION

MiRNAs are small non-coding RNAs that control gene expression in many cellular processes (He and Hannon, 2004; Grosshans and Filipowicz, 2008). In 1993, *lin-4* was the first miRNA that was discovered in the nematode *Caenorhabditis elegans* and was found to regulate the gene *lin-14* on posttranscriptional level during *C. elegans* development (Wightman et al., 1993). Shortly after, a second small miRNA involved in worm development, *let-7*, was identified. However, at this time it was assumed that these RNAs are rare exceptions and only present in nematodes. In 2001, three independent publications reported the existence of several hundreds of these small non-coding RNAs not only in nematodes but also in murine and human cells. In the meantime, more than 1000 miRNAs have been identified (<http://www.mirbase.org>) and have emerged as important regulators of gene expression. They play a key role in many physiological processes such as hematopoiesis, cell proliferation, tissue differentiation, cell type maintenance, apoptosis, signal transduction, organ development (Carissimi et al., 2009) but also in tumorigenesis (Farazi et al., 2010).

Recent reports showed that miRNAs are important control elements in the mammalian immune system. Distinct expression patterns in various hematopoietic organs and cell types points to an important role in immune system development, homeostasis and response, and dysregulation can result in pathological inflammatory responses and cancer (Baltimore et al., 2008; Liang and Qin, 2009; Sonkoly and Pivarcsi, 2009). Here, we will give a short overview about miRNAs important in the development of the immune system and immune and inflammatory responses with a special focus on eicosanoids as important mediators of the inflammatory reactions.

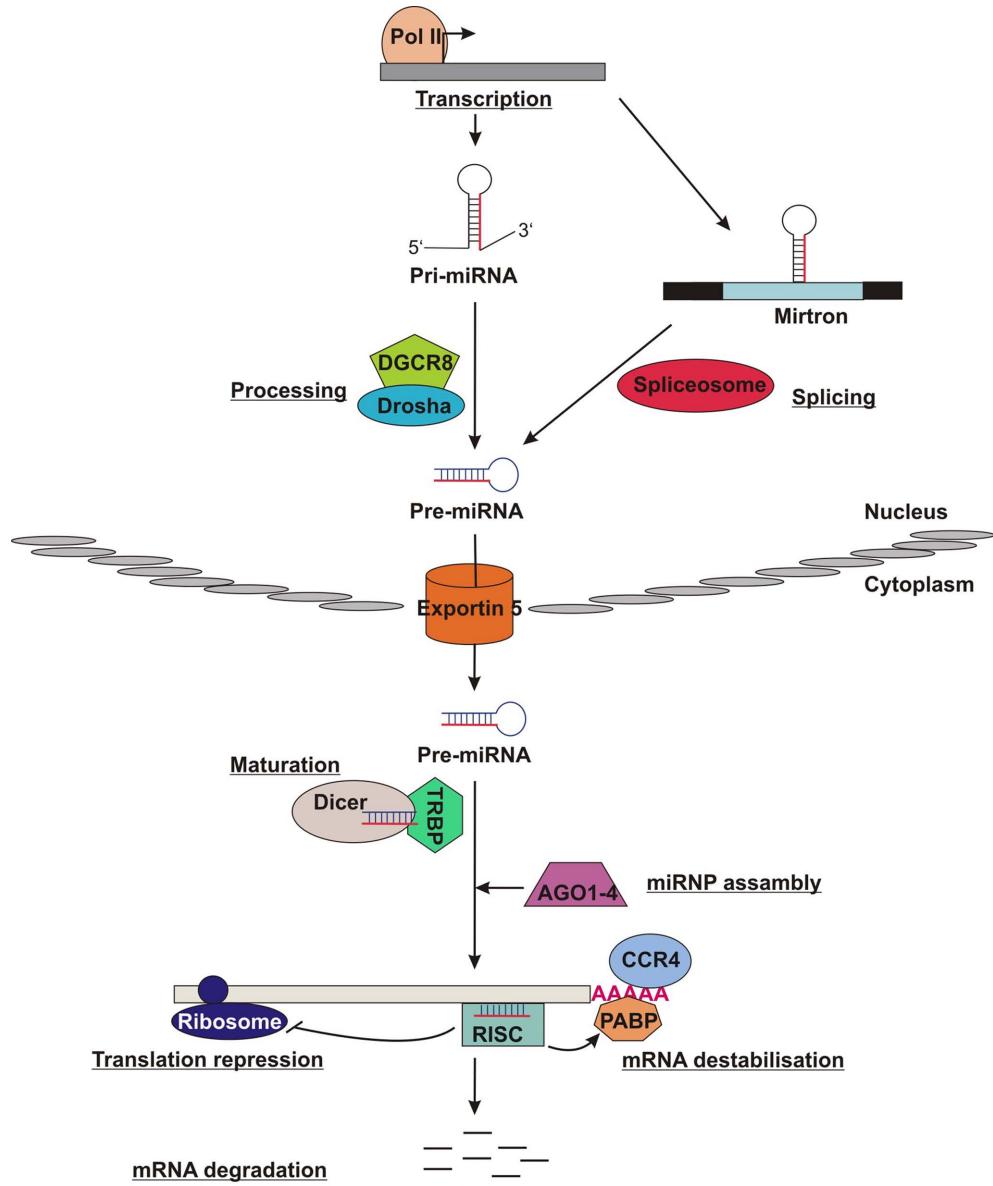
## BIOGENESIS PATHWAY OF miRNAs

MiRNAs are transcribed as long primary transcripts by RNA polymerase II to produce pri-miRNAs (Figure 1). Canonical miRNAs

are subsequently cleaved by Drosha, a member of the RNase-III enzyme family, together with DGCR8, a double-stranded RNA-binding protein (dsRBP). It results in ~70-nucleotide precursors (pre-miRNAs) containing imperfect stem loop structures (Lee et al., 2003; He and Hannon, 2004; Carissimi et al., 2009). About 40% of the currently known miRNAs are located within introns (mirtrons) which are processed by the spliceosome. Pre-miRNAs are exported from the nucleus to the cytosol by a RanGTP-dependent export complex containing Exportin 5 (Lund et al., 2004; Du and Zamore, 2005). In the cytosol, pre-miRNAs are subsequently processed by Dicer, another member of the RNase-III enzyme family, together with the dsRBP TRBP to short 21–23 nucleotide miRNA duplexes (Hutvagner et al., 2001). Only one strand of the miRNA duplex is being incorporated into a ribonucleoprotein complex (miRNP), also known as RNA-induced silencing complex (RISC) whose core components are the Argonaute family proteins (Ago1–4). The other strand is rapidly degraded (Khvorova et al., 2003; Schwarz et al., 2003). MiRNPs are then directed to their binding sites in the 3' untranslated region (UTR) of the target mRNA and mediate either translational repression by interaction with the translational machinery or mRNA destabilization through interaction with CCR4. In contrast, miRNAs direct mRNA cleavage when their sequences perfectly match the target mRNA (Lewis et al., 2005; Baek et al., 2008).

## miRNAs INVOLVED IN IMMUNE SYSTEM DEVELOPMENT

Recently, various miRNAs involved in the regulation of the mammalian immune system have been identified. First evidence came from the overexpression of miRNAs in hematopoietic stem cells which strongly affected B cell development after transplantation in mice (Chen et al., 2004). This observation was further supported by inactivation of components of the miRNA machinery which severely compromises lymphocyte development (Xiao and Rajewsky, 2009). Conditional inactivation of Dicer in T and B



**FIGURE 1 | MiRNAs pathway.** MiRNAs are transcribed as precursors (pri-miRNA) by RNA polymerase II. The pri-miRNA are cleaved by Drosha together with DGCR8 into 70-nucleotide stem loop known as pre-miRNA, in contrast mirtrons are processed by the spliceosome. The pre-miRNAs are transported to the cytosol by Exportin 5, where they are further processed by

Dicer together with TRBP to mature miRNA. The mature miRNA, indicated in red, is incorporated into a ribonucleoprotein complex (miRNP), also known as RNA-induced silencing complex (RISC) whose core components are the Argonaute family proteins (Ago1–4). miRNPs either mediate mRNA destabilization, translational repression, or mRNA cleavage.

lymphocytes resulted in an up to 10-fold reduction in total lymphocyte amount where regulatory T cells were mostly affected (Cobb et al., 2005). A conditional deletion of Ago2 compromises the development of B and erythroid cells (O'Carroll et al., 2007) whereas Dicer knockout seems to completely block the pro- to pre-B cell transition (Koralov et al., 2008). Subsequently, expression profiling combined with bioinformatic analyses attempted to identify individual miRNAs responsible for these phenotypes to explain the effect of miRNAs on B and T cell homeostasis and response.

One prominent example is miR-150. The miRNA shows significant changes in the expression levels during lymphoid development. It is highly expressed in mature B and T cells, but not in their progenitors (Monticelli et al., 2005; Zhou et al., 2007). One of the predicted targets of miR-150 was c-Myb, which is a transcription factor controlling multiple steps of lymphocyte development. Rajewsky and coworkers have demonstrated that miR-150 controls c-Myb expression *in vivo*. They further showed that the partial block of B cell development through miR-150 expression was indeed due to downregulation of c-Myb (Xiao et al., 2007).

These findings highlight that miR-150 is responsible for the transition to the pro-B to the pre-B cell stage during B cell differentiation (Xiao et al., 2007). In addition, miR-150 overexpression can restore correct T cell differentiation in Dicer deficient T cells.

MiR-155 is a further example of a miRNA with a specific function in lymphoid differentiation. Its expression is increased during activation of B and T cells as well as in activated monocytes (Vasilatou et al., 2010). Rajewsky and coworkers analyzed the role of miR-155 in regulating T helper cell differentiation into T helper cell 1 ( $T_{H}1$ ) and  $T_{H}2$ . Furthermore, miR-155 knockout mice indicated that miR-155 is required for an optimal T cell-dependent antibody response. MiR-155 exerts this control by regulation of cytokine production, e.g., interleukin-4 (IL-4) and interferon- $\gamma$  (IFN- $\gamma$ ; Thai et al., 2007). Bradley and coworkers performed a transcriptome analysis of mice deficient for B cell integration cluster/miR-155 (*bic/miR-155*) and identified miR-155 regulated genes, including various cytokines, chemokines, and transcription factors (Rodriguez et al., 2007).

MiR-181a has also been identified as a positive regulator of B lymphocyte differentiation. Furthermore, it is involved in thymic T cell differentiation by activation of the T cell receptors (Chen et al., 2004; Li et al., 2007).

A regulatory circuit involving miR-17-5p, miR-20a, miR-106a, and the transcription factors AML1 and M-CSF have been shown to control monocytogenesis. The miRNAs are downregulated in unilineage monocytic cultured cells, whereas AML1 is upregulated at protein level but not on mRNA level. Overexpression of miR-17-5p, miR-20a, and miR-106a downregulates AML1 protein expression, leading to downregulation of the M-CSF receptor, enhanced blast proliferation, inhibition of monocytic differentiation, and maturation. Additionally, AML1 inhibits transcription of the miR-17-5p-92 and the miR-106a-92 cluster as a negative feedback (Fontana et al., 2007).

The number of miRNAs with specific function in immune system constantly increases and is comprehensively reviewed elsewhere (Baltimore et al., 2008; Sonkoly and Pivarcsi, 2009). It demonstrates impressively that miRNAs display a highly important, but previously unrecognized level of control of gene expression in the immune system.

## miRNAs AND EICOSANOIDS

Beyond their crucial role in immune system development, miRNAs are also involved in immune and inflammatory responses. It became apparent that several miRNAs are induced by inflammatory stimuli (reviewed in Sheedy and O'Neill, 2008). Of note, miR-146 and miR-155 induce pro-inflammatory stimuli like interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF- $\alpha$ ), and toll like receptor (TLR) and have been involved in the onset of several inflammatory diseases like psoriasis or rheumatic arthritis (Sheedy and O'Neill, 2008).

Eicosanoids like leukotrienes and prostaglandins are biologically active lipids and important pro-inflammatory mediators involved in pathological processes such as chronic inflammation and carcinogenesis. Biosynthesis of eicosanoids starts with the release of arachidonic acid from membranes by phospholipases followed by the metabolism of the released arachidonic acid by cyclooxygenases, lipoxygenases, and P450 epoxygenase pathways

(Figure 2; Wang and Dubois, 2010). Recent findings indicate that several miRNAs are also involved in the control of key enzymes of the eicosanoid production.

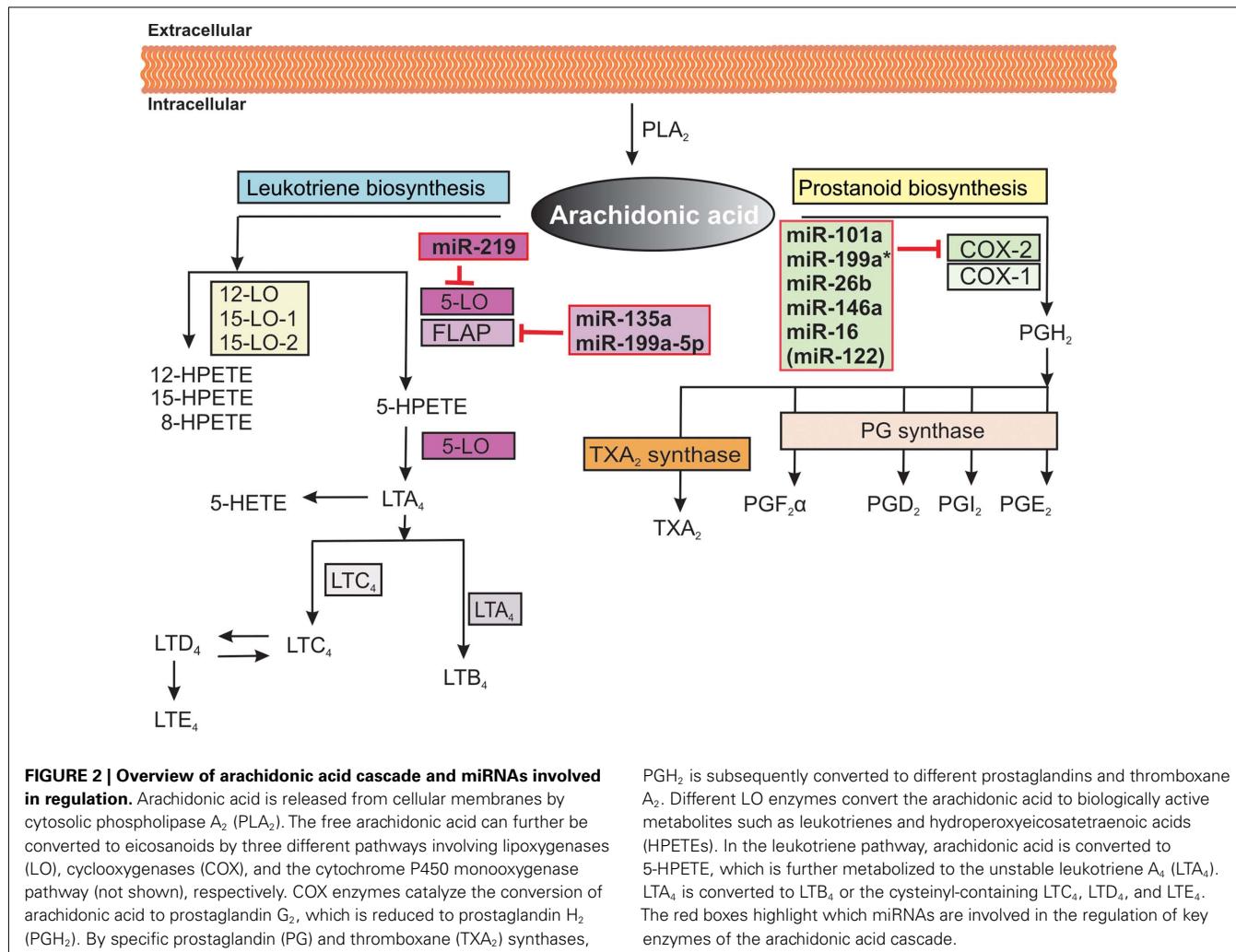
## miRNAs REGULATION IN PROSTANOID BIOSYNTHESIS

Cyclooxygenases (COX) exist in two isoforms, COX-1 and COX-2, both of which catalyze the conversion of arachidonic acid to prostaglandin H<sub>2</sub> which is further metabolized to the various prostaglandins and thromboxane A<sub>2</sub>. Prostaglandins and leukotrienes exert their biological effects in an autocrine or paracrine manner by binding to their cognate cell surface receptors (Wang and Dubois, 2010). COX-1 was thought to be a housekeeping enzyme responsible for maintaining basal prostanoïd levels that are important for tissue homeostasis. By contrast, COX-2 is undetectable in most tissues, but is strongly induced in response to hypoxia, inflammatory cytokines, and other stressors.

COX-2 expression is regulated at various levels (Harper and Tyson-Capper, 2008; Mbonye and Song, 2009). Numerous transcription factors such as nuclear factor- $\kappa$ B (NF- $\kappa$ B), activator protein-1 (AP-1), or cAMP-responsive element binding protein (CREP) are involved in the transcriptional regulation (Yamamoto et al., 1995; Kang et al., 2007). Moreover, COX-2 expression is influenced by chromatin remodeling (Deng et al., 2003). An additional level of regulation is the COX-2 mRNA stability and translation efficiency. Several *cis*-acting sequences, like AU-rich elements (ARE), have been identified within the ~2000 nt long 3' UTR. *Trans*-acting proteins, like HuR or the CUG triplet repeat-RNA-binding protein 2 (CUGBP2), recognize these *cis*-acting sequences and influence the stability of the COX-2 mRNA (Mukhopadhyay et al., 2003; Subbaramaiah et al., 2003). The use of an alternative polyadenylation signal has also been shown to affect mRNA stability and translation (Hall-Pogar et al., 2005).

Recent studies identified miRNAs as additional players in the posttranscriptional control of COX-2 expression. Chakrabarty et al. (2007) observed that miR-199a\* and miR-101a share similar temporal and spatial expression profiles like COX-2 in the mouse uterus during implantation. Further studies revealed that COX-2 expression is post-transcriptionally regulated by a direct interaction of miR-101a and miR-199a\* with COX-2 3' UTR in mice (Chakrabarty et al., 2007). The authors showed a similar dependency during endometrial carcinogenesis. An elevated COX-2 level correlated with a decreased expression of miR-101a and miR-199a\* suggesting that these two miRNAs are involved in the regulation COX-2 expression in endometrial cancer in mice (Daikoku et al., 2008).

An inverse correlation between COX-2 and miR-101a expression has also been observed in human colon cancer cells. Strillacci et al. (2009) showed that miR-101 decreases COX-2 protein levels in HT-29 cells by translational repression. This coincides with the observation that reduced miR-101 expression correlates with high levels of COX-2 protein expression in colon cancer tissues and liver metastases derived from colorectal cancer patients (Strillacci et al., 2009). Hiroki et al. (2010) found a similar relationship between COX-2 expression and miR-101. They identified a strong positive immunoreactivity of COX-2 which significantly correlated with the downregulation of miR-101 in patients with endometrial serous carcinoma (Hiroki et al., 2010). A similar inverse



relationship between COX-2 and miR-101a expression have been observed in mammary gland indicating that miR-101 regulates cell proliferation via COX-2 expression (Tanaka et al., 2009).

The expression pattern of the miR-26b is also inversely correlated with COX-2 expression in desferrioxamine (DFOM)-treated carcinoma cells of the nasopharyngeal epithelium. Here, a feedback of COX-2 expression on the miRNA level has been demonstrated (Ji et al., 2010).

Lukiw and coworkers reported that herpes simplex virus-1 (HSV-1) infection of human brain cells induces miR-146a that is associated with pro-inflammatory signaling in stressed brain cells and Alzheimer's disease. It has been shown that HSV-1 infection leads to the upregulation of pro-inflammatory markers such as cytosolic phospholipase A<sub>2</sub>, COX-2, and IL-1 $\beta$ , but also of miR-146a, coupled to a decreased expression of the immune system repressor complement factor H (CFH). The authors suggest that the miR-146a mediated downregulation of CFH and the subsequent upregulation of key members of the arachidonic acid cascade contribute to Alzheimer-type neuropathological changes (Hill et al., 2009). Recent studies demonstrate that fibroblasts from patients with chronic obstructive pulmonary disease

(COPD) produce increased amounts of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) in response to the inflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$ . The decreased expression level of miR-146a leads to reduced degradation of COX-2 mRNA and overproduction of PGE<sub>2</sub>. This specific miR-146a overexpression in COPD fibroblast is a new pathophysiological mechanism contributing to the abnormal inflammatory response in COPD patients (Sato et al., 2010).

Jing et al. (2005) have shown that miR-16 induces TNF- $\alpha$  and COX-2 mRNA degradation. Interestingly, this regulation is not only dependent on the enzymes of miRNA dependent decay but also on tristetraproline (TPP) which binds ARE and directs rapid mRNA decay (Jing et al., 2005). A direct interaction for miR-16-1 with its binding site on the target mRNA as well as an influence on ARE-mediated mRNA stability has been demonstrated. MiR-16 contains an 8-nt-sequence (UAAAUAUU) that is complementary to the ARE. MiR-16 is required for ARE-mediated decay in a sequence specific manner and requires the ARE-binding protein TPP which is involved in the formation of the decay complex. This shows that miRNAs which target ARE appear to be an essential step in ARE-mediated regulation (Jing et al., 2005; Calin et al., 2008; von Roretz and Gallouzi, 2008).

Another miRNA involved in ARE-mediated decay was recently identified by Filipowicz and coworkers. They showed that HuR was able to rescue translation of repressed cationic amino acid transporter-1 (CAT-1) mRNA, probably by interfering with the association of miR-122 with AREs within the 3' UTR of CAT-1 mRNA (Bhattacharyya et al., 2006). Since the stability of COX-2 mRNA is also dependent on HuR (Subbaramaiah et al., 2003) it can be speculated that miR-122 can also be involved in COX-2 regulation.

A further interesting aspect is if and how miRNAs itself are controlled by prostanooids. In a recent report, Ruan and coworkers found that prostacyclin (PGI<sub>e</sub>) influence expression of several miRNAs (upregulation of miRNA-711, miRNA-744, and miRNA-148b, downregulation of miR-466f-3p, miR-148a, miR-7a, miR-374). The regulation was mediated via the PGI2 receptor and was found to inhibit insulin-mediated lipid deposition in a mouse adipose tissue derived primary culture cell line (Mohite et al., 2011). This study indicates that miRNA regulation may be involved in a wide range of pathophysiological processes.

#### miRNA REGULATION IN LEUKOTRIENE BIOSYNTHESIS

The 5-lipoxygenase (5-LO) enzyme interacts with a 5-LO activating protein (FLAP) to convert arachidonic acid to the unstable leukotriene A<sub>4</sub> (LTA<sub>4</sub>). FLAP is essential for cellular leukotriene biosynthesis since it binds arachidonic acid and presents it to 5-LO. LTA<sub>4</sub> is subsequently converted into biologically active leukotriene B<sub>4</sub> (LTB<sub>4</sub>) by LTA<sub>4</sub> hydrolase or to leukotriene C<sub>4</sub> (LTC<sub>4</sub>) by LTC<sub>4</sub> synthase. LTC<sub>4</sub> can be enzymatically converted to leukotriene D<sub>4</sub> which is metabolized to leukotriene E<sub>4</sub> (Figure 2; Wang and Dubois, 2010).

Gonsalves and Kalra examined the effect of hypoxia on FLAP expression in human pulmonary vascular endothelial cells and in a transformed human brain endothelial cell line. They could demonstrate that hypoxia-mediated FLAP expression is regulated at the level of transcription. Furthermore, FLAP expression is negatively regulated by miR-135a and miR-199a-5p which provides a novel mechanism for the fine tuning of leukotriene production (Gonsalves and Kalra, 2010).

Recchiuti et al. (2011) identified a set of miRNAs that were temporally regulated in a self-limited acute inflammatory response and influenced by the lipid mediator resolving D1 (RvD1) which is involved in the resolution of inflammation. MiR-21, miR-146b, and miR-219 were upregulated by RvD1. They analyzed the effect

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of miRNA overexpression on genes involved in inflammatory and immune response and identified a plethora of candidates including cytokines and proteins involved in immune reactions like NF-κB. Based on these data the authors create networks of target genes of RvD1-regulated miRNAs (Recchiuti et al., 2011). 5-LO was among the identified targets of miR-219. Overexpression of miR-219 gave a reduction of 5-LO protein by 20% and a reduced leukotriene production. However, since no direct binding site for miR-219 is predicted within the 5-LO 3' UTR the effect may be indirect (Recchiuti et al., 2011).

An interesting report by Dincbas-Renqvist et al. (2009) showed that 5-LO can interact with the C-terminal domain of human Dicer. The interaction between the 5-LO binding domain with a dicer fragment was shown to enhance 5-LO enzymatic activity *in vitro*, whereas 5-LO modified the processing activity of Dicer. These results suggest that the processing of specific miRNAs by Dicer might be regulated by 5-LO/Dicer interaction in inflammatory and cancer cells (Dincbas-Renqvist et al., 2009).

#### CONCLUSION AND PERSPECTIVES

Eicosanoids including leukotrienes and prostaglandins are important biologically active lipids regulating immune responses in the body (Wang and Dubois, 2010). Recent evidence suggests that miRNAs are indeed involved in inflammatory signaling, yet research is clearly still at the beginning and the extent and importance of miRNA mediated regulation remains to be discovered. The number of identified miRNAs involved in eicosanoid pathway continues to grow and the examples reviewed here may just be the tip of an iceberg with the complexity of their possible role in the inflammatory processes not yet been clarified. Many questions are still open like how many miRNAs are involved in eicosanoid signaling, how these RNAs are regulated, which steps in the signaling cascade are targeted, are they associated with acute, chronic, or resolving inflammation, are they key regulators or just involved in fine tuning? A better understanding of the regulation of lipid mediator formation by miRNAs will be of interest not only for the further elucidation of lipid signaling but may open a new avenue in the development of new therapeutic concepts for treatment of inflammation and cancer.

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# Annexin A1 N-terminal derived peptide Ac2-26 exerts chemokinetic effects on human neutrophils

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It is postulated that peptides derived from the N-terminal region of Annexin A1, a glucocorticoid-regulated 37-kDa protein, could act as biomimetics of the parent protein. However, recent evidence, amongst which the ability to interact with distinct receptors other than that described for Annexin A1, suggest that these peptides might fulfill other functions at variance to those reported for the parent protein. Here we tested the ability of peptide Ac2-26 to induce chemotaxis of human neutrophils, showing that this peptide can elicit responses comparable to those produced by the canonical activator formyl-Met-Leu-Phe (or FMLP). However, whilst disruption of the chemical gradient abolished the FMLP response, addition of peptide Ac2-26 in the top well of the chemotaxis chamber did not affect (10  $\mu$ M) or augmented (at 30  $\mu$ M) the neutrophil locomotion to the bottom well, as elicited by 10  $\mu$ M peptide Ac2-26. Intriguingly, the sole addition of peptide Ac2-26 in the top wells produced a marked migration of neutrophils. A similar behavior was observed when human primary monocytes were used. Thus, peptide Ac2-26 is a genuine chemokinetic agent toward human blood leukocytes. Neutralization strategies indicated that engagement of either the GPCR termed FPR1 or its cognate receptor FPR2/ALX was sufficient to sustain peptide Ac2-26 induced neutrophil migration. Similarly, application of pharmacological inhibitors showed that cell locomotion to peptide Ac2-26 was mediated primarily by the ERK, but not the JNK and p38 pathways. In conclusion, we report here novel *in vitro* properties for peptide Ac2-26, promoting neutrophil and monocyte chemokinesis; a process that may contribute to accelerate the resolution phase of inflammation. We postulate that the generation of Annexin A1 N-terminal peptides at the site of inflammation may expedite the egress of migrated leukocytes thus promoting the return to homeostasis.

**Keywords:** inflammation, annexin A1, resolution, neutrophil, migration

## INTRODUCTION

Annexin A1 (AnxA1) is a glucocorticoid-regulated 37-kDa protein that exerts important actions on fundamental processes in inflammation. Highly abundant in myeloid cells, AnxA1 is rapidly mobilized upon cell activation (non-genomic externalization) or subsequent to *de novo* synthesis (genomic activation, e.g., after glucocorticoid treatment or pro-inflammatory cytokine application; Perretti and D'Acquisto, 2009). Once on the cell surface the protein is exposed to extracellular fluids and in the presence of calcium undergoes structural re-organization, consequent to interaction with phospholipids via the core region of the protein (~280 amino acid long), which leads to the exposure of the N-terminal region (~50 amino acid long; Gerke et al., 2005). This conformational change is thought to lead to the interaction of the AnxA1 N-terminus with specific receptors (Hu et al., 2008). It is worth recalling here that both human recombinant AnxA1 and the peptide Ac2-26 exert anti-inflammatory and pro-resolving effects in a variety of experimental models (Perretti and Dalli, 2009). Moreover, AnxA1 null mice are viable and do not have an appreciable phenotype unless challenged with inflammatory stimuli whereby a stronger and often prolonged reaction is then observed (Yang et al., 2004; Damazo et al., 2006; Babbin et al., 2008).

In addition to representing the pharmacophore of the protein affording interaction with counter-ligands, the N-terminus is also a highly regulated region. It can undergo phosphorylation on specific Tyrosine or Serine sites, a pre-requisite for secretion in certain cell types, or can be cleaved by serine proteases (Solito et al., 2006; Vong et al., 2007; D'Acquisto et al., 2008). In fact, both elastase and proteinase 3 have been shown to cleave at specific sites within the AnxA1 N-terminal region (Rescher et al., 2006; Vong et al., 2007) and it is plausible that AnxA1 can be a substrate for many other proteases. Cleavage of this protein has also been reported in human inflammatory samples including bronchoalveolar lavage fluids (Tsao et al., 1998) and blister exudates (Perretti et al., 1999) suggesting that this process is not an *in vitro* artifact but of biological significance.

Gerke and colleagues published a break-through study showing that peptides derived from the AnxA1 N-terminus activate the formyl peptide receptor type 1 (FPR1; Walther et al., 2000). Subsequently, we showed that full-length AnxA1 can bind and activate a related receptor termed FPR2/ALX (the lipoxin A<sub>4</sub> receptor). This interaction was of physiological relevance since a direct association between AnxA1 and FPR2/ALX could be shown in human and mouse activated neutrophils (Perretti et al., 2002).

Subsequent observations indicated that peptide Ac2-26 activated all three of the human formyl peptide receptors (Ernst et al., 2004). Parallel studies from our group showed that whilst peptide Ac2-26 could bind both FPR1 and FPR2/ALX, the full-length protein displayed specific binding only toward FPR2/ALX (Hayhoe et al., 2006).

It is believed that AnxA1 cleavage can represent a catabolic event, terminating the “AnxA1 mediated anti-inflammatory tone” (Vong et al., 2007). This hypothesis is backed by the observation that a cleavage-resistant species of the protein afforded higher potency in inflammatory settings (Pederzoli-Ribeil et al., 2010). In addition to this, however, it is also possible that AnxA1 cleavage could release N-terminal derived sequences that would then interact with FPR1 eliciting chemotactic responses. This could be particularly true outside the vasculature where inflammatory exudates rich in serine proteases can cleave AnxA1 generating such peptides.

To address this hypothesis in the present study we assessed (i) the chemotactic response of human neutrophils (PMN) elicited by peptide Ac2-26, (ii) the involvement of FPR1 and/or FPR2/ALX in the observed effects, and (iii) the intracellular pathways engaged by this peptide.

## MATERIALS AND METHODS

### NEUTROPHIL AND MONOCYTE ISOLATION

Experiments using healthy volunteers were approved by the local research ethics committee (P/00/029 East London and The City Local Research Ethics Committee 1). Informed written consent was provided according to the Declaration of Helsinki. Blood was collected into 3.2% sodium citrate and diluted 1:1 in RPMI-1640 before separation through a double-density gradient using Histopaque 10771 and 11191 (Sigma-Aldrich, Poole, UK). After centrifugation at 1340 rpm for 30 min polymorphonuclear (PMN) and mononuclear cells were collected from lower and upper layers respectively. Contaminating erythrocytes in the PMN cell suspension were removed by hypotonic lysis. Cells were finally washed and resuspended in RPMI-1640. Monocytes were isolated from the mononuclear cell suspension by immunomagnetic positive selection using the EasySep® Human CD14 Positive Selection Kit (STEMCELL Technologies, Grenoble, France).

### CHEMOTAXIS ASSAY

For neutrophil chemotaxis, cells were resuspended at a concentration of  $4 \times 10^6$  cells/ml in RPMI-1640 containing 0.1% BSA. The assay was performed using 3- $\mu$ m pore size ChemoTx™ 96 well plates (Neuro Probe Inc., Gaithersburg, USA) by adding chemotactic stimuli to the bottom wells, and placing 25  $\mu$ l of cell suspension on top of the filter. Plates were incubated for 90 min at 37°C with 5% CO<sub>2</sub>. After this period filters were washed with PBS and plates centrifuged at 1200 rpm for 30 s. Migrated cells were analyzed by taking 20- $\mu$ l from the bottom wells and incubating with Alamar Blue (Invitrogen Ltd, Paisley, UK) and comparing with a standard curve constructed with known cell numbers. Plates were read after 3 h in a fluorescence spectrophotometer at EX560-EM5 90 nm. For monocyte chemotaxis, the assay was performed following the same protocol outlined above incubating cells for 120 min to allow migration.

In some cases, compounds (see below) were added to freshly prepared neutrophils for 10 min at 37°C prior to cell addition to the top well of the chemotactic plates. Anti-FPR1 or anti-FPR2 antibodies were handled in a similar manner.

### PHARMACOLOGICAL TOOLS

Peptide Ac2-26 (Ac-AMVSEFLKQAWFIENEEQEYVQTVK; Tocris, Bristol, UK) or FMLP (Sigma-Aldrich, Dorset, UK) were used as chemoattractants. In initial experiments, also serum amyloid protein A (SAA; Peprotech, London, UK) was used, since it induces chemotaxis via FPR2 (He et al., 2003; Ye et al., 2009; Dufton et al., 2010). Anti-FPR1 or anti-FPR2 monoclonal antibodies (10  $\mu$ g/ml final concentration in either case) were obtained from R&D System (Abingdon, UK) or Genovac (Brussels, Belgium), respectively.

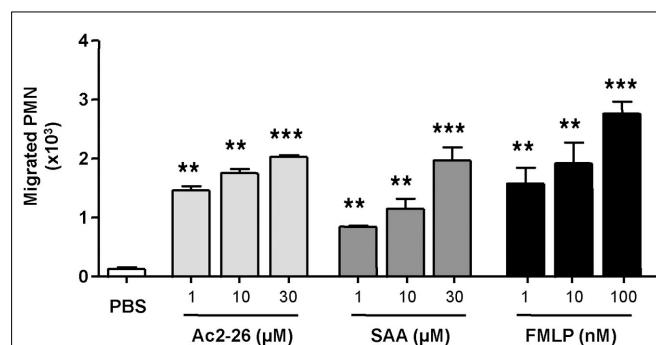
Inhibitors of mitogen-activated phosphokinase were tested to learn about the signaling pathway(s) activated by peptide Ac2-26 in these experimental settings. The compounds PD98059 (ERK inhibitor; Maiti et al., 2008), SB203580 (p38 inhibitor; Maiti et al., 2008), and SP600125 (JNK Inhibitor – SP600125; Tokuda et al., 2003) were obtained from Cell Signaling Technologies (Hertfordshire, UK) and used in the concentration range of 3–30  $\mu$ M as based on published data (Gallicchio et al., 2009). In selected experiments the ERK inhibitor (10  $\mu$ M) was tested against peptide Ac2-26 validating blockade of phospho-ERK accumulation by Western blotting using a described methodology (Hayhoe et al., 2006).

### STATISTICS

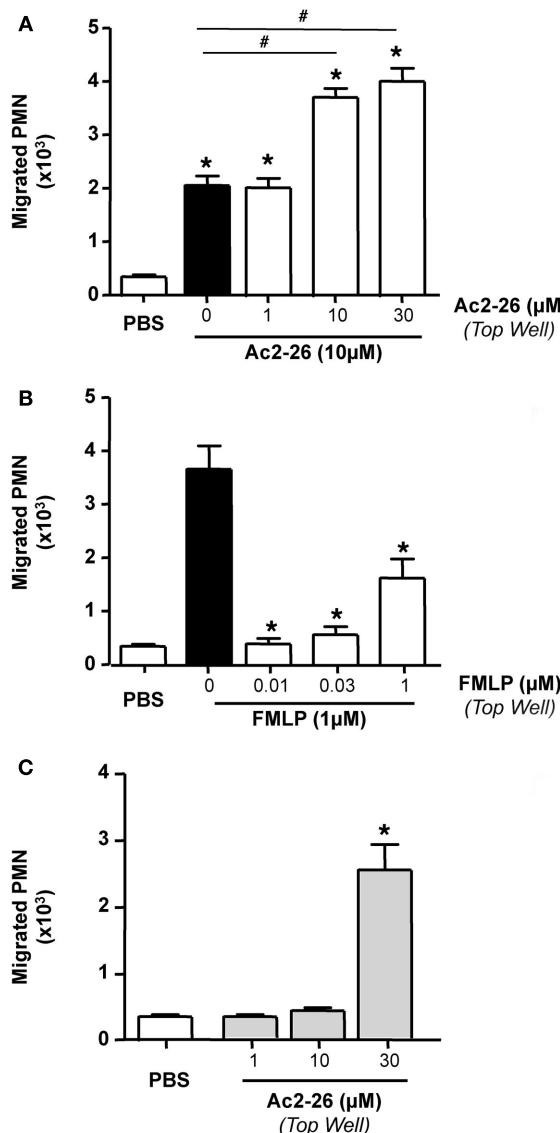
Results are presented as Mean  $\pm$  SEM of n experiments performed in triplicate or quadruplicate. Statistical differences were determined by analysis-of-variance followed by Dunnet's or Bonferroni's *post hoc* tests, taking a P-value less than 0.05 as significant.

## RESULTS

In initial experiments we tested the effect of peptide Ac2-26 on human PMN chemotaxis in comparison to the FPR1 agonist FMLP and the FPR2/ALX agonist SAA. **Figure 1** demonstrates

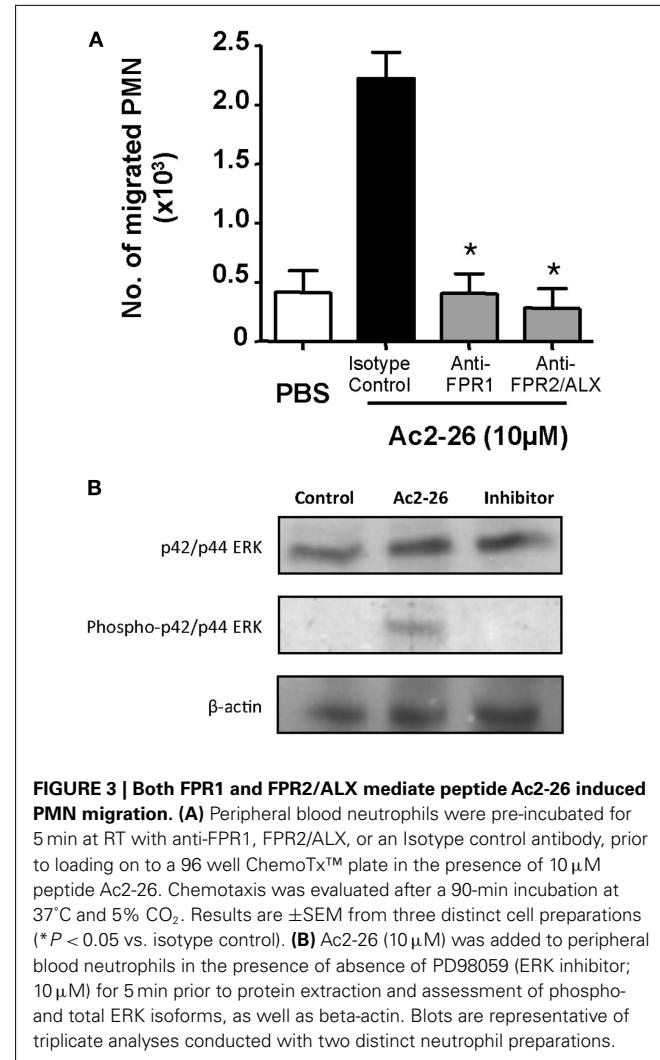


**FIGURE 1 | Peptide Ac2-26 provokes migration of human PMN: comparison to FMLP and SAA.** Neutrophils isolated from peripheral blood of healthy volunteers were resuspended at  $4 \times 10^6$  cells/ml and chemotaxis to peptide Ac2-26 (1–30  $\mu$ M), SAA (1–30  $\mu$ M), or FMLP (1–100 nM) was assessed over a 90-min period. Results are  $\pm$ SEM from three to four cell preparations (\*\*P < 0.01, \*\*\*P < 0.001 vs. PBS group).



**FIGURE 2 | Peptide Ac2-26, but not FMLP, induces PMN chemokinesis.** Peripheral blood neutrophils were incubated with either **(A)** peptide Ac2-26 (1–30  $\mu\text{M}$ ) or PBS prior to loading on to 96 well ChemoTx™ plate where the bottom wells were either loaded with peptide Ac2-26 (10  $\mu\text{M}$ ) or **(B)** FMLP (0.01–1  $\mu\text{M}$ ) prior to loading on to a chemotaxis plate in the presence of 1 nM FMLP. **(C)** Neutrophils were incubated with peptide Ac2-26 (1–30  $\mu\text{M}$ ) prior to loading on to the chemotaxis plate. Results are  $\pm$ SEM from three to four cell preparations [(A,B)] \* $P < 0.05$  vs. stimulus alone (dose 0 group); (C) \* $P < 0.05$  vs. PBS.

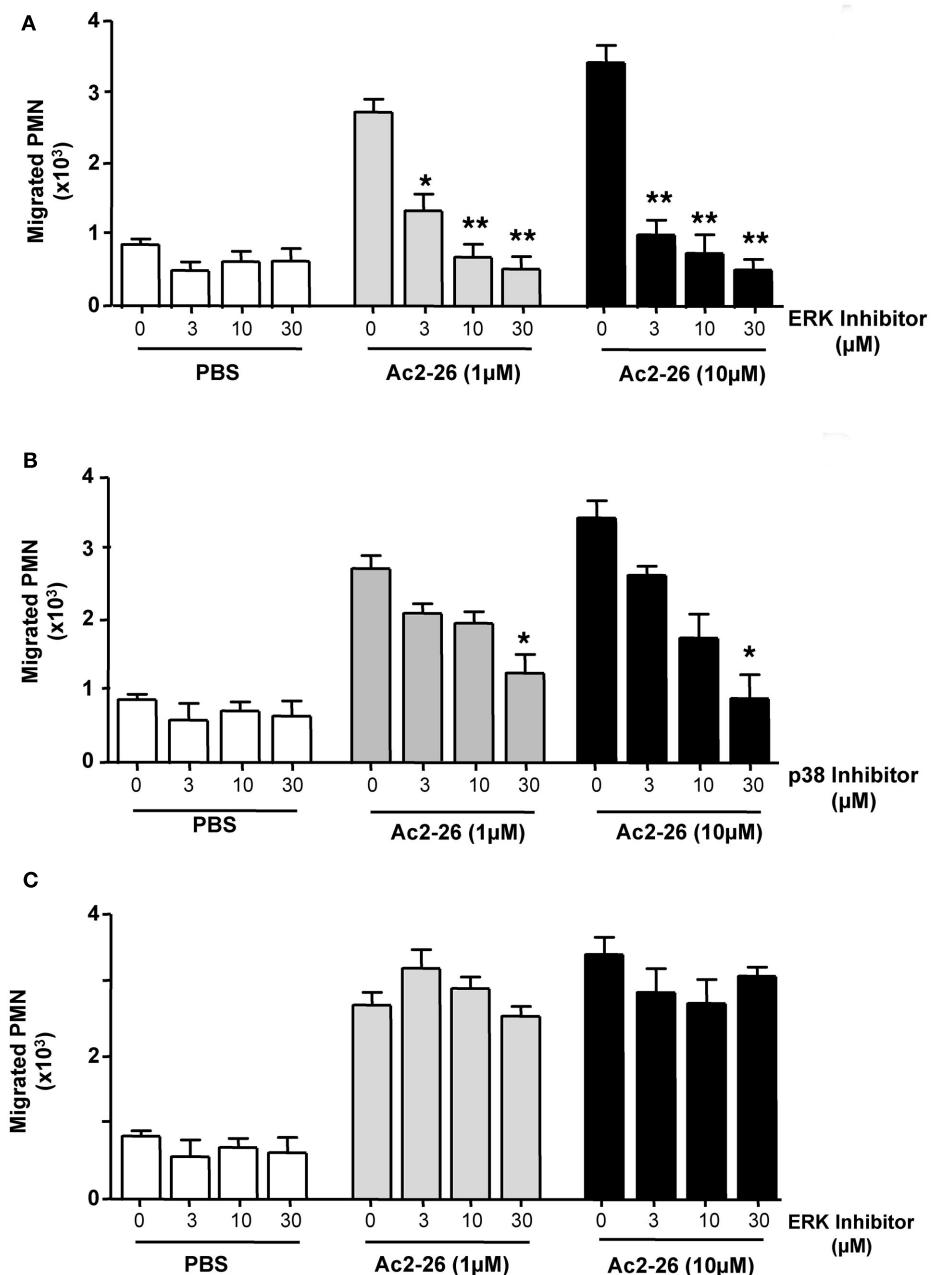
that peptide Ac2-26 can elicit a chemotactic response in the same order as FMLP, with a near maximal effect at 10  $\mu\text{M}$ , which corresponds to 3  $\mu\text{g}/\text{ml}$  final concentration. The positive control FMLP induced a marked response between 1 and 100 nM concentration range, and similarly SAA, applied in the bottom well of the chamber at 1–30  $\mu\text{M}$  range (Figure 1). Thus, peptide Ac2-26 causes PMN chemotaxis to a comparable level as produced by FMLP or SAA.



**FIGURE 3 | Both FPR1 and FPR2/ALX mediate peptide Ac2-26 induced PMN migration.** **(A)** Peripheral blood neutrophils were pre-incubated for 5 min at RT with anti-FPR1, FPR2/ALX, or an Isotype control antibody, prior to loading on to a 96 well ChemoTx™ plate in the presence of 10  $\mu\text{M}$  peptide Ac2-26. Chemotaxis was evaluated after a 90-min incubation at 37°C and 5% CO<sub>2</sub>. Results are  $\pm$ SEM from three distinct cell preparations (\* $P < 0.05$  vs. isotype control). **(B)** Ac2-26 (10  $\mu\text{M}$ ) was added to peripheral blood neutrophils in the presence of absence of PD98059 (ERK inhibitor; 10  $\mu\text{M}$ ) for 5 min prior to protein extraction and assessment of phospho- and total ERK isoforms, as well as beta-actin. Blots are representative of triplicate analyses conducted with two distinct neutrophil preparations.

Next we tested whether peptide Ac2-26 elicited genuine PMN chemotaxis or a chemokinetic response. To this end, we used 10  $\mu\text{M}$  peptide Ac2-26 as the chemoattractant concentration, and added in the top wells, vehicle, or Ac2-26 from 1 to 30  $\mu\text{M}$ . Figure 2 illustrates these results, where Ac2-26 provoked ~4-fold increase in PMN migration above control wells, and this effect was not modified by adding 1  $\mu\text{M}$  Ac2-26 in the top well. However, when 10 or 30  $\mu\text{M}$  peptide Ac2-26 were added to the top wells, the overall PMN migration was markedly augmented yielding, in essence, a doubling effect as compared to 10  $\mu\text{M}$  Ac2-26 in the bottom well alone (Figure 2A). Of interest, the chemotactic response elicited by 1 nM FMLP was abrogated by adding a higher concentration of the tri-peptide in the top well (Figure 2B). Finally, when peptide Ac2-26 was tested in the top well only, it did not alter the extent of PMN migration above control responses, except for the 30- $\mu\text{M}$  concentration that produced a remarkable neutrophil mobilization (Figure 2C).

The concentrations of peptide Ac2-26 used in the experiments are congruent with its apparent binding affinities for human FPR1 and FPR2/ALX (Walther et al., 2000; Perretti et al., 2002;

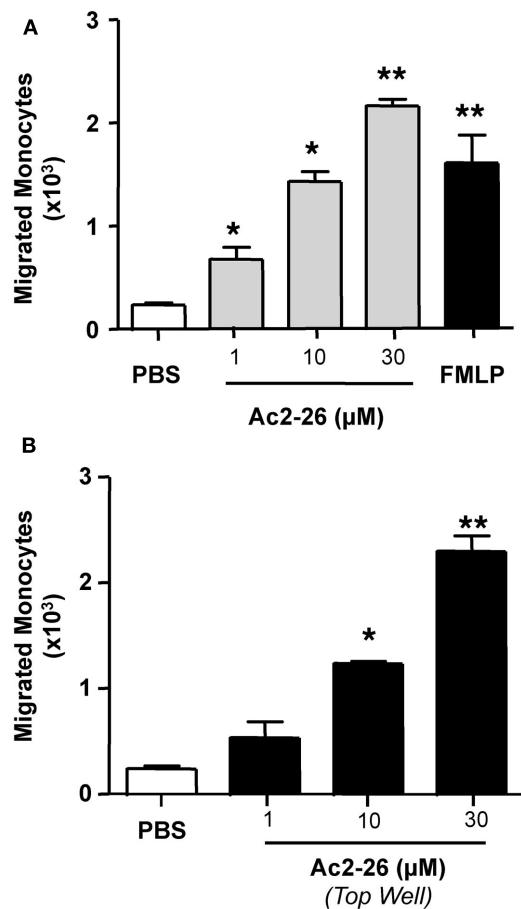


**FIGURE 4 | Peptide Ac2-26 induced PMN migration relies primarily on the ERK signaling pathway.** Neutrophils were pre-incubated for 15 min at 37°C with either (A) PD98059 (ERK inhibitor; 3–30  $\mu\text{M}$ ), (B) SB203580 (p38 inhibitor; 3–30  $\mu\text{M}$ ), or (C) SP600125 (JNK inhibitor; 3–30  $\mu\text{M}$ ) prior to loading

on to a 96 well ChemoTx™ plate in the presence of PBS or peptide Ac2-26 (3–30  $\mu\text{M}$ ). Chemotaxis was evaluated after a 90-min incubation at 37°C and 5%  $\text{CO}_2$ . Results are  $\pm\text{SEM}$  from three distinct cell preparations [ $*P < 0.05$ ,  $**P < 0.01$  vs. stimulus alone (dose 0 group)].

Hayhoe et al., 2006). Next, we tested if FPR1 or FPR2/ALX was responsible for the observed effect, using again 10  $\mu\text{M}$  peptide Ac2-26 to induce human PMN migration. To this end, we used specific antibodies against either of the human receptors. Here the cells were incubated with 10  $\mu\text{g}/\text{ml}$  (final concentration) of either antibody. Blockade of either formyl peptide receptor was able to abrogate the chemotactic effect elicited by the Ac2-26 (Figure 3A).

Peptide Ac2-26 engagement of human FPR1 and FPR2/ALX has been shown to activate the MAPK signaling in an uneven way, with phosphorylation of ERK but not JNK or p38 (Walther et al., 2000; Ernst et al., 2004; Hayhoe et al., 2006). Thus, we next evaluated whether this observation also held true in our experimental conditions showing that incubation of freshly prepared PMN with Ac2-26 led to ERK phosphorylation, an effect that could be effectively blocked upon incubation with an ERK specific



**FIGURE 5 | Peptide Ac2-26 induced human monocytes chemokinesis.** Monocytes were isolated from peripheral blood of healthy volunteers as indicated in the Section “Materials and Methods” and resuspended at  $4 \times 10^6$  cells/ml. **(A)** Chemotaxis toward peptide Ac2-26 (1–30  $\mu\text{M}$ ) was evaluated after a 90-min incubation at 37°C and 5% CO<sub>2</sub>. FMLP (1 nM) was used as positive control. **(B)** Chemokinesis induced by peptide Ac2-26 was assessed by incubating the cells with the peptide prior to loading on to the top well of the ChemoTx™ plate, the number of migrated cells to the bottom chamber was evaluated after 90 min incubation at 37°C and 5% CO<sub>2</sub>. Results are  $\pm$ SEM of three to four distinct monocyte preparations. (\* $P < 0.05$ , \*\* $P < 0.01$  vs. PBS group).

inhibitor (Figure 3B). We next assessed whether this pathway was also involved in mediating the chemokinetic effects exerted by the Ac2-26 peptide. Here we treated freshly prepared neutrophils with selective pharmacological inhibitors over an extended concentration range in order to account for any unspecific effects of the inhibitors. Pre-treatment of neutrophils with the ERK inhibitor, compound PD98059, abolished the cellular response to peptide Ac2-26, even at the lowest concentration tested of 3  $\mu\text{M}$  (Figure 4A). Intermediate effects were achieved with the p38 inhibitor which was mildly effective at concentrations of 3 and 10  $\mu\text{M}$ , yet it only significantly attenuated peptide Ac2-26 mediated effects at the highest concentration of 30  $\mu\text{M}$  (Figure 4B). On the other hand pre-treatment of PMN with the JNK inhibitor, did not have any influence on the extent of PMN migration (Figure 4C).

We next extended our observations to another cell type that is also important in the regulation of the inflammatory response, the monocyte. Here we tested whether peptide Ac2-26 elicited chemotactic/chemokinetic responses in this cell type, using a concentration range of 1–30  $\mu\text{M}$  and adding peptide Ac2-26 either to the bottom wells of the chemotactic plates, or in the top wells. As seen in Figure 5, addition of the peptide to either the top or bottom compartment of the chemotaxis plate elicited a marked response as measured by number of cells mobilized to the bottom well. We observed an increment of  $\sim$ 10-fold over controls when either the chemotactic (Figure 5A-addition of the peptide to the bottom well) or the chemokinetic (Figure 5B-addition of the peptide to the top well) effects were tested.

## DISCUSSION

We demonstrate here that the AnxA1-derived peptide Ac2-26 exerts chemokinetic, rather than chemotactic, effects on human PMN by acting through either FPR1 or FPR2/ALX and engaging primarily the ERK signaling pathway.

The seminal work of Gerke’s lab over a decade ago indicated that peptides derived from the AnxA1 N-terminal could activate human PMN by engaging the FMLP receptor and in this manner attenuate cell responses such as trans-endothelial migration (Walther et al., 2000). For a long time peptide Ac2-26 has been used as an AnxA1 surrogate in view of its ability to replicate the anti-inflammatory (Perretti and Dalli, 2009) and pro-resolving properties (Maderna et al., 2005, 2010; Scannell et al., 2007) of the native protein. Therefore, clarification of the receptor mechanism engaged by peptide Ac2-26 is of importance as it may have bearing on the pharmacological exploitation of this line of research for the development of novel anti-inflammatory therapeutics.

The model that emerges is that peptide Ac2-26 can activate all three human formyl peptide receptors, promoting calcium fluxes, and cell locomotion (Ernst et al., 2004). Such an effect may be part of a reparatory process as demonstrated with intestinal epithelial cells (Babbin et al., 2006) where addition of the peptide engaged FPRs to activate the cytoskeletal machinery favoring cell migration and repair of the epithelial monolayer. It was therefore surprising when we conducted binding studies and demonstrated that whilst full-length AnxA1 bound, and activated, the human FPR2/ALX, this protein was unable to bind human FPR1 (Hayhoe et al., 2006). In the same study we confirmed the activation of mitogen-activated phosphate kinases as an early post-receptor event, with phosphorylation of ERK but not p38 or JNK, at least within the time frame of our analyses (30 min). It is now clear that in human monocytes peptide Ac2-26 can activate the JAK/STAT/SOCS signaling requiring  $\geq$ 60 min incubation (Pupjalis et al., 2011). How can we explain this disparate receptor engagement between AnxA1 and its N-terminal pharmacophore?

One reason behind this dichotomy of behavior could be that AnxA1 exerts its inhibitory role in the inflamed vasculature exclusively through FPR2/ALX, whilst the peptide – which might be generated at the site of inflammation or tissue damage – could exert chemokinetics or chemoattractant properties via FPR1. We discussed earlier the generation of N-terminal derived peptides from AnxA1 in the inflammatory exudates by the action of serine

proteases and other proteolytic activities. In particular, PMN-derived elastase and proteinase 3 have been advocated as pivotal enzymes that could truncate the N-terminal region of AnxA1, which is exposed following calcium binding to the protein (Gerke and Moss, 2002; Gerke et al., 2005), leading to the release of biologically active peptides. Formation or release of AnxA1-derived peptides in an inflammatory exudate could contribute to resolution (Perretti and D'Acquisto, 2009) by promoting phagocytosis of apoptotic PMN by macrophages (Scannell et al., 2007; Maderna et al., 2010) and dampening inflammatory monocyte activation (Pupjalis et al., 2011). We propose that the data presented here, would argue for another pro-resolving property of peptide Ac2-26; the removal of immune cells (PMN and monocytes) from the site of inflammation favoring their exit back into the blood stream or through the lymphatic circulation and thus promoting tissue restoration and regain of its pre-inflammatory status. If confirmed in *in vivo* settings, this biological property of peptide Ac2-26 would explain: (i) the apparent discrepancy between AnxA1 and peptide Ac2-26 binding to human FPR1 and FPR2/ALX and (ii) the need for generating these short peptides, whereby AnxA1 would act as a pro-drug. In this respect recent observations made using a super-AnxA1, resistant to serine protease cleavage, would seem to argue against the second hypothesis, since this protein displayed higher anti-inflammatory activity than native AnxA1 (Pederzoli-Ribeil et al., 2010). However, here one needs to make a distinction between the anti-inflammatory properties exerted by AnxA1 (and super-AnxA1) in the vasculature and/or in the initial phases of acute inflammation, and those that may be operative during the onset of resolution, especially at the level of the exudate/tissue site, where these peptides could be generated.

Walther et al. (2000) reported that ~2 nM circulating AnxA1 can be measured in normal plasma that could augment by 10- to 100-fold in inflammatory settings, yielding concentration that can activate FPR2/ALX (Perretti et al., 2002; Hayhoe et al., 2006). At very high concentrations, (100–500 μM), likely not physiological, peptide Ac2-26 activates human PMN with production of reactive oxygen species (Walther et al., 2000) whilst inhibitory effects are predominant within the 10-μM range (Perretti et al., 1995; Walther et al., 2000). Therefore in our chemotaxis experiments, we selected concentrations of peptide Ac2-26 in the 1- to 30-μM range since we deem that concentrations higher than these would be of little biological significance. This especially in consideration of the fact that the molar ratio AnxA1:peptide Ac2-26 is 1:1, therefore it is really impossible to envisage pathophysiological settings where AnxA1 concentrations ≥30 μM could be reached.

Peptide Ac2-26 promoted a clear chemokinetic response in human PMN being, in this way, clearly distinguishable from FMLP – which produced the expected chemotactic effect (Ye et al., 2009) hence abolished when the chemical gradient was disrupted. In line with our initial data on early signaling responses (Hayhoe et al., 2006), peptide Ac2-26 induced PMN migration was highly reliant on ERK phosphorylation but not p38 or JNK phosphorylation. We reason that the mild effect of the p38 inhibitor, though

significant, was solely detected at 30 μM concentration alluding to a possible non-selective response since at concentrations above 10 μM this inhibitor has been shown to also inhibit ERK phosphorylation (Lian et al., 1999). Conversely, the ERK specific inhibitor markedly affected peptide Ac2-26 elicited PMN migration even at the 3-μM concentration. The JNK inhibitor acted as negative control, since it was unable to modulate this cellular response to peptide Ac2-26 to any appreciable extent, at any of the concentration tested, in line with previous observations (Walther et al., 2000; Ernst et al., 2004; Hayhoe et al., 2006).

Finally, the issue of the receptor engaged by peptide Ac2-26 in these experimental settings was addressed. Human PMN express FPR1 and FPR2/ALX but not FPR3, which is more relevant to macrophages and dendritic cells (Ye et al., 2009). Neutralizing antibodies to FPR1 (Yazid et al., 2010) or FPR2/ALX (Hayhoe et al., 2006) abrogated the cellular response to peptide Ac2-26. This is reminiscent of the data produced in human PMN under flow upon incubation with cromones, though in that study each antibody produced a partial effect and the combination gave total abrogation (Yazid et al., 2010). In the current experiments peptide Ac2-26 could engage either FPR1 or FPR2/ALX to promote PMN migration and each receptor seemed sufficient to govern this biological response. There are no doubts that human FPR1 is a chemotactic receptor and the same applies to human FPR2/ALX (Le et al., 2005). Our data suggests that simultaneous functional activation is important in eliciting the observed responses, an observation that favors a mechanism involving cross-talk between the two receptors, potentially resulting from heterodimerization, upon peptide Ac2-26 application. Future studies will clarify the potential molecular interlink between FPR1 and FPR2/ALX that these results indicate. It is noteworthy however that peptide Ac2-26 can bind and activate FPR1 and FPR2/ALX with similar affinities (Ernst et al., 2004; Hayhoe et al., 2006).

In conclusion, we provide *in vitro* evidence that peptide Ac2-26 can promote locomotion of human primary cells, PMN, and monocytes. Such an effect is at least in part due to a chemokinetic rather than chemotactic effect elicited by this peptide. This suggests that generation of AnxA1 N-terminal derived peptides during the resolution phase of the inflammatory response may play an important role in expediting the removal of blood-borne cells from the tissue. This in turn would favor the restoration of homeostasis. As often the case, these novel results incite new questions that must be addressed in future studies, both in terms of *in vivo* relevance as well as in the remit of molecular pharmacology, hence the potential modulation of FPR1 and FPR2/ALX localization upon peptide Ac2-26 application.

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# Targeting peripheral opioid receptors to promote analgesic and anti-inflammatory actions

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Mechanisms of endogenous pain control are significant. Increasing studies have clearly produced evidence for the clinical usefulness of opioids in peripheral analgesia. The immune system uses mechanisms of cell migration not only to fight pathogens but also to control pain and inflammation within injured tissue. It has been demonstrated that peripheral inflammatory pain can be effectively controlled by an interaction of immune cell-derived opioid peptides with opioid receptors on peripheral sensory nerve terminals. Experimental and clinical studies have clearly shown that activation of peripheral opioid receptors with exogenous opioid agonists and endogenous opioid peptides are able to produce significant analgesic and anti-inflammatory effects, without central opioid mediated side effects (e.g., respiratory depression, sedation, tolerance, dependence). This article will focus on the role of opioids in peripheral inflammatory conditions and the clinical implications of targeting peripheral opioid receptors.

**Keywords:** pain, inflammation, peripheral opioid receptors, opioids, immune cells, analgesia, anti-inflammatory

## INTRODUCTION

Inflammation is the series of highly coordinated events that is the response of vascular tissues to detrimental stimuli which results in heat, swelling, redness, and pain (Machelska et al., 2003). It is a vital protective response that functions to provide a rapid response to injuries to stimulate repair and prevent further disturbance to the affected tissue (Stein et al., 2003). Acute peripheral inflammatory pain is associated with immediate immune cell infiltration following tissue damage. Several disorders are correlated with peripheral inflammatory pain, such as arthritis and soft tissue injuries. Hyperalgesia, which is a lowered threshold to pain, is also associated with inflammatory pain. After tissue damage, resident macrophages generate an inflammatory response via release of mediators. These cause local inflammatory changes as well as attract more leukocytes to the site of inflammation which amplify these changes. Inflammatory mediators that are released and tissue acidification activate nociceptive primary afferent neurons as well as lower their signaling threshold in order to stimulate the sensation of pain and cause hyperalgesia (Hua and Cabot, 2010).

## ROLE OF ENDOGENOUS OPIOID SYSTEMS IN INFLAMMATION

An excess of pain or long-lasting pain (not necessarily "chronic") is counterproductive and so the body utilizes endogenous analgesic compounds to alleviate this sensation (Stein, 2013). Endogenous pain control mechanisms are not limited to the central nervous system (CNS). Although central mechanisms play a prominent role, evidence in the literature also suggests a significant involvement of peripheral mechanisms in counteracting pain. In fact, peripheral analgesic mechanisms have been demonstrated both in animals and humans, especially under inflammatory conditions. Most of these involve the release of opioid peptides, endocannabinoids,

somatostatin, or anti-inflammatory cytokines (Przewlocki et al., 1992; Luster et al., 2005; Hua and Cabot, 2010). Opioid peptides and their roles in anti-nociception have been extensively examined and their clinical relevance has been demonstrated in both human and animal studies (Busch-Dienstfertig and Stein, 2010; Stein, 2013).

Opioid-mediated analgesia is instigated in both the CNS and periphery through the release of endogenous opioid peptides. Evidence from the literature has shown that the central and peripheral mechanisms of endogenous opioid analgesia are interconnected, particularly in the early stages of inflammation (Binder et al., 2004; Brack et al., 2004d; Labuz et al., 2007; Hua and Cabot, 2010). The later stages have shown to have an increased role for peripheral opioid antinociception (Labuz et al., 2006, 2007). In the periphery, immune cells have been demonstrated to contain and release the opioid peptide  $\beta$ -endorphin in inflamed tissues which then acts upon opioid receptors present on primary afferent neurons to block pain transmission and thus provide analgesia (Mousa et al., 2000; Vetter et al., 2006). Labuz et al. (2006) demonstrated that treatment of inflamed tissue with anti- $\beta$ -endorphin antibody attenuated endogenous antinociceptive effects in this tissue. This local opioid-mediated effect is limited to the periphery and thus does not have the adverse systemic effects of centrally mediated opioid analgesia (Labuz et al., 2006).

## OPIOID PEPTIDES AND RECEPTORS IN INFLAMMATION

There are three families of opioid peptides that have been extensively studied: the endorphins, enkephalins and dynorphins, each of which is derived from a distinct gene and the precursors, pro-opiomelanocortin (POMC), pro-enkephalin (PENK) and prodynorphin (Hua and Cabot, 2010; Busch-Dienstfertig and Stein, 2010). Once released from immune cells opioid peptides act upon opioid receptors that are located on primary afferent neurons

and have been shown to be co-localized with nociceptors (Joseph and Levine, 2010; Wang et al., 2010; Stein, 2013). Opioid peptides render nociceptors less sensitive to excitation and thus impede the action of multiple excitatory mediators in one step. Each opioid peptide does not bind exclusively to one unique opioid receptor but instead exhibits affinity for various opioid receptors including  $\mu$ -,  $\delta$ - and  $\kappa$ -opioid receptors. During states of inflammation the numbers of opioid receptors in the periphery have been noted to be increased. For example, Jeanjean et al. (1995) showed that a single intraplantar injection of interleukin-1 $\beta$  (IL-1 $\beta$ ) was able to enhance the axonal transport of mu and kappa opioid receptors in the injected paw for as long as 6 days following a single injection. This increase in receptors is due to an increase in receptor synthesis, not an increase in speed of axonal transport, and it is theorized to occur through IL-1 $\beta$  stimulated retrograde axonal transport of cytokines and nerve growth factor from the inflamed tissue that alter neuronal gene expression in dorsal root ganglia neurons (Jeanjean et al., 1995; Puehler et al., 2004; Busch-Dienstfertig and Stein, 2010).

Enzymes within neurons such as phosphokinase C can phosphorylate opioid receptors, which leads to an increased affinity for arrestin molecules. Opioid-arrestin complexes have a decreased sensitivity for extracellular opioid peptides and are likely to be internalized via clathrin-dependent pathways (Law et al., 2000; Busch-Dienstfertig and Stein, 2010). From here, the receptors can either be recycled by reinsertion into the membrane, or they can be degraded. Recycling of peripheral opioid receptors has been shown to avoid the development of tolerance to opioids in the periphery via prevention of desensitization (Koch et al., 2005). Opioid peptides also work to prevent vesicular release from neurons via inhibition of ion channels, which prevents the release of excitatory neuropeptides such as noradrenaline and substance P (Stein, 2013). As tolerance is a significant problem associated with systemic administration of opioids, the targeting of peripheral opioid receptors, which have innate mechanisms to prevent desensitization, is clinically relevant (Joseph et al., 2010).

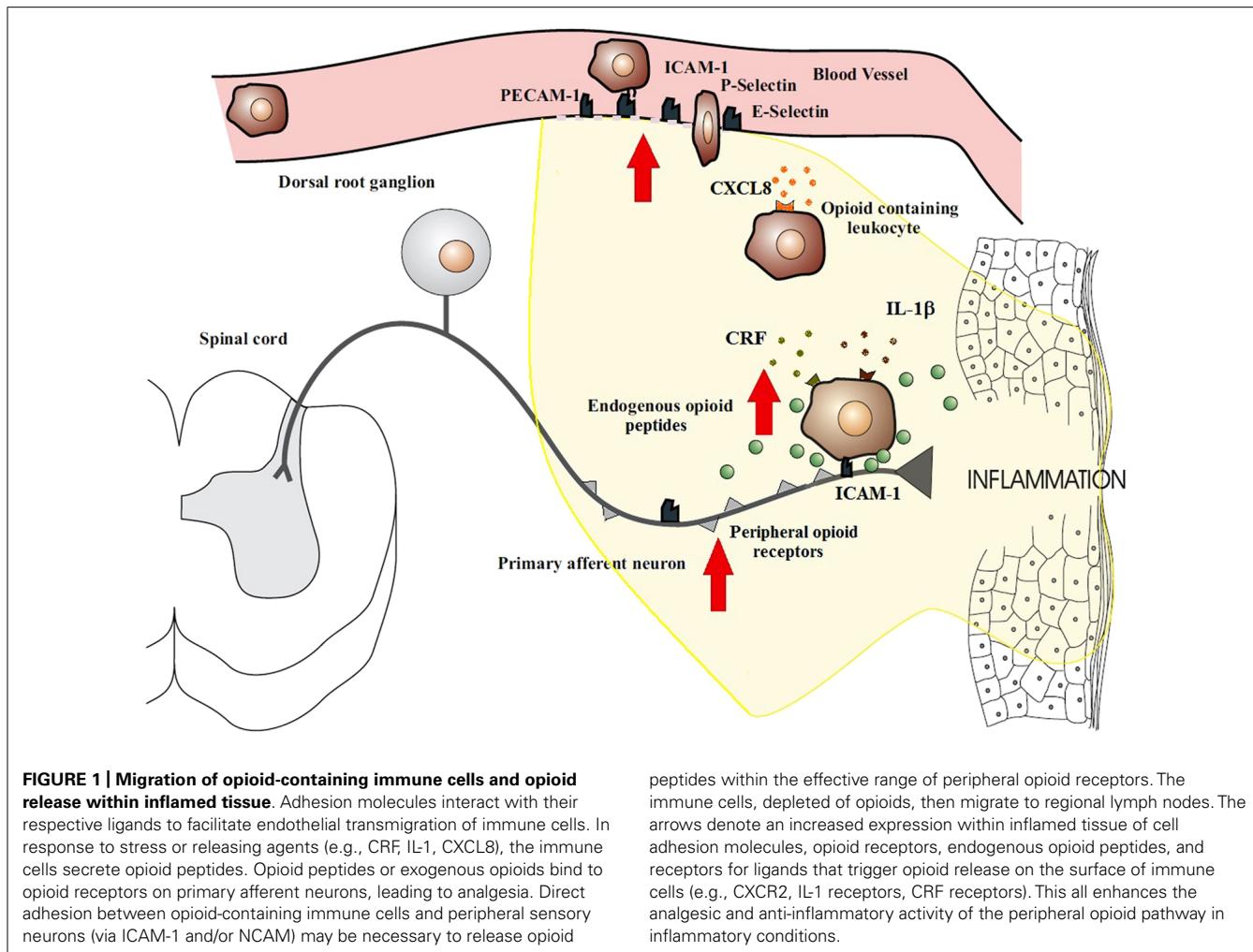
## OPIOID-CONTAINING IMMUNE CELLS IN INFLAMMATION

Inflammation rapidly stimulates immune cell extravasation and migration into tissues (Machelska et al., 2004; Rittner and Stein, 2005). Quantitative analysis has revealed that in early inflammation granulocytes (esp. neutrophils) are the major opioid-containing leukocyte, whereas at later stages of inflammation, monocytes/macrophages and lymphocytes (esp. activated T- and B-cells) predominate (Rittner et al., 2001; Brack et al., 2004a,b; Machelska et al., 2004). This is consistent with their order of infiltration to inflamed tissues (Luster et al., 2005). Inflammation has been shown to increase the expression of opioid peptides within these cells. In fact all opioid peptides as well as their mRNA transcripts encoding their precursor proteins have been identified within immune cell, with  $\beta$ -endorphin from POMC being the most prominent (Cabot et al., 1997; Cabot, 2001; Mousa et al., 2001). However, it has been suggested that only a finite number of the total immune cell population actually produce opioid peptides and home to lymph nodes (Stein et al., 2003; Rittner et al., 2005). This is supported by the observation that  $\beta$ -endorphin and POMC mRNA were less abundant in circulating lymphocytes than

in those in lymph nodes (Cabot et al., 1997). Similarly, Przewlocki et al. (1992) demonstrated the presence of immune cells containing the opioid peptide precursors, POMC and PENK, in inflamed tissue and the absence of these mRNA in non-inflamed tissue. Labuz et al. (2006) identified that the majority of immune cells infiltrating inflamed tissue contained  $\beta$ -endorphin. Therefore with the duration of inflammation, the number of infiltrating immune cells as well as total opioid peptide content at the site of tissue injury increases. These opioid peptides are translated and processed at the site of inflammation within immune cells.

## MIGRATION OF OPIOID-CONTAINING LEUKOCYTES TO INFLAMED TISSUE

Peripheral tissue injury causes a migration of opioid peptide-containing immune cells to the inflamed site. This migration appears to be both centrally and locally regulated. Exogenously stimulated systemic inhibition of pain has been shown to decrease the recruitment of  $\beta$ -endorphin containing immune cells in inflamed tissue (Schmitt et al., 2003; Heurich et al., 2007), thus suggesting a role for central regulation. Local regulation has been more extensively studied and involves a series of binding events to endothelial cells. The trafficking of opioid-containing immune cells occurs in a site-directed manner since they express adhesion molecules that govern their recruitment to damaged tissue (Schmitt et al., 2003). The homing of leukocytes to inflamed tissues involves a precise series of events which begin with circulating leukocytes tethering to and rolling along the vascular endothelial cell wall in a process mediated by P-, E-, and L-selectins (Koning et al., 2002; Stein et al., 2003; **Figure 1**). Upregulation of these cell adhesion molecules has been noted in many models of inflammation (Schurmann et al., 1995; Krishna et al., 1997; Navarro et al., 2002; Almulki et al., 2009; Svensson et al., 2009). Leukocytes are then activated by chemokines released from inflammatory cells and presented on the luminal surface of the endothelium (Koning et al., 2002; Machelska et al., 2002; Stein et al., 2003). This causes an increase in the avidity as well as an up-regulation of leukocyte integrins, in particular CD49d/CD29 and CD18, which mediate the firm adhesion of leukocytes to endothelial cells by interacting with intercellular adhesion molecule-1 (ICAM-1; Koning et al., 2002; Machelska et al., 2002; Stein et al., 2003). Leukocytes then migrate through the endothelium with the aid of platelet-endothelial cell adhesion molecule-1 (PECAM-1) expressed on endothelial cells at intercellular junctions. All these molecules are constitutively expressed and are upregulated in inflammation, except L-selectin, which is rapidly shed upon activation (Koning et al., 2002; Muro and Muzykantov, 2005). The up-regulation of these necessary components of leukocyte transmigration in inflammation results in the increased influx of immune cells. The preferential increase in opioid-containing immune cells can be explained by the co-expression of L-selectin, integrin- $\beta$  and the chemokine receptor CXCR2 on opioid-containing leukocytes (Mousa et al., 2000; Brack et al., 2004a,b,c,d; Machelska et al., 2004). Pre-treatment of rats with antibodies against these molecules have been shown to significantly decrease the number of opioid-containing immune cells that accumulate in inflamed tissue (Machelska et al., 1998, 2002, 2004).



## RELEASE OF OPIOID PEPTIDES FROM LEUKOCYTES

Opioid-containing immune cells release opioid peptides within peripheral inflamed tissue before moving to the local lymph node (Hua and Cabot, 2010). The release of opioids has been demonstrated to be governed by various factors. The chemokine CXCL8 (also known as IL-8) in humans which acts upon CXCR2 has likewise been demonstrated to increase release of opioid peptides *in vitro*. CXCL8-mediated opioid release occurs via binding of CXCL8 to its receptor on the leukocyte, which causes vesicular release of opioid peptides in a calcium-dependant manner (Rittner et al., 2006). This is the same process that is used by other inflammatory factors such as corticotropin-releasing factor (CRF) and IL-1 $\beta$ .

Schafer et al. (1997) demonstrated the ability of CRF to cause opioid release *in vitro* from immune cells obtained from inflamed tissue. Labuz et al. (2006) through the use of anti- $\beta$ -endorphin antibody *in vivo* showed that release of  $\beta$ -endorphin from immune cells is mediated by CRF interacting with its respective receptor on leukocytes. CRF and its respective receptors on the surface of leukocytes are upregulated in inflammation (Schafer et al., 1997). Schafer et al. (1997) further demonstrated that IL-1 $\beta$  influences the release of  $\beta$ -endorphin from immune cells both *in vitro* and

*in vivo* when administered locally via receptor binding. IL-1 $\beta$  levels increase during inflammation and enacts a positive feedback loop in order to increase its own concentration, whilst also having pro-inflammatory effects by upregulating the release of inflammatory factors (Schafer, 2003; Jimbo et al., 2005). Therefore as inflammation progresses, the level of endogenous opioid release also increases.

$\beta$ -endorphin containing inflammatory cells situated in close proximity to sympathetic nerve fibres within inflamed tissue have also been observed to possess increased numbers of  $\alpha$ 1- and  $\beta$ 2-adrenergic receptors. Destruction of these receptors was demonstrated to abolish endogenous opioid analgesia, thus suggesting that noradrenaline release by neuronal cells may also stimulate the release of opioid peptides (Binder et al., 2004; Machelska et al., 2004).

## THE EFFECT OF INFLAMMATORY CONDITIONS ON OPIOID EFFICACY

The rate of metabolic degradation of opioid peptides is increased in inflamed tissues (Vetter et al., 2006; Schreiter et al., 2012). Release of inflammatory factors such as hydrogen ions from damaged cells, cytokines and chemokines from resident cells

and peptidases from immune and neuronal cells create a hostile environment that acts to quickly break down opioids (Vetter et al., 2006; Schreiter et al., 2012). In order for opioid peptides to reach their receptors on nociceptive primary afferent neurons, interaction between neurons and immune cells may be required.

Recent reports have described bidirectional communication between immune and neuronal cells as well as physical contact between these cells. Of note is the observation of peripheral nerves and opioid-containing immune cells being closely associated (Przewlocki et al., 1992; Hua et al., 2006; Hua and Cabot, 2010). *In vitro* studies have demonstrated consistent alliance between lymphocytes containing opioids and cultured DRG nerves (Hua et al., 2006) whilst *in vivo* studies has observed this same phenomena in peripheral inflamed tissues with primary afferent nerves (Przewlocki et al., 1992). The mechanics of this association are yet to be elucidated, but is likely to be either by a synaptic-like connection being formed or paracrine opioid release. Adhesion molecules come into play with both of these processes in order to form intercellular interactions that are stable and cell specific (Dustin and Colman, 2002; Yamagata et al., 2003). Inhibition of ICAM-1 and neural cell adhesion molecules (NCAM) have both been demonstrated to result in a decrease lymphocyte–DRG neuronal cell interactions (Hua et al., 2006), which suggests that these play an important role in immune and neuronal cell adhesion. Whilst the exact mechanisms of these interactions are not yet understood, they may play a necessary role in the development of effective endogenous analgesia by ensuring delivery of opioids to peripheral sensory neurons before they are degraded by the extracellular environment within inflamed tissues (**Figure 1**).

## ANTI-INFLAMMATORY EFFECT OF PERIPHERAL OPIOIDS

Endogenous opioids may enact anti-inflammatory effects as well as analgesia through their actions on neuronal cells through prevention of vesicular release of noradrenaline and substance P. The function of noradrenaline in inflammation is contested, with evidence being provided for both a positive and negative role. Schlachetzki et al. (2010) observed enhanced expression of COX-2, which is heavily implicated in inflammation, following treatment with noradrenaline. However, data has also been presented that correlates noradrenaline release in Alzheimer's disease with suppression of neuroinflammation (Heneka et al., 2010). Substance P conversely has a well-reviewed pro-inflammatory action (O'Connor et al., 2004). Walker (2003) suggests that an opioid-mediated reduction of tumour necrosis factor (TNF) production and release also contributes to anti-inflammatory actions. TNF is a known regulator of inflammation and its inhibition has been shown to be an effective anti-inflammatory treatment (Esposito and Cuzzocrea, 2009). As the process of blocking the release of these neuropeptides is due to opioid receptor mediated hyperpolarisation of neuronal cells, exogenous opioids theoretically should have the same anti-inflammatory effect.

An opioid receptor independent mechanism may also be involved in opioid-mediated anti-inflammation. Gavalas et al. (1994) showed that experimentally induced mouse paw oedema was significantly inhibited after the administration of opioids and

this effect was not reversed by naxolone. Conversely, Philippe et al. (2003) noted that naxolone was able to reverse the mu-opioid receptor mediated reduction in inflammation in two *in vivo* models of colitis. These results suggest that a variety of complex regulatory activities may be performed by opioid agonists in various tissues of the body that may be naloxone-sensitive or naloxone insensitive and these pathways may directly or indirectly inhibit the release of cytokines and mediators involved in inflammation (Gavalas et al., 1994).

Fecho et al. (2007) demonstrated an anti-inflammatory action of morphine through the reduction of swelling and accumulation of neutrophils in carrageenan-induced peripheral inflammation. This effect was not dose-dependent and was not reversed by naloxone. The anti-inflammatory effect displayed by morphine is likely due to modulation of the adherence of immune cells to the endothelium by affecting the expression of cell adhesion molecules, and consequently affecting leukocyte transmigration. In comparison, administration of liposomes loaded with loperamide HCl and conjugated with antibody to intercellular adhesion molecule-1 (anti-ICAM-1), exerted analgesic and anti-inflammatory effects exclusively in peripheral painful inflamed tissue when administered intravenously in the complete Freund's adjuvant (CFA) model of acute inflammatory pain (Hua and Cabot, 2013). It was demonstrated previously that this adhesion between opioid-containing lymphocytes and cultured sensory neurons was opioid receptor dependent, with naloxone inhibiting the reduced neuroimmune adhesion in the presence of beta-endorphin (Hua et al., 2006). Opioids have been found to have significant anti-inflammatory effects in peripheral inflamed tissues, thus they are of clinical significance in relation to the treatment of peripheral inflammatory pain.

## CLINICAL IMPLICATIONS AND PERSPECTIVES

### TOPICAL OPIOID DELIVERY

Topically applied opioids have already been employed as a way of instigating peripheral nociception, however further studies are required to ascertain the anti-inflammatory activity of topical opioids. Case studies have reported the effectiveness of morphine when combined with pre-made gels such as IntraSite® for use in ulcers. van Ingen et al. (2010) recorded a case where topical morphine gel was applied to a cutaneous ulcer that had previously been treated unsuccessfully with zinc oil and surgical debridement. Within 3 days of the start of morphine gel treatment, the visual analog scale (VAS; 0–100) for pain, as determined by the patient, was halved from 80 to 40 (van Ingen et al., 2010). Welling (2007) explored the use of topical morphine in burns patients as a way of providing analgesia and avoiding systemic side effects, however no significant evidence of analgesia was seen when compared to the control groups. This study had a low level of power with a small number of participants, and damaged neuronal cells in burn patients may also account for the lack of efficacy in comparison to studies on chronic inflammatory wounds. This may be due to damaged nerve endings that result in impaired signal transduction or receptor expression. Loperamide has been reported upon in relation to the treatment of thermal injury in animals. Topical delivery of loperamide was used by Nozaki-Taguchi and Yaksh (1999) to successfully initiate antihyperalgesia in rats after thermal

injury (Nozaki-Taguchi and Yaksh, 1999). In humans, loperamide has been used in solution for analgesia in graft-versus-host related oral pain (Nozaki-Taguchi et al., 2008). Nozaki-Taguchi et al. (2008) studied four patients who were suffering from oral pain following blood stem cell transplantation. Utilizing the loperamide mouthwash resulted in oral analgesia and reduction of hyperalgesia in these patients. Analgesia was not noted in any other parts of the body, but some inhibition of gut motility was exhibited (Nozaki-Taguchi et al., 2008).

### PARENTERAL OPIOID DELIVERY

Peripheral mechanisms of opioid analgesia are well-established in the literature and have gained recognition in the clinical setting in conditions such as chronic rheumatoid arthritis and osteoarthritis, bone pain, and postoperative pain after laparoscopic, urinary bladder, and knee surgery (Busch-Dienstfertig and Stein, 2010). In patients undergoing arthroscopic knee surgery, opioid-containing immune cells are detectable in the inflamed synovium and the blockade of intra-articular opioid receptors by naloxone results in significantly increased postoperative pain for up to 4 h (Stein et al., 1993). More recently, peripheral opioid analgesia has also been reported in experimental neuropathic pain (Labuz et al., 2009, 2010).

The most extensively studied clinical situation is the intra-articular application of opioid agonists for pain control after knee surgery (Stein and Lang, 2009; Busch-Dienstfertig and Stein, 2010), which is now established in routine clinical practice (Lang et al., 2010). Intra-articular morphine was found to be as potent as dexamethasone in reducing pain as well as synovial inflammation (Stein et al., 1999). However, a limitation of intra-articular administration of opioids is that repeated injections carry a risk of infection and cannot be easily applied to more than one joint. Negative results from some reviews have been attributed to lack of

study sensitivity, lack of tissue inflammation, or the superimposition of general or local anesthetic effects (Stein and Machelska, 2011). Importantly, the use of peripherally acting opioid agonists for the prolonged treatment of inflammatory pain has not been shown to induce peripheral tolerance (Zollner et al., 2008; Lang et al., 2010), which has important implications for the treatment of pain associated with conditions such as chronic arthritis, inflammatory neuropathy, postoperative pain and cancer. Unfortunately the clinical studies to date were not designed to focus on the anti-inflammatory potential of opioids, as this is a more recent established finding of peripheral opioid therapy. Therefore, future clinical studies should incorporate both quantitative and qualitative anti-inflammatory measures into the research design.

### CONCLUSION

Increasing studies have clearly produced evidence for the clinical usefulness of peripheral opioid analgesics, in particular administration of opioids into local inflamed tissue. A major long-term goal remains to develop peripherally selective opioid compounds, suitable for oral and/or intravenous route of administration to improve clinical pain relief. Peripherally targeted opioids may be used in a co-treatment approach with other therapies (e.g., cytokine modulators or TNF- $\alpha$  antagonists) at low doses for conditions such as rheumatoid arthritis. This may provide a much sought after alternative for the management of chronic arthritic and other inflammatory conditions whilst avoiding central opioid mediated side effects and of typical side effects produced by non-steroidal and steroidal anti-inflammatory drugs.

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# Genetic architecture of human fibrotic diseases: disease risk and disease progression

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Genetic studies of human diseases have identified multiple genetic risk loci for various fibrotic diseases. This has provided insights into the myriad of biological pathways potentially involved in disease pathogenesis. These discoveries suggest that alterations in immune responses, barrier function, metabolism and telomerase activity may be implicated in the genetic risks for fibrotic diseases. In addition to genetic disease-risks, the identification of genetic disease-modifiers associated with disease complications, severity or prognosis provides crucial insights into the biological processes implicated in disease progression. Understanding the biological processes driving disease progression may be critical to delineate more effective strategies for therapeutic interventions. This review provides an overview of current knowledge and gaps regarding genetic disease-risks and genetic disease-modifiers in human fibrotic diseases.

**Keywords:** fibrosis, auto-immunity, genetics, GWAS, disease progression

## INTRODUCTION

Fibrosis arises as the result of excessive connective tissue and extracellular matrix deposition. It emerges from an aberrant or uncontrolled repair response often triggered by tissue damage that may be initiated by radiation, mechanical injury or infections and results in scar formation. In the context of auto-immunity, with sustained immune activation, the injury and repair phases persist and lead to scar tissue formation that disrupts organ architecture and function with a frequently fatal outcome (**Figure 1**).

In the last decade, the scientific community has successfully collaborated through consortia to unravel the genetic basis of susceptibility for many diseases. Genome-Wide Association Studies (GWAS) have identified numerous genetic polymorphisms that confer higher risk for diseases and have provided insights into the biological processes that contribute to disease susceptibility. One key finding is the substantial overlap of genetic loci associated with disease risk across a variety of complex immune diseases (Cotsapas and Hafler, 2013). This highlights the complexity of the etiology of clinical phenotypes that have an immune basis but are also largely influenced by environmental factors and can affect different target organs. Although the target organs may be different, a common complication of these diverse immune-mediated diseases is the abundance of fibrotic processes and scar tissue formation. This likely reflects that, when altered, many processes such as inflammation, barrier function and metabolism may result in sustained tissue injury, impaired repair processes and ultimately fibrosis (**Figure 2**).

Earlier genetic studies focused on signals that distinguish between disease and healthy status using “case-control” studies. Recent efforts have sought to identify genetic factors influencing clinical outcomes with emerging “case-case” studies, looking at patient subgroups that follow different disease courses. The hope is this will provide insights into the pathogenic processes dictating disease progression and severity. Emerging results on genetic disease-modifiers show limited overlap with genetic loci

involved in disease risk, highlighting the point that disease initiation and disease progression are not necessarily driven by the same mechanisms. Interestingly, these studies also allow us to determine how genetics might contribute to milder clinical outcomes, as illustrated by the recent discovery of a common polymorphism in *FOXO3* locus affecting the TGF- $\beta$  pathway which appears associated with improved prognosis in Crohn’s disease and rheumatoid arthritis (Lee et al., 2013a). Further investigations will define whether, across diseases involving different organ systems, genetic variants affecting a set of common key biological pathways might favor more susceptibility to fibrotic complications.

## DISEASES ASSOCIATED WITH LUNG FIBROSIS

### IDIOPATHIC INTERSTITIAL PNEUMONIAS AND IDIOPATHIC PULMONARY FIBROSIS

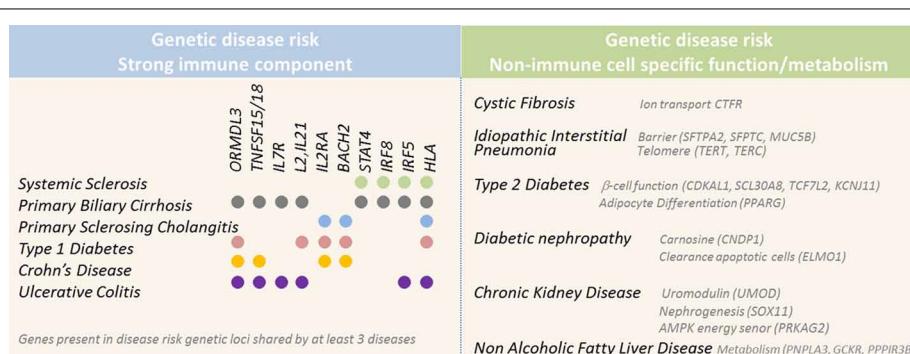
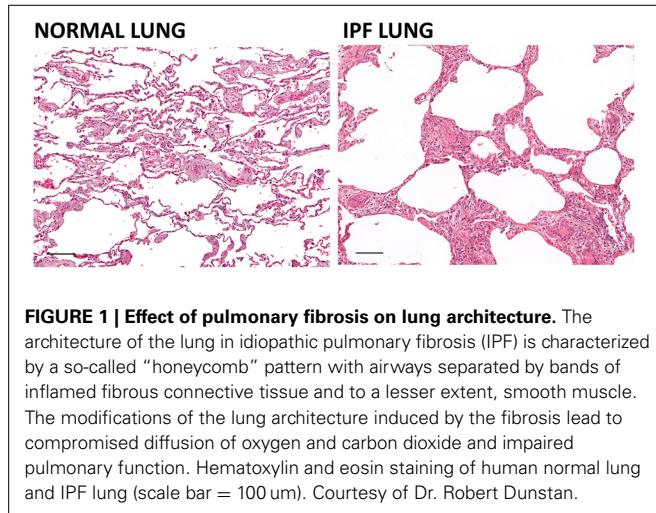
Familial forms of idiopathic pulmonary fibrosis (IPF) account for 2–20% of IPF patients, supporting a strong genetic component in the development of the disease [reviewed in Kropski et al. (2013)]. Investigations on families have identified genetic variants in *SFTPC* (encoding surfactant protein C), *SFTPA2* (encoding surfactant protein A2), *MUC5B* (encoding a mucin constituent of the mucus), as well as *TERT* and *TERC* (encoding components of the telomerase complex) to be associated with pulmonary fibrosis (Nogee et al., 2001; Thomas et al., 2002; Armanios et al., 2007; Tsakiri et al., 2007; Wang et al., 2009; van Moorsel et al., 2010; Ono et al., 2011; Seibold et al., 2011). The genetic association of genetic polymorphisms in *MUC5B-MUC2-TOLLIP* as well as *TERT* and *TERC* loci with high risk for pulmonary fibrosis has been confirmed by recent results from GWAS comparing 4683 controls and 1616 cases of fibrotic idiopathic interstitial pneumonias (IIP) including 77% of IPF cases with independent replication cohort (Fingerlin et al., 2013). These observations provide justification for investigating familial diseases with linkage studies, as well as large GWAS approaches.

The implication of genetic polymorphisms in *SFTPA2*, *SFTPC*, *MUC5B* as well as *DSP* (encoding desmoplakin) as risk factors for pulmonary fibrosis suggest that the integrity of the barrier function is critically important in maintaining lung homeostasis (Fingerlin et al., 2013). Coding mutations in *SFTPC* associated with pulmonary fibrosis lead to aberrant pro-surfactant protein C intermediate products which cause alterations in protein maturation [reviewed in Tanjore et al. (2013), Thurm et al. (2013)]. These mutations are often, but not always, detected in conjunction with activation of the Unfolded Protein Response (UPR) pathway, which has also been reported in the alveolar epithelial cells of IPF patients carrying the *SFTPC* L188Q mutation (Tanjore et al., 2012; Thurm et al., 2013). *SFTPA2* mutations induce retention of surfactant protein A in the endoplasmic reticulum and similarly lead to UPR activation (Wang et al., 2009; Maitra et al., 2010). Two proposed mechanisms link UPR activation to tissue injury and fibrosis: UPR activation likely increases the loss of epithelial cells after injury and may also be involved in promoting epithelial-to-mesenchymal (EMT) transition (Tanjore et al., 2011;

Zhong et al., 2011). Both these mechanisms likely favor the cycle of aberrant injury and repair that is typical of fibrotic responses. Recent studies have, however, also revealed that *SFTPA1* and *SFTPC* mutations induce excessive TGF- $\beta$  secretion (Maitra et al., 2012, 2013). Depending on the mutations, this effect does not always depend on UPR activation. This brings into question the importance of the role of UPR activation in promoting profibrotic phenotype associated with the polymorphisms identified by genetic studies.

The rs35705950 risk Single Nucleotide Polymorphism (SNP) for pulmonary fibrosis in the *MUC5B* region has generated a lot of interest. *MUC5B* expression is reported to be higher in the lung of IPF vs. healthy subjects, and in subjects carrying rs35705950 risk allele (Seibold et al., 2011). *MUC5B* encodes a member of the mucin family, which contains highly glycosylated proteins that are component of mucus secretions that protect the epithelial layer. Perhaps unexpectedly, rs35705950 was recently associated with improved survival in an IPF (Peljto et al., 2013). This supports the concept that underlying mechanisms of disease initiation and disease progression may be quite distinct. Given these recent revelations, it has now been proposed that the increased expression of *MUC5B* predisposes to IPF, but probably also has a beneficial role in enhancing the mucosal host defense during tissue damage. Additional SNPs in the *MUC5B* region including in the *TOLLIP* and *MUC2* loci are also associated with higher risk for IPF and the biological contribution of these SNPs in the increased risk for IPF remains to be elucidated (Fingerlin et al., 2013).

As *MUC5B*, *SFTPC*, and *SFTPA1* are expressed by alveolar type II cells, this raises the possibility that injury of these cells is a critical pathogenic mechanism in pulmonary fibrosis (Seibold et al., 2013). This hypothesis is supported by the observation that lung fibrosis occurs following ablation of alveolar type II in genetically modified mice using diphtheria toxin (DT) receptor transgene under the control of *Sftpc* promoter (Sisson et al., 2010). However, lung fibrosis was not observed in a different genetic mouse model where the DT expression is controlled by an inducible Cre recombinase knocked into the *Sftpc* locus (Barkauskas et al., 2013). This discrepancy in phenotype could reflect different levels of



**FIGURE 2 | Selected genes located in genetic risk loci associated with higher susceptibility for diseases with fibrotic complications.** Genetic studies have successfully identified numerous genetic risk loci associated with higher susceptibility for diseases associated with fibrosis. Left panel

displays diseases associated with a strong immune component and the genetic risk loci implicated in at least three of these diseases. Right panel displays diseases associated with risk loci implicating genes involved in non-immune function, such as barrier and metabolic functions.

cell ablation between the two systems or the differential contribution of additional lung cell populations that mediate the lung epithelium repair.

Different studies in human and mouse models have attempted to provide biological insights into the association of *TERT* and *TERC* polymorphisms with pulmonary fibrosis. Several reports showed shortened telomeres in IIP patients with or without mutation in genes encoding components of the telomerase complex, however, this was not replicated in a recent investigation of an IPF Mexican cohort (Alder et al., 2008; Cronkhite et al., 2008; Diaz de Leon et al., 2010; Liu et al., 2013a). In mouse models, two independent groups reported that *Tert* and *Terc* KO mice do not present spontaneous lung fibrosis and that *Terc* deficiency does not affect bleomycin-induced lung fibrosis (Liu et al., 2007; Degryse et al., 2012; Liu et al., 2013a). Degryse et al. did not observe any phenotype on bleomycin-induced lung fibrosis using *Tert* deficient mice, while Liu et al. reports a protective effect using similar disease model but a different *Tert* deficient strain. The reason of the discrepancy of these results is yet to be elucidated. However, the protection phenotype observed in *Tert* deficient mice in the bleomycin-induced lung fibrosis was similar with mice from 2nd and 4th generation despite shortening of the telomeres. This led the authors to speculate that this effect may not be dependent on telomere length (Liu et al., 2013a). Supporting the notion that *TERT* and *TERC* deficiency might contribute to pulmonary fibrosis by mechanisms dependent on telomerase activity but not necessarily telomere length, telomerase activity is induced in IPF and NSIP fibroblasts and systemic sclerosis lung compared to healthy donor samples (Fridlender et al., 2007; Liu et al., 2013a). Furthermore, telomerase activity was recently shown to regulate Wnt signaling, mitochondrial function and oxidative stress (Park et al., 2009; Sahin et al., 2011). These pathways are known to be activated in human and mouse disease tissues and the inhibition of Wnt/beta catenin pathways and oxidative stress decreases fibrosis in mouse models and are therefore currently considered as attractive therapeutic fibrosis targets (Lam and Gottardi, 2011; Hecker et al., 2012).

The examples described above show genetic studies have successfully unraveled key components of the genetic architecture of IIP and IPF by leveraging the strong genetic signals associated with disease in familial cases. Functional *in-vivo* characterizations of the genetic polymorphisms associated with pulmonary fibrosis are now starting to provide insights into potential mechanisms that remain to be further validated. Emerging efforts to evaluate the role of the susceptibility loci for pulmonary fibrosis have led to unexpected results, as demonstrated by the discovery of the association of *MUC5B* variant with disease risk, but improved disease prognosis. Additional genetic polymorphisms are proposed to affect IPF severity, such as TLR3 L412F, and FcγRIIA R131H variants, which further reflect the influence of immune mechanisms in IPF progression (Bournazos et al., 2010; O'Dwyer et al., 2013). Polymorphisms in the angiotensinogen promoter are also described to be associated with further decline of pulmonary function in IPF subjects perhaps consistent with results from mouse models in which the angiotensin pathway promotes fibrosis (Molina-Molina et al., 2008; Dang et al., 2013). However, these results are yet to be replicated in well-powered studies.

These examples clearly demonstrate the need for genetic studies of disease progression to further understand pathogenesis, alongside development of mouse models and *in-vitro/ex-vivo* models of human, cells and tissues to fully validate the leads provided by genetic studies.

### INTERSTITIAL LUNG DISEASE IN SYSTEMIC SCLEROSIS

Systemic Sclerosis (SSc) is thought to be a chronic systemic autoimmune disease with limited genetic component because of the rare familial cases and low concordance for disease in monozygotic twins (4.7%) (Feghali-Bostwick et al., 2003). However, the concordance in monozygotic twins for the presence of antinuclear antibodies in SSc is very high (90 vs. 40% for dizygotic twins), suggesting that the auto-immunity component of SSc is highly inheritable, but that the disease phenotype may be influenced by other factors that are largely not dependent on genetics. Despite limited disease heritability, several GWAS have detected genetic associations with risk for SSc and appear to have confirmed the role of the immune response in the disease risk. Many of the identified risk loci are shared with Rheumatoid Arthritis and Systemic Lupus Erythematosus, including alleles located in *MHC*, *STAT4*, *CD247*, and *IRF5* loci (Radstake et al., 2010; Allanore et al., 2011; Gorlova et al., 2011). These genes suggest that dysregulation of different components of the immune response influence auto-immunity. For example, *STAT4* regulates signaling from IL-12 and IL-23 receptors in T-cells and from IFN receptor in monocytes and NK cells, while *IRF5* is a transcription factor in the type 1 interferon pathway, and *CD247* encodes for a subunit of the T-cell receptor and modulates T-cell activation [reviewed in Romano et al. (2011)]. However, precise functional consequences of the risk alleles discovered in these loci still remain to be elucidated. These studies come with great challenges for *ex-vivo* studies using samples from patients carrying risk and non-risk alleles, and with the development of mouse models with knock-in of risk alleles for *in-vivo* studies.

Interstitial lung disease (ILD) is one complication of SSc and is most often associated with diffuse cutaneous disease and the presence of anti-topoisomerase I antibodies (Steen et al., 1988; Assassi et al., 2010). Genetic candidate approach studies (albeit with limited sample size) have identified genetic polymorphisms associated with SSc-ILD in *CTGF*, *HGF*, *MMP12*, which encode known regulators of fibrotic responses, and in *IRAK1* and *NLRP1*, which encode proteins involved innate immune responses (Fonseca et al., 2007; Manetti et al., 2010; Dieudé et al., 2011a,b; Hoshino et al., 2011; Sharif et al., 2012) (see Table 1). Similar to the above example where the *MUC5B* rs35705950 SNP is associated with IPF susceptibility but with improved prognosis, the *IRF5* SNP rs4728142 confers higher risk for SSc, but also longer survival with milder ILD (Sharif et al., 2012). Combination of the risk alleles at *STAT4* SNP rs7574865 and *IRF5* SNP rs2004640 leads to increased risk for ILD, highlighting that studies of genetic interactions may be relevant for disease (Dieudé et al., 2009). Observations such as this reflect the complexity of these diseases.

While IPF and SSc-ILD present with distinct clinical features, they are both characterized by the presence of fibrotic lesions in the lung at end stage disease. Similar gene expression profiles are detected in lung explants from IPF and SSc patients, suggesting

**Table 1 | Genetic polymorphisms proposed to be associated with SSc-ILD.**

Variants	Genes	Population	Discovery	Replication	Replication	Odd ratio	p = value	Association with phenotypes			
								Anti-SLC70	SSc	Expression	Ref
rs2276109	MMP12	Italian	250/263			2.94 (95% CI 1.25–6.95)	p = 0.01	Yes	ILD	higher level of MMP12	Manetti et al., 2010
CTGF -945GG	CTGF	UK	200/188	300/312		3.1 (95% CI, 1.9–5.0)	p < 0.001	Yes	ILD	higher level of CTGF	Fonseca et al., 2007
HGF TT -1652	HGF	Japanese	159/103	155/0		8.1 (95% CI 2.5–26.0)	p = 0.0004	NA	ESLD	lower level of HGF	Hoshino et al., 2011
rs1059702	IRAK1	EU	849/625	495/509	466/1083	2.09 (95% CI 1.35–3.24)	p = 0.0009	Yes	ILD		Dieudé et al., 2011a, 2009
rs8182352	NLRP1	EU	870/962	532/324	527/301	1.19 (95% CI 1.05–1.36)	p = 0.0065	Yes	ILD		Dieudé et al., 2011b
rs2004640	IRF5	French	# 179/374	# 134/374		1.786 (95% CI 1.25–2.58)	p = 0.002	NA	ILD		Dieudé et al., 2009
rs7574865	STAT4										
<b>VARIANTS ASSOCIATED WITH IMPROVED PROGNOSIS</b>											
rs4728142	IRF5	Caucasian	914 cases	529 cases		0.75 (95% CI 0.62–0.90)	p = 0.002		Longer survival	Lower level of IRF5	Sharif et al., 2012

*Candidate gene approach studies with limited power but increasing sample sizes have reported several candidate polymorphisms that may confer risk for SSc-ILD. Discovery and Replication stages show numbers of case and control patients. ## symbol indicate a case-case study.*

some overlap in pathogenic mechanisms (Hsu et al., 2011; Murray et al., 2012). This hypothesis was tested with three independent studies that investigated the *MUC5B* SNP rs35705950 risk allele for IPF in SSc-ILD. There was no association with SSc-ILD, while the association with IPF was confirmed by all groups (Peljto et al., 2012; Borie et al., 2013; Stock et al., 2013). This result further highlights the differences in pathogenic mechanisms associated with IPF and SSc-ILD, even when the disease tissue gene expression profiles may be similar.

Understanding the genetic architecture associated with SSc-ILD will be crucial to provide biological insights into the pathogenic mechanisms driving this debilitating disease. Breakthrough discoveries will require well-powered studies and comprehensive genetic analysis with meta-analysis of genome-wide data rather than candidate gene studies.

## DISEASES ASSOCIATED WITH RENAL FIBROSIS

Tubulointerstitial fibrosis is a feature of progression of chronic kidney diseases (CKD) and diabetic nephropathy (DN). The incidence of end stage renal disease in African Americans is known to be 3–4-fold higher compared to non-African Americans (Li et al., 2004). This excess risk is thought to be mainly due to genetic polymorphisms in the *MY9H/APOL1* region with a non-synonymous SNP in *APOL1* locus (Kao et al., 2008; Kopp et al., 2008). Interestingly, this polymorphism appears to result from a positive selection in population of African ancestry, due to a functional advantage over sleeping sickness (Genovese et al., 2010).

## CHRONIC KIDNEY DISEASE

GWAS have identified genetic polymorphisms associated with renal function and susceptibility to CKD. Genetic polymorphisms in *UMOD*, *SOX11*, and *PRKAG2* loci appear associated with CKD

(Köttgen et al., 2009, 2010; Gudbjartsson et al., 2010). Mutations in *UMOD* are linked to familial kidney diseases, and common polymorphisms in the *UMOD* locus were shown to be associated with risk for CKD in two GWAS scans (Köttgen et al., 2009, 2010; Vyletal et al., 2010). *UMOD* encodes uromodulin, which is released in the urine and plays a protective role against urinary tract infections and ischemia-induced acute kidney injury, as shown in studies of *Umod*-deficient animals (Bates et al., 2004; Mo et al., 2004; El-Achkar et al., 2008). The underlying mechanisms are unclear as uromodulin appears to have cell-specific effects that could be both pro-inflammatory (on macrophages and neutrophils) or anti-inflammatory [reviewed in El-Achkar and Wu (2012)].

*SOX11* appears essential for embryonic development as *Sox11*-deficient mice die at birth with many malformations (Hargrave et al., 1997; Sock et al., 2004). *Sox11* was shown to control the expression of *Wnt4* in *Xenopus* (Murugan et al., 2012). *Wnt4* signaling is known to play a key role in nephrogenesis, as its activation promotes renal fibrosis in mouse models (Kispert et al., 1998; Surendran et al., 2002). In addition, missense mutations in *WNT4* locus are associated with renal hypodysplasia in humans (Vivante et al., 2013). Thus, one may speculate that *SOX11* genetic variants associated with CKD might affect renal function through dysregulation of *Wnt4* pathway; this hypothesis remains to be tested.

*PRKAG2* encodes a subunit of the energy sensor AMP-activated protein kinase (AMPK) whose role in renal homeostasis has been extensively studied (Hallows et al., 2010). In the context of tissue injury, activation of AMPK inhibits Epithelial-to-Mesenchymal Transition EMT and Reactive Oxygen Species (ROS) production induced by known pro-fibrotic factors in renal fibrosis, such as TGF-β, angiotensin II and high glucose (Lee et al., 2013b). It is also reported to promote Monocyte-to-Fibroblast

transition (Yang et al., 2013). AMPK activity was shown to be protective in non-diabetic and in high fat diet-induced renal disease models (Declèves et al., 2011; Satriano et al., 2013). The beneficial effects of metformin, an AMPK activator, on renal function are recognized, but its use in CKD is currently at the center of controversial debates due to potential risk of lactic acidosis in the context of renal deficiency, (Ekström et al., 2012; Rocha et al., 2013).

### DIABETIC NEPHROPATHY

DN is a common complication of type 1 and type 2 diabetes, which have been associated with very distinct disease risk loci [Figure 2 and reviewed in Ntzani and Kavvoura (2012), Polychronakos and Li (2011)]. Results of genetics studies are extensively discussed in two recent reviews (Gu and Brismar, 2012; Palmer and Freedman, 2012), we therefore will focus only on the genetic association of *ELMO1*, *CNDP1*, and *FRDM3* loci with DN risk, as they were detected in both GWAS and candidate gene approach studies.

*ELMO1* encodes Engulfment and cell motility 1 and regulates Rac signaling and biological processes linked actin cytoskeleton remodeling. *ELMO1* plays an established role in the clearance of apoptotic cells (Park et al., 2007; Elliott et al., 2010; van Ham et al., 2012), leading to the hypothesis that *ELMO1* regulates homeostasis upon kidney injury by ensuring clearance of apoptotic cells and that impairment of this function might promote DN. *ELMO1* is also known to contribute to the development of vasculature and to the production of extracellular matrix protein (ECM), which both may affect renal fibrosis (Shimazaki et al., 2006; Epting et al., 2010).

*CNDP1* encodes carnosinase that hydrolyzes carnosine, an anti-oxidant molecule. Carnosine is a protective factor in several animal models of renal disease and was shown to inhibit TGF- $\beta$  and ECM production by mesangial cells in hyperglycemic conditions (Köppel et al., 2011; Riedl et al., 2011; Menini et al., 2012). While *CNDP1* polymorphisms are suspected to affect the level of its substrate carnosine, this remains to be demonstrated.

Little is known about the biological function of *FRDM3*, however, its locus is proposed to be associated with defective renal function in rats, based on Quantitative Trait Loci analysis (Garrett et al., 2010). It was recently suggested that *FRDM3* risk SNP for DN may affect BMP signaling, a hypothesis that remains to be validated (Martini et al., 2013).

Genetic studies have identified many genetic polymorphisms that confer risk for CKD and DN using the gene candidate approach, but GWAS often have not confirmed these associations (Gu and Brismar, 2012; Palmer and Freedman, 2012). Current studies include only cross-sectional measurements of renal function, and genetic factors affecting disease progression of renal diseases are yet to be elucidated.

## DISEASES ASSOCIATED WITH LIVER FIBROSIS

### AUTOIMMUNE LIVER DISEASES

The genetic architecture of autoimmunue liver diseases such as Primary Sclerosing Cholangitis (PSC) and Primary Biliary Cirrhosis (PBC) was recently studied through a series of GWAS (Hirschfield et al., 2009; Liu et al., 2010; Mells et al., 2011; Melum et al., 2011; Liu et al., 2013a,b). Inflammation and tissue damage is thought to trigger sustained aberrant tissue repair responses

that ultimately lead to the replacement of the organ by scar fibrotic tissue. Susceptibility loci largely overlap with the loci detected in other complex immune diseases affecting different organs: PSC and PBC shared common risk loci with multiple sclerosis, celiac disease, inflammatory bowel disease (IBD), rheumatoid arthritis and type 1 diabetes (Mells et al., 2013). Concurrent autoimmune disorders are commonly present in PSC and PBC patients (PSC is often seen in patients with IBD, type 1 diabetes and autoimmune thyroid disease and PBC is often seen in patients with Sjogren's syndrome, Raynaud Phenomenon, autoimmune thyroid disease and rheumatoid arthritis (Mells et al., 2013), which may explain the result of the genetic studies. Due to this co-occurrence of auto-immune diseases, case-case studies focusing on disease progression will be especially critical in PBC and PSC in order to identify pathogenic mechanisms that could be targeted by therapies.

### NON-ALCOHOLIC FATTY LIVER DISEASE

Non-Alcoholic Fatty Liver Disease (NAFLD) is strongly associated with obesity, type 2 diabetes and dyslipidemia. The disease is characterized by steatosis with an increased hepatic Free Fatty Acid flux and cellular damage that trigger inflammatory and fibrotic responses. Genetic polymorphisms in the *PNPLA3* locus that encodes for adiponutrin have been associated with NAFLD in many genetics studies using the candidate approach, and with well-powered GWAS (Daly et al., 2011). Adiponutrin is a triacylglycerol hydrolase, and the I148M variant associated with NAFLD induces accumulation of triacylglycerol and hepatic steatosis (He et al., 2010; Li et al., 2012). In independent studies, *PNPLA3* locus has also been associated with NAFLD progression and fibrosis (Speliotes et al., 2011; Kitamoto et al., 2013). Additional potential genetic disease-modifiers associated with fibrosis reported in these studies are *NCAN*, *GCKR*, *LYPLAL1*, *SAMM50*, and *PARVB* loci. *LYPLAL1* encodes a lysophospholipase and *GCKR* encodes glucokinase regulatory protein that regulates both glucose metabolism and lipogenesis. *GCKR* and *NCAN* variants affect circulating triglyceride levels (Gorden et al., 2013; Shen et al., 2013). Altogether, this suggests that risk for NAFLD and its progression could be largely influenced by genetic factors regulating lipid metabolism.

Genetic variants of angiotensin II receptor 1 have also been reported to be linked to fibrosis in NAFLD in two different studies, but with some inconsistencies in effects of *AGTR1* variants/alleles perhaps due to cohort ethnicities (Yoneda et al., 2009; Zain et al., 2013). The use of blockers of angiotensin receptor in patients with liver fibrosis has yielded different results, therefore their beneficial effect in NAFLD remains controversial (Yokohama et al., 2004; Abu Dayyeh et al., 2011; Hirata et al., 2013). Thus, understanding the role of *ATGR1* genetic polymorphisms in the progression of NAFLD liver fibrosis, and defining the relevant patient population, might be crucial to evaluate the potential beneficial role of angiotensin receptor blockers in NAFLD progression.

## DISEASES ASSOCIATED WITH INTESTINAL FIBROSIS

Intestinal fibrosis is a common complication occurring with intestinal inflammation such as that seen with IBD, which comprises both Crohn's Disease (CD) and Ulcerative Colitis (UC)

(Speca et al., 2012). The GWAS approach was very successful in identifying more than a hundred genetic risk factors for IBD (Franke et al., 2010; Anderson et al., 2011). These discoveries highlighted a major role for inflammatory pathways controlling innate and adaptive immune responses, mucosal barrier function, endoplasmic reticulum stress and oxidative stress in the disease pathogenesis (Khor et al., 2011). The chronic inflammatory injury in IBD triggers unrelenting mucosal injury/repair processes, and this ongoing damage/repair cycle is thought to underlie the intestinal fibrosis and strictures that are commonly seen in CD patients. In a subset of CD patients, the fibrosis and strictures can lead to intestinal obstruction and thus surgery. Immunosuppressive and anti-inflammatory treatments have little effects on intestinal fibrosis once the process has started, suggesting that non-immune pathways must be playing a role in the progression of fibrosis.

It is somehow surprising that there are only a few reports on genetic polymorphisms associated with higher risk for intestinal fibrosis, despite the strong success in recruiting a large number of patients for the IBD GWAS efforts. Candidate-gene approach studies with small size cohorts have implicated *NOD2* and *CX3CR1* polymorphisms as higher risk factors for structuring CD and the subsequent need for surgery (Abreu et al., 2002; Lesage et al., 2002; Brand et al., 2006; Seiderer et al., 2006; Sabate et al., 2008). *NOD2* is a known risk factor for CD and is presumed to primarily control innate immune response to bacterial products, while *CX3CR1* is a chemokine receptor involved in leukocyte recruitment. In addition, GWAS to identify genetic polymorphisms associated with CD severity has recently implicated several loci including *IL12B*, *RXRA/COL5A1*, *AHR*, and *FOXO3* loci in severe clinical phenotypes defined by need for surgery (Dubinsky et al., 2013; Lee et al., 2013b). However, their specific association to structuring CD is unclear. It is the hope that future studies will be able to identify genetic risk factors associated with the structuring CD to better understand the pathogenesis of the development of intestinal fibrosis in IBD.

## DISEASES ASSOCIATED WITH SKIN FIBROSIS

Skin fibrosis represents a cardinal feature of several diseases with debilitating skin pathologies, including keloid disease/hypertrophic scars, systemic sclerosis and nephrogenic systemic fibrosis. While the etiology of skin fibrosis remains poorly understood, growing evidence supports the hypothesis that fibrosis arises from aberrant tissue injury (e.g., vasculopathy) and repair (e.g., wound healing) responses.

A number of studies in recent years have investigated the genetic basis of skin fibrosis, especially in keloid disease (KD) where higher prevalence in ethnicities with darker pigmentation. Linkage studies in familial keloids have been reported, with suggested candidate genes involved in fibroblast proliferation (*EGFR*), inflammation (*TNFAIP6*), and TGF- $\beta$  signaling (*SMADs*) (Marneros et al., 2004; Yan et al., 2007). Supporting the potential role of inflammation and/or immune activation in the pathogenesis of KD, polymorphisms in the HLA region are associated with increased risk for KD in Caucasian, Chinese and Black populations (Brown et al., 2008, 2010; Lu et al., 2008). More recently, two independent GWAS of KD in Japanese and Chinese

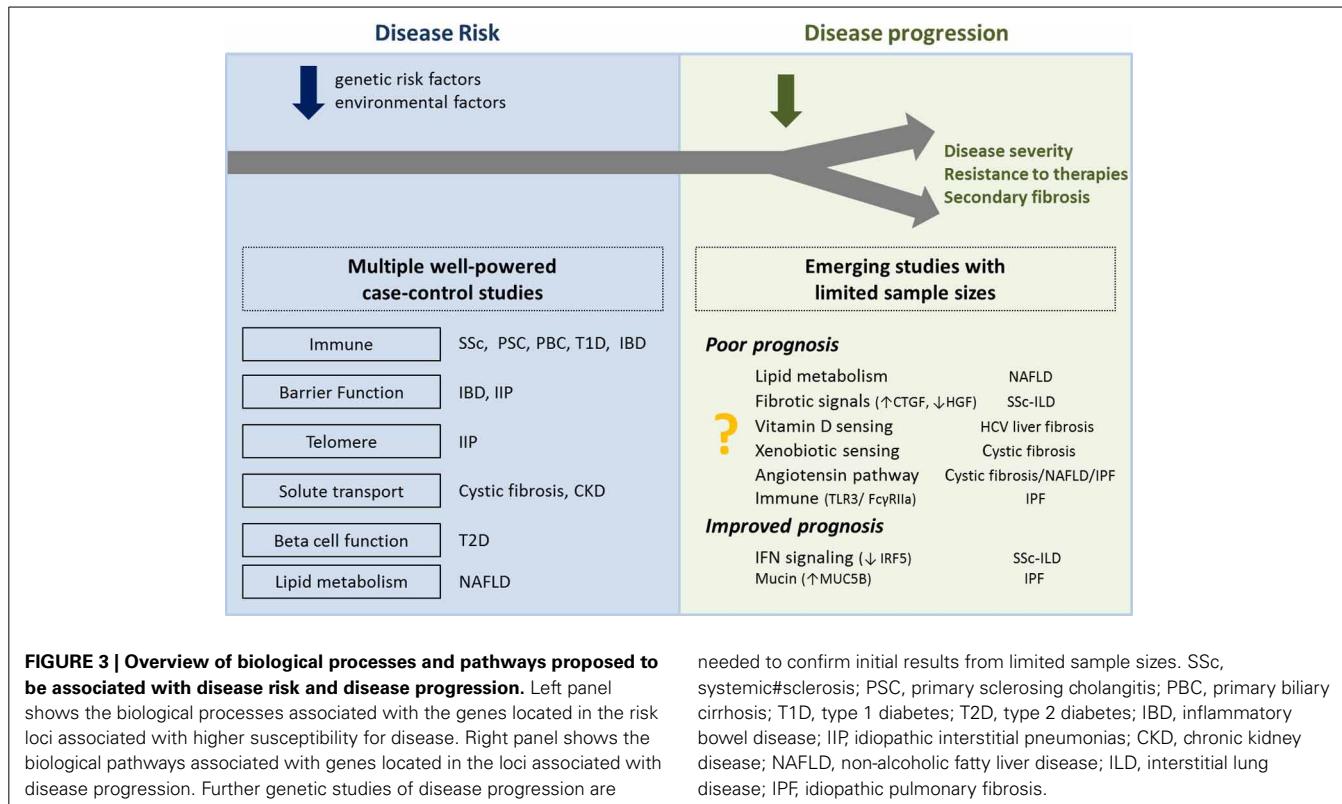
populations identified risk SNPs in chromosomal regions 1q41 and 15q21.3 (*NEDD4* locus) (Nakashima et al., 2010; Zhu et al., 2013). It will be of great interest to understand how these loci may confer disease susceptibility for KD whose etiology is poorly understood.

Several candidate gene and GWAS have been carried out in systemic sclerosis (SSc), with most of the genetic variants identified being immune regulatory genes as mentioned earlier in this review. While these findings clearly support a major role of autoimmunity in SSc genetics, it is less clear whether these SSc susceptibility loci are directly involved in SSc skin fibrosis. SSc can be divided in two subtypes and the extent of the skin fibrosis is greater in diffuse SSc than in limited SSc. GWAS in SSc detected only one locus in *ZC3H10/ESYT1* region conferring susceptibility preferentially for the diffuse clinical phenotype (Gorlova et al., 2011). It remains to be elucidated whether there is relationship between this risk locus and any pathogenic mechanisms linked to the skin fibrosis in diffuse SSc. Since 90% of the SSc patients have Raynaud's syndrome preceding their onset of skin hardening by several years, and vasculopathy is often viewed that may be a key disease-driving cause of SSc, it is somewhat surprising that no vasculature-related genes have been described from the SSc GWAS studies so far.

In addition to risk factors for SSc, an allele in *CAV1* locus (encoding caveolin 1) was recently shown in a French cohort and replicated in an Italian cohort to confer protection against SSc and in particular limited SSc (Manetti et al., 2012). This protective allele was shown to be associated with an increased expression of caveolin 1 in skin from both healthy subjects and SSc patients. Caveolin 1 is a component of membrane caveolae that is proposed to regulate TGF- $\beta$  receptor degradation (Del Galdo et al., 2008a). Confirming an anti-fibrotic role of caveolin 1, *Cav1*-deficient mice develop spontaneous lung and skin fibrosis (Drab et al., 2001; Del Galdo et al., 2008a,b). Caveolin 1 expression is decreased in many human fibrosis tissues including SSc skin and lung, IPF lung and keloid-derived fibroblasts, which suggest that the caveolin 1-mediated regulatory pathway may represent a new therapeutic opportunity in fibrotic diseases (Wang et al., 2006; Del Galdo et al., 2008a,b; Zhang et al., 2011).

## FIBROSIS PROGRESSION: PROMISING STUDIES IN CYSTIC FIBROSIS AND HCV-INDUCED FIBROSIS

Cystic fibrosis arises as the result of an abnormal transport of salt due to mutations in *CFTR*. Although this is a Mendelian disorder, additional genetic factors are emerging as disease modifiers due to their influence on disease severity. Polymorphisms in *MUC5AC* may affect the severity of cystic fibrosis lung disease highlighting further the role of mucin in maintaining lung homeostasis (Guo et al., 2011). A recent GWAS meta-analysis including more than 3000 patients detected a SNP in a large intergenic region near *EHF* and *APIP* to be associated with disease severity (Wright et al., 2011). Additional suggestive (close to genome-wide significance) associations were reported in *AGTR2* and in *AHRR* regions, indicating a role for angiotensin and xenobiotic sensing pathways in the severity of cystic fibrosis. As mentioned previously, the angiotensin pathway may be involved in the progression of NAFLD. Strikingly, angiotensin receptor blockade protects



from experimental lung fibrosis and *Ahr*-deficient mice develop hepatic fibrosis (Fernandez-Salgueiro et al., 1995; Andreola et al., 2004; Waseda et al., 2008; Yaguchi et al., 2013). Further studies will be required to test the hypothesis that these pathways may be critical in influencing disease progression in cystic fibrosis and may lead to additional therapeutic approach for this Mendelian disorder.

Genetic disease-modifiers in HCV-induced fibrosis were recently identified in a GWAS meta-analysis including more than 2000 patients (Patin et al., 2012). Genetic polymorphisms in *RNF7* and *MERTK* were associated with fibrosis progression and also point to the previously mentioned involvement of oxidative stress and the clearance of apoptotic cells in fibrotic diseases (Duan et al., 1999; Scott et al., 2001; Zizzo et al., 2012). Different candidate gene approach studies detected rs12785878 near *DHCR7* to be associated with 25-hydroxyvitamin D [25(OH)D] serum levels, liver stiffness in chronic liver diseases, and progression of liver fibrosis in HCV patients (Grönhage et al., 2012; Petta et al., 2013). The same SNP was associated with development of hepatocarcinoma, but not with progression rate of liver fibrosis in HCV patients (Lange et al., 2013). Strikingly, vitamin D receptor was demonstrated to be key in the control of liver fibrosis by affecting SMAD3-mediated transcriptional response in mouse model, supporting the notion that this pathway might be essential in the control of liver fibrosis (Ding et al., 2013).

Together these studies confirm that genetics may play a critical role in influencing disease progression independently of the cause of the fibrosis (Mendelian or infectious disease). Understanding the underlying biological pathways associated with these disease

needed to confirm initial results from limited sample sizes. SSc, systemic sclerosis; PSC, primary sclerosing cholangitis; PBC, primary biliary cirrhosis; T1D, type 1 diabetes; T2D, type 2 diabetes; IBD, inflammatory bowel disease; IIP, idiopathic interstitial pneumonias; CKD, chronic kidney disease; NAFLD, non-alcoholic fatty liver disease; ILD, interstitial lung disease; IPF, idiopathic pulmonary fibrosis.

modifiers, and how they influence fibrosis, may lead to new leads for therapeutic strategies.

## CONCLUDING REMARKS

Genetic studies have successfully identified polymorphisms associated with susceptibility for diseases with fibrotic complications. On-going functional studies attempt to elucidate the underlying pathogenic mechanisms. After a decade of human genetics studies focusing on disease risk, emerging results from genetic studies of disease progression suggest a multi-hit paradigm in which disease initiation and disease progression are not necessarily driven by the same mechanisms (Figure 3). Early discoveries on fibrosis progression point to pathways already shown in mouse models to control fibrotic responses, such as vitamin D and xenobiotic sensing pathways. Perhaps future genetic studies on disease progression will identify more genes and pathways identified in mouse models to control fibrotic responses.

The identification of genetic disease modifiers comes with great challenges with a requirement for clinical annotations to inform on disease progression or severity with well-powered case-case studies rather than case-control studies to understand disease progression in human fibrotic diseases. However, elucidating the genetic basis of disease severity is crucial to understand pathogenic mechanisms and may be even more relevant to highlight biological pathways for therapeutic interventions.

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# Fibroblasts in fibrosis: novel roles and mediators

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Fibroblasts are the most common cell type of the connective tissues found throughout the body and the principal source of the extensive extracellular matrix (ECM) characteristic of these tissues. They are also the central mediators of the pathological fibrotic accumulation of ECM and the cellular proliferation and differentiation that occurs in response to prolonged tissue injury and chronic inflammation. The transformation of the fibroblast cell lineage involves classical developmental signaling programs and includes a surprisingly diverse range of precursor cell types—most notably, myofibroblasts that are the apex of the fibrotic phenotype. Myofibroblasts display exaggerated ECM production; constitutively secrete and are hypersensitive to chemical signals such as cytokines, chemokines, and growth factors; and are endowed with a contractile apparatus allowing them to manipulate the ECM fibers physically to close open wounds. In addition to ECM production, fibroblasts have multiple concomitant biological roles, such as in wound healing, inflammation, and angiogenesis, which are each interwoven with the process of fibrosis. We now recognize many common fibroblast-related features across various physiological and pathological protracted processes. Indeed, a new appreciation has emerged for the role of non-cancerous fibroblast interactions with tumors in cancer progression. Although the predominant current clinical treatments of fibrosis involve non-specific immunosuppressive and anti-proliferative drugs, a variety of potential therapies under investigation specifically target fibroblast biology.

**Keywords:** fibroblast, myofibroblast, fibrosis, scleroderma, idiopathic pulmonary fibrosis, extracellular matrix, endostatin

## INTRODUCTION

Fibroblasts are the workhorse of the most important tissue that holds the human body together—connective tissue. Connective tissue joins and supports all other tissues, including the parenchymal tissues of organs. This connective tissue is made of fibroblasts widely-spaced in a vast extracellular matrix (ECM) of fibrous proteins and gelatinous ground substance. Fibroblasts produce the ECM's structural proteins (e.g., fibrous collagen and elastin), adhesive proteins (e.g., laminin and fibronectin), and ground substance (e.g., glycosaminoglycans, such as hyaluronan and glycoproteins). However, fibroblasts also play various additional roles beyond ECM production. For example, fibroblasts serve pivotal roles in ECM maintenance and reabsorption, wound healing, inflammation, angiogenesis, cancer progression, and in physiological as well as pathological tissue fibrosis. Ancillary to these various biological roles, fibroblasts produce and respond to a broad array of paracrine and autocrine signals, such as cytokines and growth factors. Targeting these ancillary signaling events is the main strategy underlying multiple lines of research for a new generation of treatments for fibroblast-related disorders.

Fibroblasts are mesenchymal cells derived from the embryonic mesoderm tissue, and they are not terminally differentiated. They can be activated by a variety of chemical signals that promote proliferation and cellular differentiation to form myofibroblasts with an up-regulated rate of matrix production. Fibroblast activation plays a vital role in wound healing however, in some cases and for reasons that remain to be fully elucidated, their activation becomes uncontrolled, producing a pathological fibrotic response

that promotes multiple diseases and affects a variety of organs. Indeed, fibrosis plays a significant contributory role in most cases of organ failure. Examples are wide ranging: systemic sclerosis (SSc); idiopathic pulmonary fibrosis (IPF); liver cirrhosis; kidney fibrosis; and the cardiac fibrosis observed in cardiac hypertrophy resulting in heart failure. The essential role of fibroblasts in lung fibrosis was validated using lineage-specific deletion of the type II TGF $\beta$  receptor (Hoyle et al., 2011).

Here we review a variety of fibroblast functions that illustrate a central role for fibroblasts in the pathology of fibrosis. We review ECM production in relation to fibrosis, some examples of critical chemical signaling, myofibroblast differentiation, the role of fibroblasts in stromal–cancer interactions, and potential clinical therapies targeting fibroblasts.

## FIBROBLAST ROLE IN MATRIX PRODUCTION AND MAINTENANCE

Fibroblasts' most well-known biological role is the production of the rich ECM of connective tissues. Fibroblasts produce and secrete all components of the ECM, including the structural proteins, adhesive proteins, and a space-filling ground substance composed of glycosaminoglycans and proteoglycans.

## STRUCTURAL PROTEINS

Fibroblasts produce an interconnecting meshwork of extracellular protein fibers and connector proteins that provide structure to tissue. Reciprocally, these proteins can promote the differentiation of profibrotic myofibroblasts through positive feedback

regulation during fibrosis (Blaauwber et al., 2013). The variety of ECM structures serve roles as diverse as forming lamina that delineate borders separating distinct cell types with different functions, to serving as interstitial regions that connect cells of a common type with a common biological function. Fibroblasts provide these specialized ECMs in different tissues by expressing and secreting a robust and variable repertoire of structural proteins with distinct properties. For example, the fibril rigidity provided by collagen type I, the most abundant protein in mammals, is due to its rope-shaped, triple-stranded helical tertiary protein structure that reinforces its tensile strength, preventing overstretching. In contrast, elastin proteins form highly crosslinked, yet unstructured, elastic networks that allow for expansive stretching without breaking. Tissues requiring differing degrees of rigidity vs. flexibility, such as skin and lung, differ in the relative expression of collagen subtype and elastin proteins. Moreover, the pathological histology of fibrosis includes an increase in the relative balance of collagen (Gilbane et al., 2013; McKleroy et al., 2013). A large number of genes that express different collagen subtypes also provide unique matrix properties. Fibroblast-mediated formation of basement membrane (composed of a layer of basal lamina and a layer of reticular lamina) serve as structural scaffolds critical for tissue regeneration in wound healing; cell barriers segregating epithelial from endothelial tissues; barriers preventing malignant escape or invasion of cancerous cells; filtration devices found in the glomerular filtration of blood in the kidney; and filtration of the alveoli-capillary interface in the lung. Basement membrane ECM is composed largely of collagen type IV, and in this environment, the presence of collagen type I promotes the up-regulation of TGF $\beta$  and epithelial-to-mesenchymal transdifferentiation (EMT), producing, for example, fibroblasts with a profibrotic phenotype in kidney fibrosis (Zeisberg et al., 2001).

### ADHESIVE PROTEINS

Adhesive ECM proteins such as fibronectin and laminin form the connection between cells and the ECM. Fibronectins bind to ECM fibers and contain RGD domains required for interacting with the integrin transmembrane cell adhesion proteins. It is ironic that fibronectin is essential for collagen assembly into ECM, yet a conditional knock out of fibronectin expression in the liver was found to increase collagen production, TGF $\beta$  signaling, and hepatic fibrosis (Kawelke et al., 2011). Therefore, locally produced fibronectin in ECM also regulates the amount of active TGF $\beta$  and serves to protect tissue from TGF $\beta$  hyperstimulation (Kawelke et al., 2011). Laminins also interact with integrin receptors to regulate cell attachment to the basal lamina. In fact, laminin has been proposed as a biomarker for hepatic fibrosis (Santos et al., 2005).

### GROUND SUBSTANCE

The ground substance of ECM is a hydrated gel of proteoglycans that is interspersed among the structural proteins. The ground substance forms a final pathway for nutrient flow beyond the reach of blood vessel transport into tissues as well as a pathway for intercellular communication. This cell-free medium forms an avenue for cell migration of immune cells,

fibroblasts, and myofibroblasts. It is also an essential avenue for endothelial cell migration during angiogenesis. The proteoglycans of ground substance are proteins that are posttranslationally modified by the addition of unbranched polysaccharides called glycosaminoglycans (GAGs), or descriptively as mucopolysaccharides. These GAGs are composed of repeating disaccharide units that contain an amino sugar of either N-acetylglucosamine or N-acetylgalactosamine and are often sulphated. The family of GAG posttranslational modifications found in ECM includes hyaluronan (non-sulfated), heparin and heparan sulfate, chondroitin and dermatan sulfate, and keratan sulfate.

Proteoglycans directly interact with many chemical signals, such as growth factors and TGF $\beta$ . Through these interactions, the ECM proteoglycans can regulate chemical signaling by either inhibiting or augmenting a signal's responsiveness. The proteoglycan tenascin-C (TN-C) interacts with ECM (Chung et al., 1995), cell surface receptors (Schnapp et al., 1995; Midwood et al., 2009; De Laporte et al., 2013), and is secreted in response to TGF $\beta$  stimulation (Pearson et al., 1988). TN-C is highly expressed during embryonic development, chronic inflammation, and fibrosis. Our group has characterized the role of insulin-like growth factor binding protein (IGFBP)-3, which is overexpressed in fibrotic skin and lungs, in mediating the induction of TN-C by TGF $\beta$  (Brissett et al., 2012). Exposure of lung fibroblasts *in vitro* to IGFBP-3 directly induces TN-C production and secretion. Further, SSc patients with pulmonary fibrosis have significantly higher levels of circulating TN-C compared with SSc patients without pulmonary fibrosis (Brissett et al., 2012). Another ECM proteoglycan, decorin, antagonizes TGF $\beta$  activity (Yamaguchi et al., 1990). Decorin is a small proteoglycan that contains a single GAG chain of either chondroitin sulfate or dermatan sulfate. Decorin directly interacts with TGF $\beta$  and inhibits its profibrotic biological activity. Recombinant expression of decorin in the lung airways of mice inhibits bleomycin-induced pulmonary fibrosis (Kolb et al., 2001). In addition to the secreted proteoglycans of the ECM, there are also a number of cell membrane proteoglycans that can interact with chemical signals and function as co-receptors. An important example is the syndecans. Syndecan 2 (SDC2) is over-expressed during fibrosis and is induced in fibroblasts in response to both TGF $\beta$  and IGFBP-3 (Ruiz et al., 2012). Similar to the above examples, proteoglycans can also interact with membrane-bound and secreted proteases such as MMP-7 (Yu and Woessner, 2000) as well as secreted protease inhibitors such as TIMP-3 (Yu et al., 2000), regulating their biological activities in signaling and ECM processing.

### ECM MAINTENANCE AND REABSORPTION

In addition to extracellular matrix production, fibroblasts are also responsible for its maintenance and reabsorption. Unlike bone, where a specialized cell type, osteoblasts, produces bone (a highly mineralized extracellular matrix) and a distinct cell type, osteoclasts, reabsorbs bone, there are no known fibroblast cells that specialize in matrix reabsorption. As far as we know, the same fibroblasts that produce ECM are responsible for matrix maintenance and degradation. As such, these cells have important roles in resolving pathological fibrosis. Collagen

maturation is controlled by the enzyme lysyl oxidase (Lox), which is produced by fibroblasts, cross-links collagen fibers, and thereby strengthens ECM. Interestingly, inhibiting LOX-mediated collagen cross-linking antagonizes both fibrosis and tumor metastasis (Cox et al., 2013). Collagen turnover (catabolism) is regulated by a multitude of secreted extracellular proteases. Fibroblasts produce both matrix degrading enzymes (such as metalloproteinases, aka MMPs) as well as their inhibitors (the tissue inhibitors of metalloproteinases, aka TIMPs). Curiously, MMP expression in IPF is increased, and experiments with various MMP knockout mice demonstrate protection from bleomycin-induced lung fibrosis (McKleroy et al., 2013).

## CONCOMITANT AND INTERWOVEN BIOLOGICAL ROLES OF FIBROBLASTS

### WOUND HEALING

Fibroblasts have a pivotal role in wound healing in response to tissue injury. First and foremost, fibroblasts respond to wound healing by proliferating and by chemotaxing to the sites of tissue injury to rebuild the ECM as a scaffold for tissue regeneration. Fibroblast to myofibroblast transitioning enables the contraction of the matrix to seal an open wound in the event of the loss of tissue (Gabbiani, 2003; Midwood et al., 2004). Fibroblasts also play a role in blood clotting, such as in the production of urokinase plasminogen activators (PAs) and their inhibitors (PAIs). Fibroblasts express the protease activated receptor PAR1 that enables fibroblast responsiveness to activated thrombin. PAR1 receptor expression is upregulated in IPF (Howell et al., 2005) and in lung tissue of SSc patients (Bogatkevich et al., 2005). Further, PAR1 knockout mice resist bleomycin-induced lung fibrosis (Howell et al., 2005). PAR1 receptors have well documented roles in the process of fibrosis, and PAR1 receptor antagonists as well as thrombin inhibitors could be beneficial for treating SSc and IPF (Atanelishvili et al., 2014).

### INFLAMMATION

Fibroblasts serve roles in inflammation and immune cell recruitment to sites of tissue injury. Furthermore, fibroblasts produce and are responsive to many inflammatory cytokines. Fibroblasts are responsive to cytokines such as TGF $\beta$ 1, IL-1 $\beta$ , interleukin-6 (IL-6), IL-13, IL-33 (Feghali and Wright, 1997; Scotton and Chambers, 2007; Savinko et al., 2012), as well as prostaglandins (Keerthisingam et al., 2001; Stratton et al., 2002; Maher et al., 2010) and leukotrienes (Mensing and Czarnetzki, 1984; Chibana et al., 2003; Eap et al., 2012). Fibroblasts are stimulated chemically by inflammatory agents to differentiate into myofibroblasts that have a greatly up-regulated rate of matrix production (discussed in more detail below). In turn, fibroblasts produce and secrete cytokines such as TGF $\beta$ 1, IL-1 $\beta$ , IL-33, CXC, and CC chemokines (Feghali and Wright, 1997; Gharaei-Kermani et al., 2003, 2012; Scotton and Chambers, 2007), as well as reactive oxygen species (Amara et al., 2010; Bondi et al., 2010). These factors allow fibroblasts to assist in the activation and migration of resident immune cells such as macrophages. Moreover, the recruitment of non-resident immune cells is facilitated by the fibroblast-mediated production and maintenance of the relatively spacious, non-solid ground substance of the extracellular matrix, which plays an

important role as a thoroughfare for the extravasation of immune cells into connective tissue. These tools endow fibroblast roles in chemical (non-specific) and cell-mediated immunity, acute and chronic inflammation, and inflammation resolution. Fibroblasts can contribute to chronic inflammation (Flavell et al., 2008), and reciprocally, inflammatory cytokines promote fibroblast to myofibroblast transition, facilitating fibrosis.

### ANGIOGENESIS

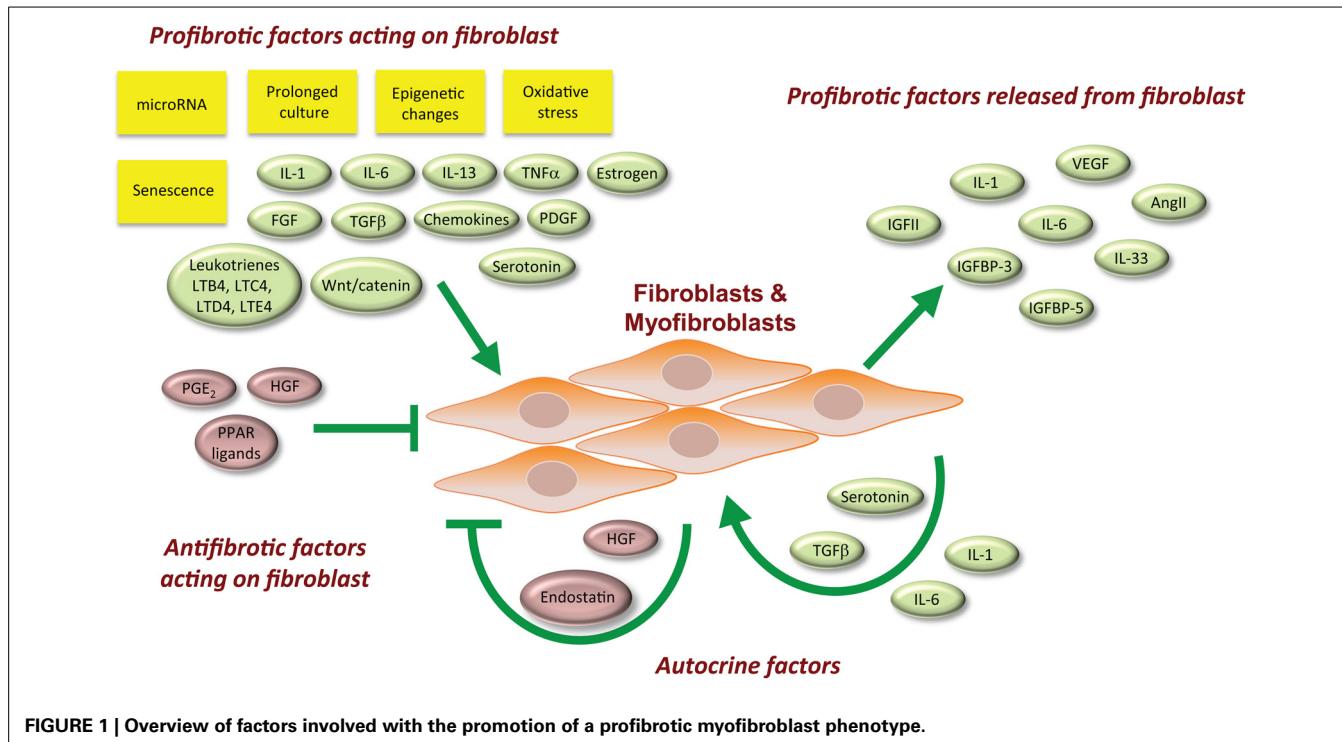
Fibroblasts have close interactions with endothelial cells and facilitate angiogenesis into tissues beyond the reach of existing blood vessels. This response requires the migration of endothelial cells to construct tubes through the ground substance of connective tissue (Dunn et al., 2000). Fibroblasts play a critical role in angiogenesis; fibroblast-derived matrix proteins and cytokines are essential for endothelial cell-mediated lumen formation (Newman et al., 2011). A major mechanism for this phenomenon is the fibroblast-mediated production and release of vascular endothelial growth factor (VEGF), which acts on VEGF receptors expressed on endothelial cells to promote angiogenesis. Interestingly, dermal fibroblasts from SSc patients overexpress VEGF in response to autocrine TGF $\beta$  signaling (Kajihara et al., 2013). This VEGF could play a role in the vascular damage that in turn promotes fibroblast activation, thereby supporting fibrosis (Kajihara et al., 2013).

## ANCILLARY SIGNALING FUNCTIONS OF FIBROBLASTS

The signaling factors associated with fibroblast biology are not necessarily specific for one biological role over another. Below we discuss these signaling factors with respect to their sources and their cellular targets (Figure 1). The net effect of multiple stimuli that recapitulate the milieu in human tissues is difficult to reproduce in the laboratory, although initial efforts using *ex vivo* organ cultures such as skin (Yasuoka et al., 2008; Yamaguchi et al., 2012) are promising.

### PARACRINE SIGNALS THAT ACT ON FIBROBLASTS

Some factors act to promote a profibrotic phenotype. Platelet-derived growth factor (PDGF) (Scotton and Chambers, 2007), IL-6 (Feghali et al., 1992), IL-13 (Fuschiotti, 2011; Fuschiotti et al., 2013), and the eicosanoid leukotrienes (Mensing and Czarnetzki, 1984; Chibana et al., 2003; Eap et al., 2012) are cytokines that promote inflammation and the development of a fibrotic response. PDGF is produced by a variety of cell types, such as platelets, endothelial cells, smooth muscle cells, and macrophages. It acts on the mesenchyme and fibroblasts to induce proliferation, differentiation, and ECM production (Scotton and Chambers, 2007). PDGF is important for wound healing, inflammation, angiogenesis, embryonic development, and fibrosis. IL-6 is a pro-inflammatory cytokine that acts on a range of cell types and is produced by T cells, macrophages, skeletal muscle cells, and fibroblasts (Feghali and Wright, 1997). IL-6 has well-known roles in inflammation, cellular differentiation, and fibrosis. IL-6 is elevated in the blood of SSc patients and produced in excess by SSc fibroblasts (Feghali et al., 1992; Feghali and Wright, 1997). Elevated IL-6 levels correlate with worse long-term survival, and therefore IL-6 could potentially be used as a prognostic clinical



**FIGURE 1 |** Overview of factors involved with the promotion of a profibrotic myofibroblast phenotype.

biomarker for patients with diseases such as SSc (Khan et al., 2012). IL-13 is a cytokine mainly produced by mast cells but also T lymphocytes (Wynn, 2003), and it acts on multiple cell types, including fibroblasts. With roles in both inflammation and fibrosis, IL-13 can stimulate TGF $\beta$  production, TGF $\beta$  activation, collagen production, MMP expression, fibroblast proliferation, and myofibroblast differentiation (Fuschiotti, 2011; Fuschiotti et al., 2013). The leukotriene eicosanoids are additional signals that induce fibroblast proliferation and matrix production contributing to fibrosis (Mensing and Czarnetzki, 1984; Chibana et al., 2003; Eap et al., 2012).

Of special note, the fibroblast growth factor (FGF) family of genetically related protein ligands was originally identified as mitogens and differentiation factors for fibroblasts (Armelin, 1973; Gospodarowicz, 1974). Despite the fact that this family has since been found to act on multiple cell types, FGFs require heparin or heparan sulfate proteoglycan molecules to bind their surface receptors (Ornitz and Itoh, 2001). Therefore, they require close proximity to ECM and the fibroblasts that secrete ECM. There are 22 FGF genes found in humans (Ornitz and Itoh, 2001), and these genes are alternatively-spliced (Ornitz and Itoh, 2001) generating additional FGF proteins. FGF ligands activate a family of 4 FGF receptors with differing specificities (Dionne et al., 1991). High amounts of active FGF2 are found in bronchoalveolar lavage fluid and in lung tissues of patients with pulmonary fibrosis (Henke et al., 1991; Inoue et al., 1996). Mast cell-generated FGF2 correlates with the localization and the extent of fibrosis in IPF (Inoue et al., 1996, 2002).

Paracrine factors that can antagonize fibrosis have also been described. The eicosanoids prostaglandin E2 (PGE<sub>2</sub>) (Keerthisingam et al., 2001; Maher et al., 2010) and prostacyclin

(PGI<sub>2</sub>) (Stratton et al., 2002) are two examples of paracrine signals that inhibit the fibrotic response. PGE<sub>2</sub> inhibits fibroblast proliferation and collagen production (McAnulty et al., 1997; Goldstein and Polgar, 1982; Saltzman et al., 1982). It acts on target cells by activating the G protein-coupled EP receptors 1–4. PGE<sub>2</sub> has been observed to inhibit fibroblast to myofibroblast differentiation and the TGF $\beta$ 1 stimulated expression of  $\alpha$  smooth muscle actin ( $\alpha$ SMA) in lung fibroblasts via the EP2 subtype receptors (Kolodick et al., 2003). PGE<sub>2</sub> also has an anti-apoptotic effect on the alveolar epithelium (Maher et al., 2010). Iloprost is a synthetic analog of prostacyclin eicosanoid PGI<sub>2</sub> that dilates systemic and pulmonary arterial vascular beds and is used for the treatment of patients with SSc (Erre and Passiu, 2009).

#### PARACRINE SIGNALS PRODUCED AND BROADCAST BY FIBROBLASTS

IGFBP-3 and -5, IGF-II, connective tissue growth factor (CTGF), IL-33, CXC chemokines, CC chemokines, and reactive oxygen species (ROS) are paracrine signals produced by fibroblasts that further enhance a tissue fibrotic response. Both IGFBP-3 and -5 are profibrotic factors implicated in SSc and IPF (Veraldi et al., 2009; Veraldi and Feghali-Bostwick, 2012). CTGF plays pivotal roles in wound healing, angiogenesis, and fibrosis. It is highly expressed by endothelial cells in response to a variety of stressors, but it is also expressed and released by fibroblasts. CTGF interacts with a wide variety of receptors, extracellular ligands, as well as ECM proteins (Liu et al., 2011a). CTGF knock-out mice are less responsive to the induction of fibrotic skin lesions by bleomycin (Liu et al., 2011a). Conversely, transgenic mice with fibroblast-specific expression of CTGF develop fibrosis without any other profibrotic stimulus and display an accelerated fibrotic response relative to exogenously added CTGF (Sonnyal

et al., 2010). CTGF is well-characterized to act synergistically with TGF $\beta$  to promote pathological fibrosis. Furthermore, TGF $\beta$ 1 induces CTGF in fibroblasts (Leof et al., 1986; Paulsson et al., 1987; Soma and Grotendorst, 1989; Igarashi et al., 1993; Mori et al., 1999). IL-33 is a cytokine produced primarily by endothelial and smooth muscle cells in the vasculature and the lungs. However, its production by dermal fibroblasts can also be induced by inflammation and in cardiac fibroblasts by mechanical stress. Knocking down IL-33 expression in mice is cardioprotective in the face of chronic angiotensin II stimulation (Sanada et al., 2007). IL-33 facilitates inflammation and fibrosis (Savinko et al., 2012). CXC and CC chemokines are broadcast by fibroblasts and play significant roles in the chemotaxis of various leukocytes to sites of tissue injury (Gharaee-Kermani et al., 2003, 2012). TGF $\beta$  also induces NADPH oxidase 4 (NOX4) in fibroblasts (Amara et al., 2010; Bondi et al., 2010), implicating reactive oxygen species (ROS) and oxidative stress in the pathology of fibrosis. NOX4 generates a basal level of extracellular H<sub>2</sub>O<sub>2</sub>. NOX4 mRNA expression is increased in rat kidney fibroblasts in culture (Bondi et al., 2010) and in primary lung fibroblasts derived from the lungs of patients with IPF (Amara et al., 2010).

Fibroblasts do not typically express and release the angiogenic hormone angiotensin II (AngII), however both activated macrophages and myofibroblasts produce AngII during the pathology of fibrosis (Berk et al., 2007). Although the phenomenon is not well characterized in most tissues, AngII is known to promote TGF $\beta$ -mediated cardiac remodeling (Rosenkranz, 2004) and the fibrosis observed in left ventricular cardiac hypertrophy (Rosenkranz, 2004). These findings suggest that AngII could contribute to fibrosis in other tissues.

### AUTOCRINE ACTIONS AMONG FIBROBLASTS

TGF $\beta$  (Scotton and Chambers, 2007), IL-1 $\beta$ , and IL-6 (Feghali and Wright, 1997) are examples of cytokines that are both produced by fibroblasts (as well as other cell types) and that act on fibroblasts to promote inflammatory and fibrotic responses. TGF $\beta$ s are named after their ability to act on fibroblasts to induce oncogenic transformation. The three known TGF $\beta$  ligands assert their biological actions by activating TGF $\beta$  receptors, cell surface serine/threonine kinase receptors. TGF $\beta$  is the prototypic profibrotic cytokine. It acts on fibroblasts and myofibroblasts to promote proliferation, migration, matrix production, the production of chemotactic signals promoting leukocyte recruitment to the site of injury, fibrosis, and the differentiation of fibroblasts to myofibroblasts. Inhibitors of each TGF $\beta$  receptor antagonize fibrosis development in experimental models (Bonniaud et al., 2005; Fu et al., 2011). The cytokine IL-1 $\beta$ 's expression and release is induced by a wide variety of cells, including fibroblasts, in response to inflammation (Feghali and Wright, 1997). IL-1 $\beta$  is a potent pro-inflammatory cytokine that also induces production of the profibrotic cytokines PDGF and TGF $\beta$ .

Some autocrine fibroblast factors counteract fibrosis, such as the hepatocyte growth factor (HGF). HGF is a protein produced by fibroblasts and acts by binding the receptor tyrosine kinase c-Met expressed on nearby epithelial cells, endothelial cells, and fibroblasts (Bogatkevich et al., 2007a,b; Crestani et al., 2012).

HGF also plays an important role in wound healing, angiogenesis, and tumorigenesis.

### SOURCES OF FIBROBLASTS

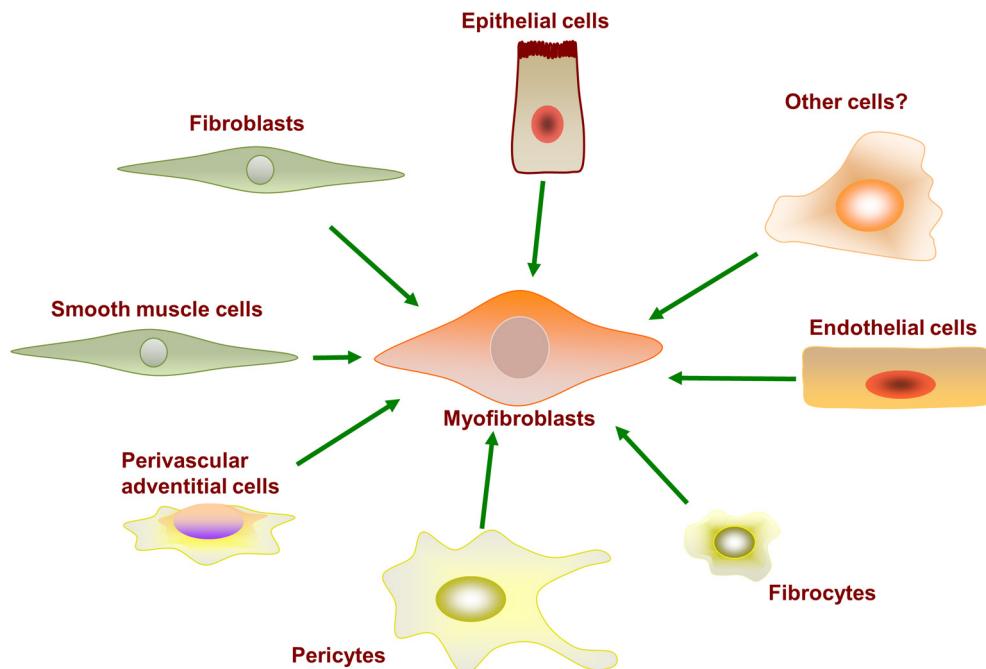
Fibroblasts are a heterogeneous cell population that consists of subsets with different capacities to produce ECM components such as collagen. Fibroblast subpopulations have been described in the skin (Jelaska and Korn, 2000) and lung (Pechkovsky et al., 2010). Furthermore, fibroblast phenotypes may differ across sites of the same organ, as has been shown in skin. In fact, fibroblasts from different skin sites retain positional memory with distinct gene expression profiles (Chang et al., 2002). Furthermore, fibroblasts are chemotactic and can migrate and accumulate in new areas in response to secreted cytokines, a behavior well characterized in the wound healing response after tissue injury. Although the parenchyme of the liver, pancreas, and lung are derived from endothelial tissue and the parenchyme of the skin and brain are derived from epithelial tissue, the stromal connective tissue found in all organs is derived from mesothelial tissue. Yet, due to the differing mixture of neighboring tissue types, there is the potential for differential fibroblast phenotypes and differential fibrotic responses in each of these organs.

Fibroblasts are not a terminally differentiated cell type and retain the potential to be activated for differentiation into subtypes of fibroblast-like cells. Myofibroblasts are rarely found in healthy human physiology; they become vastly up-regulated after injury and play a critical role in the wound healing response (Gabbiani, 2003; Midwood et al., 2004). Myofibroblasts can originate from a variety of precursor cells, as shown in **Figure 2**. Major developmental signaling factors such as the Wnt (Liu et al., 2012), Notch (Kavian et al., 2012), and Sonic hedgehog ligands (Stewart et al., 2003; Ding et al., 2012) have well-described roles in myofibroblast differentiation from precursor cells. Myofibroblasts are well characterized to differentiate from resident fibroblasts *in vivo* and *in vitro* in response to profibrotic cytokine stimulation. However, myofibroblasts have also been observed to differentiate from various other precursor cells, including epithelial cells, endothelial cells, pericytes, multipotent monocytes, and fibrocytes. Whether different cell origins are the source of myofibroblasts in different tissues remains to be determined.

The myofibroblast phenotype is characterized as: (i) expressing  $\alpha$ SMA; (ii) contractile; (iii) having a greater rate of ECM synthesis and secretion; and (iv) resistant to apoptosis. The contractile biology of myofibroblasts facilitates ECM fiber manipulation and wound contraction at sites of injury. Myofibroblasts display a heightened and constitutive expression of cytokines, chemokines, and cell surface receptors (Strehlow and Korn, 1998; Abraham and Varga, 2005). These differentiated myofibroblasts also display epigenetic changes (Wang et al., 2006a).

### EPITHELIAL CELLS

The EMT has been observed as a source of myofibroblasts in kidney fibrosis (Ng et al., 1998; Jinde et al., 2001; Liu, 2004); however, subsequent attempts to trace such phenomena in the skin, lung, heart, and liver have been largely unsuccessful *in vivo*, suggesting that EMT-mediated formation of myofibroblasts is



**FIGURE 2 | Myofibroblasts can differentiate from a variety of precursor cell types.**

not substantial. Recent evidence argues against epithelial cells as the main source of myofibroblast differentiation in idiopathic lung fibrosis. Using lineage tracing with recombinant td-Tomato and f-GFP tags and confocal microscopy resolution, Rock et al. demonstrated focal accumulation of fibroblasts derived from the proliferation of stromal cells (Rock et al., 2011). Recent transgenic lineage tracing studies in mouse kidney also indicate that most myofibroblasts are not derived from epithelial cells (Lin et al., 2008; Humphreys et al., 2010). Although these studies using rodents contradict findings suggesting an epithelial origin to myofibroblasts, they do not exclude the possibility that EMT may occur in human tissues.

### ENDOTHELIAL CELLS

Another source of myofibroblasts during fibrosis has been hypothesized to originate from endothelial cells by a process of endothelial-mesenchymal transition (EndoMT) (Piera-Velazquez et al., 2011). This event has been characterized in animal models of fibrosis; however, similar phenomena in human fibrosis patients have not yet been well characterized.

### VASCULAR SMOOTH MUSCLE CELLS (VSMC)

The many similarities between myofibroblasts and VSMCs promote the question of a potential lineage relationship between the two cell types (Yoshida and Owens, 2005). For example, these cells are contractile and they share multiple markers such as  $\alpha$ SMA, SM22 $\alpha$ , desmin, and vimentin (Yoshida and Owens, 2005). Furthermore, myofibroblasts express proteins, such as osteopontin, that can be induced in VSMCs by phenotypic modulator ligands (Yoshida and Owens, 2005). However, the biological roles of these two cell types in disease are different (Gan et al.,

2007). Furthermore, myofibroblasts and VSMCs have distinct transcriptional mechanisms for  $\alpha$ SMA expression (Gan et al., 2007). Observations such as the induction of  $\alpha$ SMA expression in myofibroblasts and the reduction in  $\alpha$ SMA expression in VSMCs during vascular injury (Yoshida and Owens, 2005) suggest the reduction of a contractile phenotype in VSMCs could also promote another source of myofibroblasts differentiation.

### PERICYTES

Pericytes are mesenchyme-derived cells that wrap around small blood vessel walls and closely associate with the outer surface of the endothelial cell layer. Pericytes regulate the permeability of the blood vessel wall; for example, pericytes are the principal cell type that regulates the permeability of the blood-brain barrier (Armulik et al., 2010). Pericytes physically interact with endothelial cells and chemically via paracrine signaling to control the tight junctions and transvascular trafficking across endothelial cells. As such, malfunctioning pericytes can promote vascular leakage (Schrimpf et al., 2012), and the observation that pericytes can be induced to differentiate into myofibroblasts, losing their pericyte functionality, suggests that this differentiation may contribute to both tissue fibrosis and vascular leakage in IPF (Hung et al., 2013). Pericytes are not a terminally differentiated cell type; they can further differentiate into fibroblasts, osteoblasts, and smooth muscle cells. Pericytes endogenously express  $\alpha$ SMA and have a functioning contractile apparatus; therefore, distinguishing between these cells and other cell types is challenging. One genetic fate mapping study investigating the potential contribution of pericytes in mice to bleomycin-induced lung fibrosis utilized the nerve/glial antigen 2 (NG2) protein, a chondroitin sulfate cell surface-associated proteoglycan, as a marker for this cell type. This strategy found

no appreciable contribution of pericytes to the expansion of myofibroblasts and the resulting fibrosis even though the pericyte population itself did expand significantly (Rock et al., 2011). A different investigation utilized the forkhead transcription factor Foxd1 as a marker for lung pericytes and found that up to 68% of  $\alpha$ SMA-expressing cells in fibrotic lungs are pericyte-derived (Hung et al., 2013). These studies suggest that multiple subpopulations of pericytes may exist in the lung and differentially contribute to myofibroblast formation and fibrosis.

### MONOCYTES AND FIBROCYTES

Fibrocytes are circulating fibroblast-like cells in the vascular system that are derived from bone marrow stem cells (Bucala et al., 1994). “Fibrocyte” is a term sometimes ascribed to a relatively inactive fibroblast-like cell, whereas the term “fibroblast” designates a fully active cell as described throughout our review. Fibrocytes can extravasate from vessels into connective tissues. In the kidney, it has been proposed that myofibroblasts in fibrotic areas could be derived from bone marrow progenitor multipotent cells via a cellular differentiation lineage: bone marrow progenitor cell to monocyte to fibrocyte to fibroblast (Quan et al., 2004; Pilling and Gomer, 2012). Circulating CD14 $^{+}$  immunophenotypic monocytes derived from the plasma of SSc patients are reported to express greater  $\alpha$ SMA (and so an increased maturation toward myofibroblasts) compared to those of healthy control individuals but with minor functional contraction properties as determined with a collagen contraction assay (Binai et al., 2012). The blood of SSc patients with interstitial lung disease is enriched for fibrocytes and monocytes with an overt profibrotic phenotype (Mathai et al., 2010). However, lineage tracing studies in rodents display differing conclusions. GFP-expressing fibroblasts derived from the multipotent, transgenic bone marrow transplanted into chimeric normal mice appear to be a unique population of cells that do not express  $\alpha$ SMA and fail to differentiate into myofibroblasts when extracted and stimulated *ex vivo* in culture (Hashimoto et al., 2004; Kisileva et al., 2006; Barisic-Dujmovic et al., 2010; Rock et al., 2011). Similarly, using an  $\alpha$ SMA promoter-driven GFP transgenic mouse, no kidney or bone marrow stromal expression was observed in chimeric wild-type mice (Yokota et al., 2006). However, the chimeric mice were only analyzed under physiologic conditions, and no profibrotic stimulus was tested in this model (Yokota et al., 2006). In contrast, in another study, bone marrow from GFP transgenic mice were transplanted into wild-type rats, and a 23% increase in the number of GFP and  $\alpha$ SMA-expressing cells in the pancreas was observed to peak in the early stage of an experimentally-induced but clinically-relevant pancreatitis rat model (Mathai et al., 2010; Akita et al., 2012). In all, despite substantial evidence that activated myofibroblasts are derived from local stromal fibroblasts, blood borne monocytes and monocyte-derived cells appear to facilitate fibrosis via the production of ECM and paracrine signaling with resident profibrotic cells (Murray et al., 2012).

### ADVENTITIAL FIBROBLASTS IN PULMONARY HYPERTENSION

A very similar role of fibroblast activation appears to promote pulmonary hypertension compounding other fibrotic disorders.

Pulmonary hypertension can be a complication of IPF and SSc and is a significant contributor to mortality (Lee et al., 1992; Arcasoy et al., 2001; King et al., 2001). Adventitial fibroblasts found in blood vessels can be activated by hypoxia and are implicated as a source of myofibroblasts that promote vascular remodeling and the associated pulmonary hypertension (Stenmark et al., 2006).

### FIBROBLASTS IN CANCER BIOLOGY

Fibroblasts have an integral role in cancer beyond sarcomas and cancers originating from fibroblastic cells. Non-cancerous fibroblasts interact with cancer cells, affecting tumor biology and pathogenesis. The biological processes of wound healing, inflammation, fibrosis, angiogenesis, and their associated biochemical machinery all play critical roles in cancer by regulating the microenvironment of a tumor, tumor size, and tumor invasion of adjacent tissues and metastasis (Mueller and Fusenig, 2004). Fibroblasts in and around tumors are persistently activated by tumor cells (Kalluri and Zeisberg, 2006). In response, fibroblasts secrete cytokines and ECM that modulate tumor progression and regulate stroma-cancer interactions (Madar et al., 2013). Non-cancerous fibroblasts surrounding tumors contribute directly to angiogenesis and tumor growth by producing multifarious signaling molecules (Madar et al., 2013). Fibroblasts also secrete the ECM that modulates tumor progression, and fibroblasts remodel the connective tissue surrounding a tumor, allowing cancerous cells to be released from a contained tumor into the vascular system, thereby promoting metastasis (Liu et al., 2011b). As such, fibroblast cells also participate in the pathogenesis of carcinomas—the more abundant cancers originating from epithelial cells. In spite of recent advances in delineating the role of fibroblasts in tumor progression, the role of these cells in earlier tumor development is still incompletely understood.

### POTENTIAL THERAPIES TARGETING FIBROBLASTS

Since fibroblasts are a central effector cell in fibrosis, they are suitable therapeutic targets. There have been some recent exciting research findings inciting potential strategies to combat the pathological fibrotic response that occurs in various disorders. The examples listed below are not intended to be a comprehensive presentation for the pipeline of future antifibrotic therapies that target the fibroblast cell lineage. There are multiple strategies being investigated that are too numerous to cover here. The following examples help to illustrate the diverse range of targeting strategies where novel therapies potentially could ensue.

### ENDOSTATIN

Endostatin is an endogenous inhibitor of angiogenesis that has completed clinical trials as an anticancer agent to prevent the growth of tumor mass. It inhibits endothelial cell proliferation and vessel formation (O'Reilly et al., 1997) but also acts on fibroblasts (Yamaguchi et al., 2012). Endostatin is a small peptide (20–30 KDa) derived from the carboxy terminus of the collagen type XVIII produced and released by fibroblasts. Endostatin is then released from this precursor protein by proteolytic cleavage by the action of cathepsin L (Felbor et al., 2000), and peptides of differing lengths have also been reported to be generated by the

matrix metalloproteases MMP-3, -7, -9, -13, or -20 (Heljasvaara et al., 2005). Endostatin concentrations are greater in the blood and lungs of patients with IPF and SSc, but these concentrations do not seem to reach “therapeutic” levels (Hebbar et al., 2000; Sumi et al., 2005; Richter et al., 2009). Interestingly, the amino terminus portion of endostatin contains the anti-angiogenic functionality (Tjin Tham Sjin et al., 2005), whereas the carboxy terminus portion contains the antifibrotic activity, suggesting that the two biological functions can be attributed to dissociable domains (Yamaguchi et al., 2012). Indeed, a 48 amino acid portion of endostatin’s carboxy terminus has strong antifibrotic actions *in vitro*, *in vivo*, and *ex vivo* in human tissue (Yamaguchi et al., 2012). This peptide ameliorates pulmonary and dermal fibrosis in an animal model and *ex vivo* in human skin in response to TGF $\beta$  (Yamaguchi et al., 2012).

### VITAMIN D3

Vitamin D is important for regulating mineral absorption in the intestines, calcium and phosphate concentrations in blood, and for maintaining bone integrity. However, vitamin D deficiency has also been found to correlate with many extra-osseous disorders. Vitamin D3 is processed by the liver to form the biologically active form 1,25-dihydroxyvitamin D3 (1,25-(OH)<sub>2</sub> vitamin D3). 1,25-(OH)<sub>2</sub> vitamin D3 activates the vitamin D receptor (VDR, a steroid hormone receptor) that heterodimerizes with retinoid hormone receptor to directly regulate gene transcription. 1,25-(OH)<sub>2</sub> vitamin D3 inhibits the expression of type I collagen in hepatic stellate cells (Potter et al., 2013). Furthermore, 1,25-(OH)<sub>2</sub> vitamin D3 has been observed to inhibit a fibrotic response in human primary lung fibroblasts (Ramirez et al., 2010), and 1,25-(OH)<sub>2</sub> vitamin D3 deficiency correlates with the severity of liver fibrosis and that of SSc (Vacca et al., 2009; Caramaschi et al., 2010; Rios Fernandez et al., 2010; Arnson et al., 2011; Slominski et al., 2013). These studies suggest that the correction of vitamin D deficiency is promising as a clinical strategy to alleviate fibrotic disorders.

### EPIGENETICS

Epigenetic changes in fibrosis are the subject of active investigation. Several epigenetic modifications, like histone posttranslational modifications and DNA methylation events, have been reported. The fact that these epigenetic modifications do not alter the DNA sequence suggests they are reversible, and as such, they represent an attractive therapeutic strategy for countering fibrosis. Cyclooxygenase 2 (COX2) expression—a biosynthetic enzyme for antifibrotic prostaglandin production—is suppressed in IPF due to decreased histone acetylation (Coward et al., 2009), and histone deacetylase 4 (HDAC4) activity mediates TGF $\beta$  stimulated myofibroblast differentiation in a response that can be inhibited by global inhibition of HDACs (Glenisson et al., 2007). HDAC7 has been proposed as a potential target for the treatment of SSc (Hemmatazad et al., 2009). DNA methylation of the gene FLI1 coding for the ETS transcription factor Fli-1 inhibits its genetic expression, resulting in enhanced collagen type 1 expression and persistent activation of fibroblasts in SSc (Wang et al., 2006a). Primary fibroblasts isolated from the adventitial layer of blood vessels following vascular injury maintain their activated,

pro-fibrotic state when grown *in vitro* (Li et al., 2011). The constitutive activation is an imprinted phenotype maintained by epigenetic alterations in key inflammatory and pro-fibrotic genes (Li et al., 2011). Epigenetic drugs have been approved for cancer, paving the way for using such agents, including DNA demethylating agents, for other diseases. The reversibility of epigenetic alterations and the ready availability of small molecule inhibitors of these enzymes provide a potential future therapeutic strategy that could antagonize the profibrotic phenotype of fibroblasts.

### WNT-LIKE SIGNAL TRANSDUCTION PATHWAY

Wnt signaling and a classical Wnt-like intracellular signal transduction pathway in fibrosis are a bustling area of research, and multiple proteins within this pathway have been proposed as druggable targets to prevent myofibroblast differentiation and fibrosis. Wnt is a paradigm developmental signaling protein that regulates proliferation, migration, and cell fate determination. Wnt ligands activate frizzled receptors—members of the G protein-coupled receptor (GPCR) superfamily of proteins that share common structure and function relationships—and promote the inhibition of glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ). Interestingly, many other GPCRs such as the lysophosphatidic acid receptor, the angiotensin type I receptor, the endothelin 1 receptor, and CC and CXC chemokine receptors regulate myofibroblast differentiation and fibrosis. GPCRs have also been demonstrated to couple to the Wnt-like intracellular signal transduction cascade Akt-GSK3 $\beta$ - $\beta$ catenin-PPAR $\gamma$  (Beaulieu et al., 2004, 2005, 2008; Kendall et al., 2011). However, it is not yet known if this cascade is the main contributor for GPCR-regulation of myofibroblast transition and fibrosis. Regardless, it is an interesting observation that multiple targets belonging to this signaling pathway are currently being actively investigated as potential targets for fibrosis therapies.

There is an apparent activation of the developmental Wnt- $\beta$ catenin program in SSc (Bhattacharyya et al., 2012). Wnt activated  $\beta$ catenin promotes fibroblast to myofibroblast transition in human skin (Liu et al., 2012) and is required for TGF $\beta$ -mediated fibrosis (Akhmetshina et al., 2012). While Akt is not required for Wnt-stimulated catenin accumulation (Torres et al., 1999; Chen et al., 2000), Akt has been implicated in sustaining the Wnt signaling cascade (Fukumoto et al., 2001). Akt is a critical node of PI3K signaling and regulates cell cycle, cell growth, and cell fate determination. PI3K-Akt signaling is also utilized as a non-canonical pathway of TGF receptors that contribute to fibrosis (Wan et al., 2013). Sustained Akt activation is critical for upregulating  $\alpha$ SMA expression and myofibroblast differentiation in the wound healing response, implicating Akt as a potential therapeutic target for fibrosis. Whereas a constitutively active Akt1 protein (myr-Akt1) induces  $\alpha$ SMA expression in fibroblasts, a dominant negative Akt1 (Akt1 K179M) inhibits  $\alpha$ SMA expression (Abdalla et al., 2013). Akt is a ubiquitous kinase that phosphorylates and inactivates the constitutive activity of GSK3 $\beta$  (Fang et al., 2000). Knocking out GSK3 $\beta$  expression in fibroblasts accelerates wound healing, increases fibrosis, enhances collagen production, decreases cell apoptosis in wound sections, increases profibrotic  $\alpha$ SMA expression, and increases myofibroblast formation (Kapoor et al., 2008). The transcriptional coregulator

$\beta$ catenin is required for the TGF $\beta$ -mediated inhibition of peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) expression in hepatic stellate cells (Qian et al., 2012). PPAR $\gamma$  is a nuclear receptor that is important for mesodermal cell fate determination. PPAR $\gamma$  expression and activity promotes adipocyte differentiation and inhibits fibroblast and hepatic stellate cell activation of the profibrotic phenotype (Qian et al., 2012). PPAR $\gamma$  agonists inhibit lung myofibroblast proliferation *in vivo* as well as TGF $\beta$ -mediated myofibroblast differentiation and collagen production in culture (Milam et al., 2008). PPAR $\gamma$  agonists also inhibit dermal fibrosis (Wei et al., 2010, 2012). Although only an associative depiction of a pathway is characterized here from research on individual proteins in this pathway, collectively they suggest that a Wnt-like pathway could have a strong influence on myofibroblast differentiation and fibrosis.

## FIBROBLAST RECEPTORS

Caveolin 1 inhibits the function of the chemotactic receptor CXCR4 in fibrocytes (Tourkina et al., 2011) and caveolin 1 is down regulated in IPF (Wang et al., 2006b). The introduction of a peptide containing the caveolin scaffolding domain of caveolin 1 is sufficient to inhibit fibrocyte accumulation in the lungs in a bleomycin-induced animal model of fibrosis, suggesting that the caveolin scaffolding domain could be employed as a potential therapy for SSc lung disease (Tourkina et al., 2011). In yet another example, the single nucleotide polymorphism L412F in the gene expressing Toll-like receptor 3 in pulmonary fibroblasts has been found to correlate with a greater risk of mortality in IPF patients (O'Dwyer et al., 2013). The L412F polymorphism could be used as a biomarker for more progressive fibrotic disease, and the resulting loss of function of TLR3 is a potential therapeutic target in IPF (O'Dwyer et al., 2013). This study highlights the importance of a personalized medicine approach to the multifactorial group of fibrotic disorders. The inhibition of TLR4 has been reported to antagonize AngII-induced cardiac fibrosis (Wang et al., 2014), the activity of TLR4 in hepatic stellate cells enhances liver fibrosis (Seki et al., 2007), and the activity of TLR4 in skin fibroblasts enhances SSc (Bhattacharyya et al., 2013). Here, the entanglement of inflammatory and ECM-producing functions of fibroblasts can be exploited to improve fibrotic therapies (Bhattacharyya et al., 2013).

Given the multifactorial etiology of various fibrotic disorders, both in different tissues and within the same tissue (Murray et al., 2012), the increasing interest in developing and employing personalized medicine, where a one-size-fits-all approach is unlikely to be as effective, offers to accelerate the development of more effective antifibrotic therapies for defined subsets of patients.

## SUMMARY

Fibrosis is the thickening of ECM that is preceded by inflammation or physical tissue injury. Fibroblasts are the principal cell type that produces, maintains, and reabsorbs ECM. These fibroblasts have the capacity to become activated by inflammatory cytokines to myofibroblasts that display up-regulated cellular migration, exaggerated ECM production, the endowment of a contractile apparatus, and increased chemical signaling secretion and responsiveness. Although various precursor cell types have

been observed to contribute to myofibroblast development, resident fibroblast stromal cells appear to be the most significant contributing source of myofibroblasts and the resulting tissue fibrotic response in diseases such as SSc and IPF. Thus, fibroblasts are a suitable therapeutic target. In view of the numerous factors at play in fibrosis, it is likely that combinatorial therapy that includes targeting fibroblasts will be more effective than single target approaches for the treatment of organ fibrosis.

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# The epithelium in idiopathic pulmonary fibrosis: breaking the barrier

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Idiopathic pulmonary fibrosis is a progressive disease of unknown etiology characterized by a dysregulated wound healing response that leads to fatal accumulation of fibroblasts and extracellular matrix (ECM) in the lung, which compromises tissue architecture and lung function capacity. Injury to type II alveolar epithelial cells is thought to be the key event for the initiation of the disease, and so far both genetic factors, such as mutations in telomerase and MUC5B genes as well as environmental components, like cigarette smoking, exposure to asbestos and viral infections have been implicated as potential initiating triggers. The injured epithelium then enters a state of senescence-associated secretory phenotype whereby it produces both pro-inflammatory and pro-fibrotic factors that contribute to the wound healing process in the lung. Immune cells, like macrophages and neutrophils as well as activated myofibroblasts then perpetuate this cascade of epithelial cell apoptosis and proliferation by release of pro-fibrotic transforming growth factor beta and continuous deposition of ECM stiffens the basement membrane, altogether having a deleterious impact on epithelial cell function. In this review, we describe the role of the epithelium as both a physical and immunological barrier between environment and self in the homeostatic versus diseased lung and explore the potential mechanisms of epithelial cell injury and the impact of loss of epithelial cell permeability and function on cytokine production, inflammation, and myofibroblast activation in the fibrotic lung.

**Keywords:** epithelium, fibroblasts, idiopathic pulmonary fibrosis, apoptosis, TGF- $\beta$

## INTRODUCTION

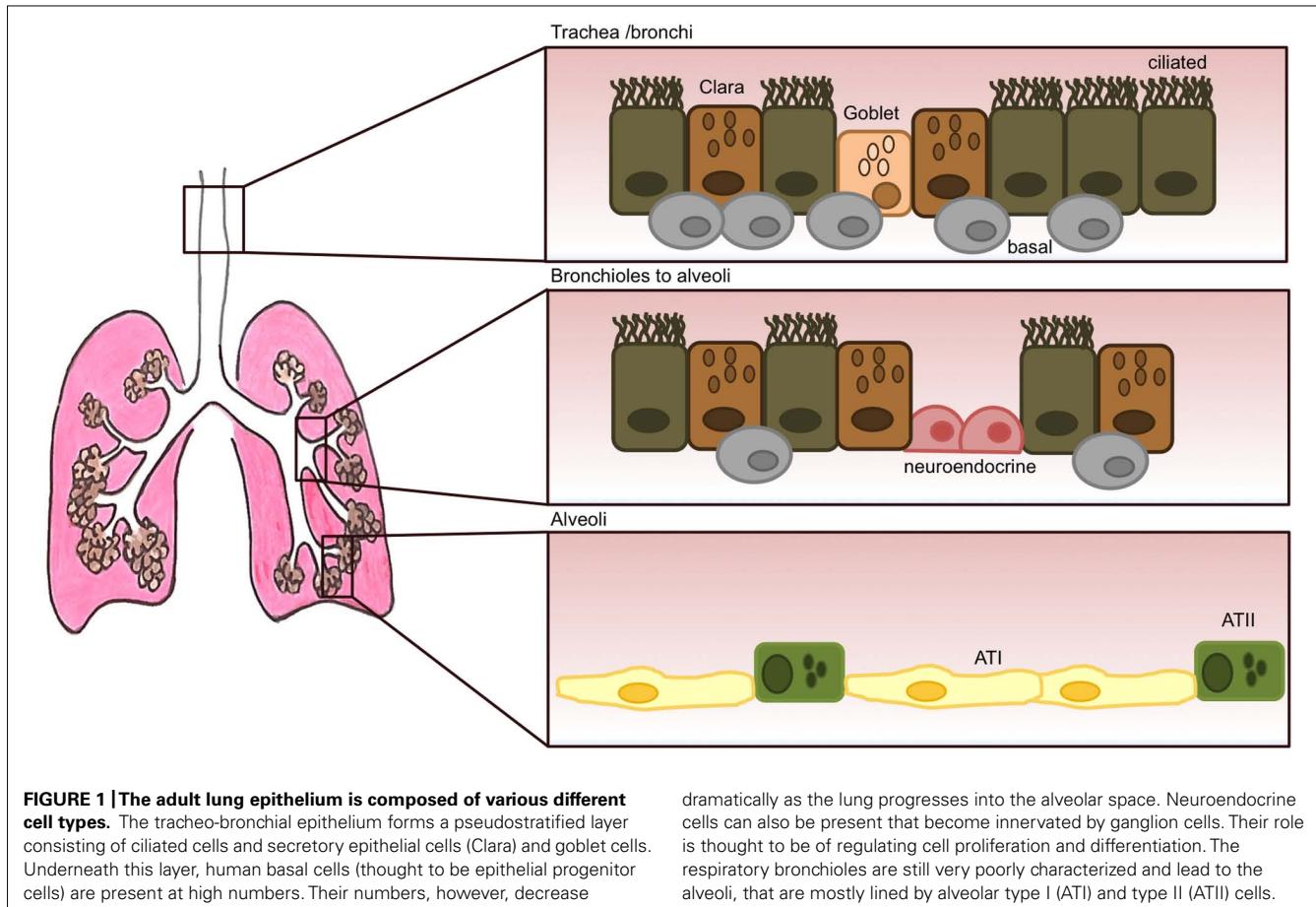
Idiopathic pulmonary fibrosis (IPF) is a devastating, fibroproliferative chronic lung disorder with complex and as yet unknown disease biology. The histopathology of IPF demonstrates a characteristic heterogeneity: areas of normal parenchyma interspersed with areas of paraseptal and subpleural fibrosis (The Idiopathic Pulmonary Fibrosis Clinical Research Network, 2012). At the cellular level, IPF is characterized by alveolar epithelial injury, initiation of inflammatory cascades, exaggerated pro-fibrotic cytokine expression, increased extracellular matrix (ECM) deposition, and the development of fibrotic lesions known as fibroblast "foci" (Wilson and Wynn, 2009). IPF has a heterogeneous clinical course, with a median survival after diagnosis of only 2.5–3.5 years (King et al., 2011). Although much of the pathogenesis is still to be elucidated, fibroblasts and epithelial cells, in particular type II alveolar epithelial cells and myofibroblasts are thought to be key drivers in the initiation and progression of the disease, respectively (Sakai and Tager, 2013). This review will focus on the epithelial cell, explore the mechanisms of cell injury and their role in repair in the fibrotic lung, as well as interactions with other key effector cells in IPF such as the fibroblasts.

## THE LUNG EPITHELIUM AS A PHYSICAL BARRIER AGAINST FOREIGN INSULTS

The airway epithelium is a pseudo-stratified mucosal barrier that consists of multiple cell types. It constitutes the first barrier of defense against environmental insults and infection by

providing not only a mechanical and physical barrier to impede entry of foreign particles but also by its ability to orchestrate both the innate and adaptive immune responses. The lower airway surface, where gas exchange takes place is mainly covered by two types of alveolar epithelial cells, alveolar type I epithelial cells (ATI) that cover 90% of the airway surface due to their large flattened phenotype and whose main function is gas interchange, and alveolar type II epithelial cells (ATII) that are the most abundant epithelial cell type and whose function is to maintain the alveolar space by secretion of several types of surfactant proteins and other ECM components (Serrano-Mollar, 2012). The production of surfactant by ATII cells enables the gas exchange to occur by lowering the surface tension within the alveoli (Rackley and Stripp, 2012).

As well as alveolar epithelial cells in the alveolar compartment, other epithelial cell types populate the lung; with secretory Clara and goblet cells, ciliated, basal and neuroendocrine cells forming the tracheo-bronchial pseudostratified epithelium (Figure 1). Ciliated and secretory cells work in concert to clear the airway passages from micro-organisms, air pollutants and other inhaled pathogens. Mucous and goblet cells produce and release mucus into the apical surface of the epithelium thus trapping foreign particles (Roche et al., 1993) which are then cleared out by the action of ciliated cells beating in a rhythmic movement in the ascending direction (Fahy and Dickey, 2010; Gras et al., 2013). Mucus is a viscoelastic gel composed mainly of highly charged glycoproteins called mucins, and also some anti-viral and



anti-inflammatory components such as lysozyme, defensins, IgA, and various cytokines (Nicholas et al., 2006). So far, 11 mucins have been identified in humans, with MUC5AC and MUC5B being predominant in human sputum (Rose et al., 2001). Mucin production importantly becomes up-regulated following viral infection to allow for better trapping and disposal of viral particles, however, over-production of mucus or over-proliferation of mucus-producing cells (goblet cell hyperplasia) can have deleterious effects by creating mucous plugs and thus leading to airway obstruction (Vareille et al., 2011). The latter is a common feature of several chronic lung diseases such as asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (Rogers, 2004; Hauber et al., 2006).

The epithelial integrity and permeability are maintained by tight connections within the epithelial cell layer, such as tight junctions, adherens junctions, and desmosomes. These cell-cell junctions provide strong adhesion, maintaining mechanical strength in the tissue, enabling communication between neighboring cells and blocking the entry of viruses, bacteria or inhaled allergens into the basolateral membrane where they can access epithelial cell receptors and/or activate antigen-presenting cells (Roche et al., 1993).

Mostly, the mature lung is non-proliferative, with prolonged survival of resident cells. However, in the event of damage to the epithelial layer following inflammation, infection, or exposure to

airway pollutants, it is critical that the site of injury is quickly closed and replaced by newly differentiated epithelial cells in order to maintain an effective physical barrier. Several studies have identified some epithelial cell types and also progenitor cells in the lung as potential key regenerative players in the replacement of the epithelium after damage, although most of these remain relatively controversial. In the alveoli, ATII cells have been shown to proliferate and differentiate into type I cells (Cardoso and Whitsell, 2008; Ghosh et al., 2013) and in the conducting airways, basal cells (Figure 1), in contact with the basement membrane but not the airway lumen were found capable of long-term self-renewal and differentiation into ciliated and non-ciliated cell types *in vivo* (Hong et al., 2004). Lastly, neuroendocrine cells form clusters called neuroepithelial bodies, and there is some evidence these may play a role in regulating epithelial cell proliferation and differentiation of neighboring cells (Hoyt et al., 1991). The signaling and transcriptional programs that are activated in this process of wound healing can resemble and somewhat recapitulate early lung developmental programs (Rackley and Stripp, 2012). These pathways typically become dysregulated during chronic lung disease.

## THE EPITHELIUM IN INTERSTITIAL LUNG DISEASE

Alteration of the phenotype of alveolar epithelial cells is a central feature in IPF, whereby continuous damage to the epithelium

and concomitant cell apoptosis are thought to contribute to the perpetuation of the fibrotic scarring (Jin and Dong, 2011). The causative event that initiates the fibrotic cascade in IPF is still unknown, although apoptosis or senescence of epithelial cells is arising as a hypothesis for the main initiator event (Chilosi et al., 2013). Indeed, recent studies found that IPF patients carry increased number of apoptotic cells in alveolar and bronchial epithelia (Plataki et al., 2005). The bleomycin mouse model supports this hypothesis by showing that inhibition of epithelial cell apoptosis prevents the development of the disease (Kuwayo et al., 1999). This model is widely used in IPF research and shows the histological features of a fibrotic lung. It does, however, have limitations, as it is steroid responsive and the fibrosis resolves itself with time (Chandler, 1990), so it does not fully replicate the extent of the human disease.

What stimuli trigger the apoptotic cascade in epithelial cells is still under scrutiny. Cell senescence and premature aging due to genetic factors may be one cause but environmental factors such as cigarette smoking, viral infections, and gastroesophageal reflux (GER) are a few of the hypothesis that are currently being investigated.

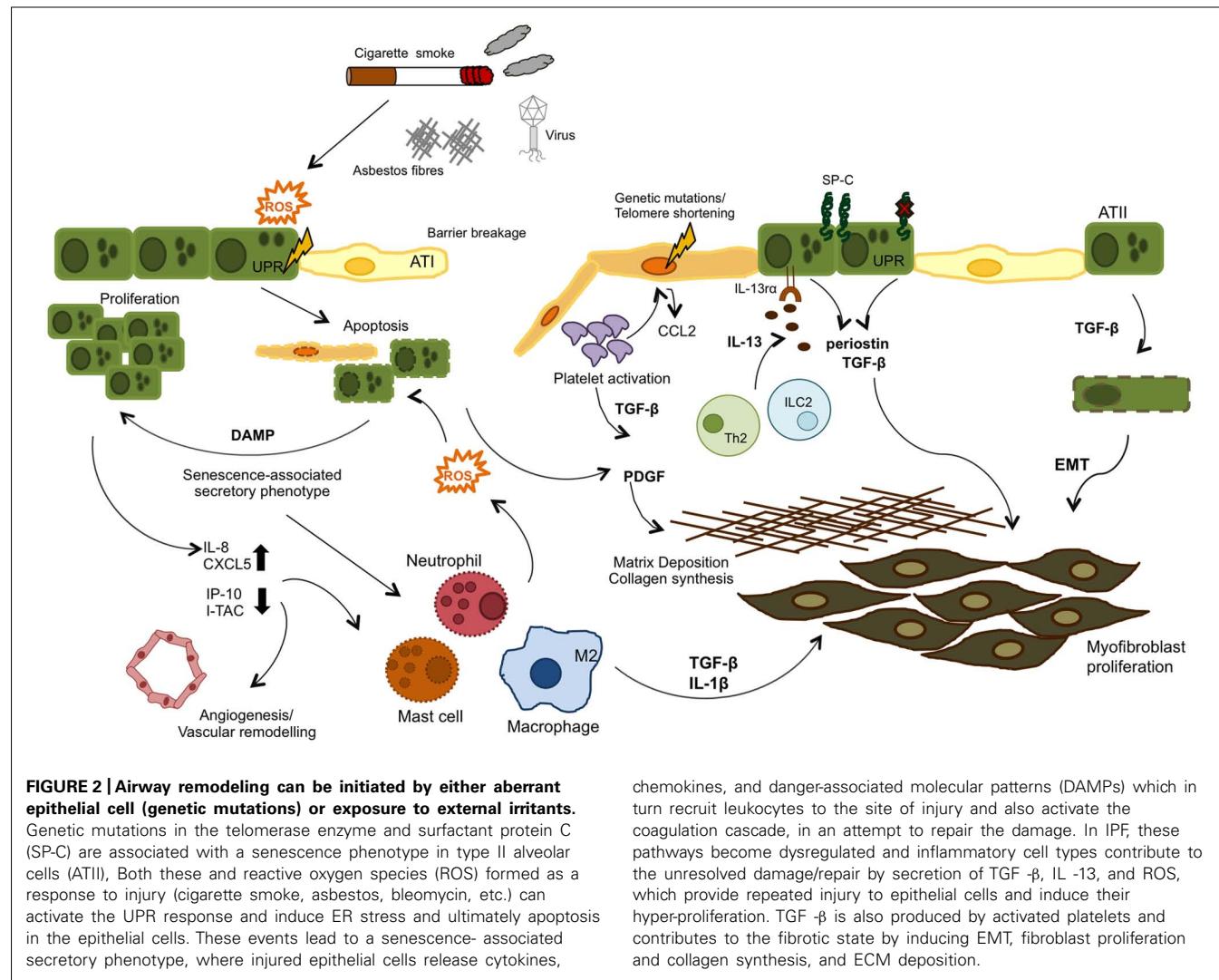
Genetic mutations of telomerase, an enzyme that adds telomere repeats to the end of linear chromosomes, occur in 10% of familial IPF (Chilosi et al., 2012). Telomerase is known to maintain the precursor function in ATII cells and dysregulation of this enzyme greatly affects their regenerative capacity. Telomere shortening is dangerous for the cell as it causes DNA damage and induces cell death. Another disease-linked mutation that may lead to alveolar epithelial cell apoptosis occurs in the surfactant protein C gene which has also been found in familial IPF (Thomas et al., 2002). This mutation results in abnormal surfactant protein folding and accumulation of misfolded protein in the cell cytoplasm which activates the unfolded protein response (UPR) in an attempt to rescue the cell from cell death by halting the protein production. When this mechanism is not resolved, the cell enters a state of stress, called endoplasmic reticulum (ER) stress which ultimately leads to apoptosis (Noble et al., 2012). Other surfactant proteins, surfactant protein A and D have also been shown to be important mediators of respiratory infection susceptibility in mice (LeVine and Whitsett, 2001), which highlights the role of these proteins in the maintenance of the epithelial barrier. Environmental factors, like the mentioned viral infections but also cigarette smoking can induce UPR and ER stress, and in this way also contribute to accelerated telomere shortening and cellular senescence in the alveolar epithelia (Tsujii et al., 2004). Polymorphisms in the promoter region of the MUC5B gene have also been linked to IPF, this time not in ATII cells, but in bronchial epithelial cells (Seibold et al., 2011), suggesting that broader epithelial cell defects can affect the onset of disease. More recently, one study has found that MUC5B promoter polymorphisms were associated with interstitial lung disease in the general population, independently of cigarette smoking (Hunninghake et al., 2013). Despite this, in IPF patients, MUC5B polymorphisms actually associated with increase survival in another study (Peljto et al., 2013). These potentially contradicting reports underline the complexity of this disease, and prompt further, more functional studies to better understand

the role of these genetic factors in inducing or driving disease pathology. A very recent study using genome-wide association (GWAS) has also identified 7 new susceptibility loci for pulmonary fibrosis associated with epithelial cell function, including DSP which encodes for desmoplakin, a component of the epithelial cell desmosome, and DDP9, also associated with epithelial cell adhesion and maintenance of its cytoskeleton (Fingerlin et al., 2013).

These studies are very important to identify genetic defects that underline the disease and not only help to increase our knowledge of the mechanisms that drive it but also to aid in finding potential therapeutic targets. This understanding may also lead to better methods of diagnostic, and increase the chances of an early diagnose that can better help patients. In fact, one study has looked at peripheral blood proteins and their association with disease and mortality and identified that high concentrations of matrix metalloproteinase 7 (MMP-7), interleukin (IL)-8, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1) were associated with poor overall survival. Altogether, these proteins were shown to be involved in alveolar epithelial cell damage, oxidative stress, macrophage activation and neutrophil recruitment, all of which have been described in IPF (Richards et al., 2012). Lastly, a study of the transcriptional profiles of lung tissue from IPF patients has recently defined new genes associated with expression of cilium in epithelial cells which define particular subtypes of the disease, in this way helping in future patient stratification for therapies. In this study, patients with high cilium gene expression showed increased microscopic honeycombing, increased tissue expression of MUC5B and MMP-7, but with no effect on the number of fibroblastic foci, compared with other patients (Yang et al., 2013).

Genetic factors alone are not however the sole cause of IPF and it is well known that oxidative stress induced by chronic exposure to toxic substances, mainly cigarette smoke is a high risk factor for these patients. Cigarette smoke can induce oxidative stress and ultimately lead to cell death by causing an excessive production of reactive oxygen species (ROS), such as hydrogen peroxide, superoxide anions, and hydroxyl radicals that cells cannot scavenge and process (Psathakis et al., 2006). In IPF, the oxidative stress is continuously present as myofibroblasts that are constitutively activated by transforming growth factor beta (TGF- $\beta$ ) also produce high amounts of hydrogen peroxide. This has been elegantly demonstrated in an *in vitro* co-culture system whereby TGF- $\beta$ -stimulated fibroblasts induced epithelial cell apoptosis through their production of hydrogen peroxide (Waghray et al., 2005). Exposure to cigarette smoke cannot, *per se*, induce IPF disease, and other chronic lung diseases such as COPD occur due to several years of cigarette smoke exposure. There are, however, differences between these two, not only different genetic susceptibilities but also the fact that the epithelial cell seem to be the main causative cell target in IPF, through senescence or apoptosis whereas in COPD, senescent markers have been more associated with mesenchymal cells like fibroblasts and endothelial cells (Chilosi et al., 2012, 2013).

Other potential initiators of lung fibrosis have been linked to viral infections, as these represent injury to the epithelium and lung and also activate the immune system (**Figure 2**). Some



studies have found both Epstein–Barr virus (EBV) and herpes simplex virus (HSV) in alveolar epithelium of IPF patients, and that their presence was associated with poor prognosis (Tsukamoto et al., 2000; Margaritopoulos et al., 2013). Some recent theories arose that point to gastric contents as another potential cause of injury to the lungs in IPF, and GER has been associated with acute exacerbations in these patients (Lee et al., 2012). To support this observation an *in vitro* study has shown that a component of bile could induce TGF- $\beta$  production by lung epithelial cells and also fibroblast proliferation, two key mechanistic features of IPF (Perng et al., 2007).

Once the epithelium is injured, the epithelial/fibroblastic pathways of wound healing become activated. The IPF lung shows a typical loss of integrity of the alveolar epithelium, with disruption of basement membrane integrity and collapse of the alveolar structure. The number of ATI cells is reduced whereas hyperplasia of ATII cells develops. These represent the progenitor cells that are able to differentiate to ATI cells and therefore re-epithelialize the site where the barrier was broken. However, the latter is seriously impaired in the IPF lung (Chapman, 2011).

Other mechanisms of lung repair other than epithelial cell proliferation involve a migration of bone marrow-derived mesenchymal stem cells (BM-MSC) into the lung through chemokine gradients and their differentiation into epithelial cells. However, there are conflicting results on whether this process actually takes place in IPF (Rojas et al., 2005; Xu et al., 2009). Epithelial damage also activates fibroblasts to differentiate into myofibroblasts that then form the characteristic fibroblastic foci. The source of these myofibroblasts is still under debate but there are currently three hypotheses of how these cells are generated in the lung. The first one is the migration of local fibroblasts to the site of injury by expression of platelet-derived growth factor (PDGF) from platelets and TGF- $\beta$  and tumor necrosis factor alpha (TNF- $\alpha$ ) from the epithelium and their subsequent differentiation into myofibroblasts (Selman and Pardo, 2006). This hypothesis seems quite appealing due to the proximity of the cells to each other, and resting resident fibroblast can be immediately exposed to the cytokine and chemokine milieu secreted in response to damage and become activated. However, this may account for the initial burst of the fibrotic response but may not be sufficient in the

full blown disease, where myofibroblast take over large sections of the lungs. The second theory proposes that myofibroblasts may be derived from circulating CXCR4-positive fibrocytes (or circulating mesenchymal cells), and that these are attracted to the lung by the high expression of chemokine CXCL12 from epithelial cells (Andersson-Sjoland et al., 2008; Antoniou et al., 2010). In favor of this hypothesis, a higher number of circulating fibrocytes have been found in the blood of IPF patients compared with healthy controls (Andersson-Sjoland et al., 2008). The third hypothesis has been given a lot of attention recently and is thought to occur through a loss of the characteristic epithelial cell phenotype, such as E-cadherin expression and the de-differentiation of the epithelial cell into a myofibroblast by gaining of mesenchymal markers like  $\alpha$ -smooth muscle actin and fibronectin, in a process called epithelial-to-mesenchymal transition (EMT; Willis et al., 2006). TGF- $\beta$ , the main cytokine regulating fibrosis, is thought to drive EMT directly and perpetuate this event in the lung (Guarino et al., 2009). A key pathway that has been found dysregulated in fibrosis involved in EMT is the Wnt-signaling pathway. Hyperplastic ATII cells overexpressing the WNT-1 inducible signaling protein or WISP-1 up-regulate the secretion of pro-fibrotic markers like MMP-7 and plasminogen-activator inhibitor 1 (PAI-1) which could induce EMT in the neighboring epithelium (Margaritopoulos et al., 2013). The Wnt pathway has been shown to be activated by cell senescence, which triggers a “senescence-associated secretory phenotype” whereby injury-induced apoptosis in epithelial cells initiates the release of damage factors or alarmins that target the neighbouring type II alveolar epithelial cells to induce their proliferation in an attempt to restore homeostasis (Chilosi et al., 2012).

In addition to above mentioned genetic and environmental factors that can induce epithelial cell damage in fibrosis and the mechanisms that it activates (apoptosis, cell proliferation, release of pro-inflammatory, and pro-fibrotic cytokines) which perpetuate the damage/repair response, the mechanical stress to the lung is another cofactor in inducing alveolar damage. The mechanical stretch focused on limited parts of lung parenchyma has detrimental effects in alveolar epithelial permeability and tissue regeneration following injury and has also been shown to increase the production of ROS (Chilosi et al., 2013; Davidovich et al., 2013). In fact, inducing mechanical stretch and compression in *in vitro* cultures of epithelial cells inhibited wound closure by inhibiting both cell spreading and cell migration and this was dependent on the duration of the stretch cycles (Savla and Waters, 1998). Moreover,  $\alpha v \beta 6$ -mediated activation of TGF- $\beta$  has been shown to require cellular tension and thus increased stiffness of the lung tissue may form a positive feedback loop promoting progression of the fibrosis (Giacomini et al., 2012).

## THE INJURED EPITHELIUM AND THE IMMUNOLOGICAL RESPONSE

Despite immune suppressors, such as steroids having had little to no effect in the clinic for the treatment of IPF, injury to the epithelium typically elicits an immune response in the lung. The coagulation cascade is the first mechanism activated in the wound-healing process, and activated platelets release pro-fibrotic factors like PDGF and TGF- $\beta 1$  (Chambers, 2008). Damaged epithelial

cells release a variety of the chemokines that recruit inflammatory monocytes and neutrophils to the site of injury. In a single injury, such as infection or allergen exposure, monocytes differentiate into phagocytic macrophages that phagocytose the fibrin clot and neutrophils remove debris and kill invading bacteria. In the case of repeated injury such as the one occurring in COPD and IPF, neutrophils and macrophages are not eliminated quickly enough and their presence can further exacerbate the fibrotic cascade by continuous production of ROS (Wynn and Ramalingam, 2012). The recruitment of neutrophils to the bronchoalveolar space is considered a predictor of early mortality in IPF patients (Kinder et al., 2008), and both macrophages and neutrophils have been identified as pro-fibrotic cell types in mouse models of pulmonary fibrosis (Pardo et al., 2000; Duffield et al., 2005). Other innate myeloid cell types that have also been suggested as having a pro-fibrotic role in the lung include eosinophils and mast cells (Wynn and Ramalingam, 2012) and, more recently a newly identified cell type, innate lymphoid cells 2 (ILC2) has also been implicated as a mediator of hepatic fibrosis (McHedlidze et al., 2013). Within the adaptive immune system, there is some evidence that CD4 $^{+}$  Th1, Th2, and Th17 subtypes may play role in pulmonary fibrosis and their plethora of cytokines such as interferon gamma (IFN- $\gamma$ ) for Th1 and IL-4 and IL-13 for Th2 cells have been linked to disease development in the lung (Wynn, 2011). Interestingly, IPF fibroblasts are hyperresponsive to IL-13, and increased expression of IL-13 and its receptor IL-13R- $\alpha 1$  correlate with disease severity (Murray et al., 2008). As well as its direct effects on fibroblast proliferation and epithelial cell apoptosis (Borowski et al., 2008), IL-13 can also target TGF- $\beta$  directly *in vivo* which further augments the fibrotic response (Lee et al., 2001). The strategy of targeting IL-13 in IPF is currently undergoing clinical trials. Regulatory T cells have also been associated with IPF, however, there is still some controversy to whether their role in IPF is pro- or anti-fibrotic (Kotsianidis et al., 2009; Liu et al., 2010).

Toll-like receptor (TLR) activation in epithelial cells could be the trigger responsible for the epithelial-induced immune cell recruitment to the lungs and TLR signaling pathways have also been linked with tissue repair, as they can promote tissue remodeling (Jiang et al., 2005). In line with the hypothesis of viral infections as one of the insults that could initiate IPF, TLR9 (that recognizes nucleic acid strands) has been found overexpressed in fibroblast of IPF patients (Margaritopoulos et al., 2010). In a more recent study, TLR2 was also found up-regulated in the lungs of IPF patients compared with healthy controls (Samara et al., 2012). This receptor has also been found to be critical for the release of pro-inflammatory cytokines and promotion of collagen and fibronectin deposition following bleomycin exposure (Razonable et al., 2006; Yang et al., 2009). In addition, in a radiation-induced lung fibrosis model, both TLR2 and TLR4 were found to have a protective effect by preventing epithelial cell injury and suppressing fibrogenesis (Pauw et al., 2010). Activation of TLR2 and TLR3 can also induce up-regulation of mucin expression (Li et al., 1997; Chen et al., 2004), and therefore a dysregulation of TLR signaling can impact not only the type of immune response initiated but also the balance of mucous production and clearance. This can have a major impact during viral exacerbations in IPF patients,

which are one of the major causes of mortality in these patients. A recent study places TLR3 as an important factor in IPF. It was found that in IPF-derived fibroblasts carrying the TLR3 L412F polymorphism, TLR3 activation resulted in abnormal cytokine production. Moreover, TLR3-deficient mice showed increased collagen production in the lungs following bleomycin-induced fibrosis and patients carrying this polymorphism had significantly greater risk of mortality and accelerated decline in forced vital capacity (O'Dwyer et al., 2013).

Though well-known sentinels for the recognition of pathogen specific patterns, TLRs have also been shown to identify some endogenous ligands, including fragmented forms of hyaluronic acid (a major component of ECM in most organs) which could signal through TLR2 and TLR4 receptors in immune cells (McKee et al., 1996; Scheibner et al., 2006). Other endogenous TLR ligands reported include HMGB1, signaling through TLR2 and TLR4. HMGB1 is a nuclear protein that can either be released by either activated immune cells following inflammation or by necrotic cells (Scaffidi et al., 2002). More recently, fibrinogen cleavage products have also been found to act as TLR4 ligands in both alveolar macrophages and epithelial cells, and that this interaction up-regulated the gene expression of IL-13R $\alpha$ 1 and MUC5AC in both these cell types (Millien et al., 2013). These endogenous patterns are usually referred to as danger-associated molecular patterns (DAMPs) and it can be easily hypothesized that they may be of importance in chronic lung diseases where the epithelial barrier is severely damaged, although further studies will be needed to identify the extent to which they may either cause it or contribute to their perpetuation.

An autoimmune response has been also suggested as part of the IPF pathology, where B cell aggregates were found in patient's lungs and circulating activated CD4 T cells in their serum (Marchal-Somme et al., 2006). Indeed some studies have found circulating auto-antibodies against epithelial antigens in the serum of IPF patients, including antibodies against cytokeratins and, more recently an anti-periplakin antibody (Fahim et al., 2012). The latter was shown to delay wound repair *in vitro*, by decreasing epithelial cell migration (Taille et al., 2011). Therefore, there is some evidence of a chronic inflammatory setting in IPF, however, further investigation is warranted to understand whether this is caused by the aberrant wound-healing and permanent secretion of pro-inflammatory modulators by epithelial cells and fibroblasts, or whether the initial lung injury provokes a deregulated immune response and the immune cell-derived cytokines perpetuate the aberrant apoptosis/cell proliferation in epithelial cells and the continuous differentiation and proliferation of myofibroblasts.

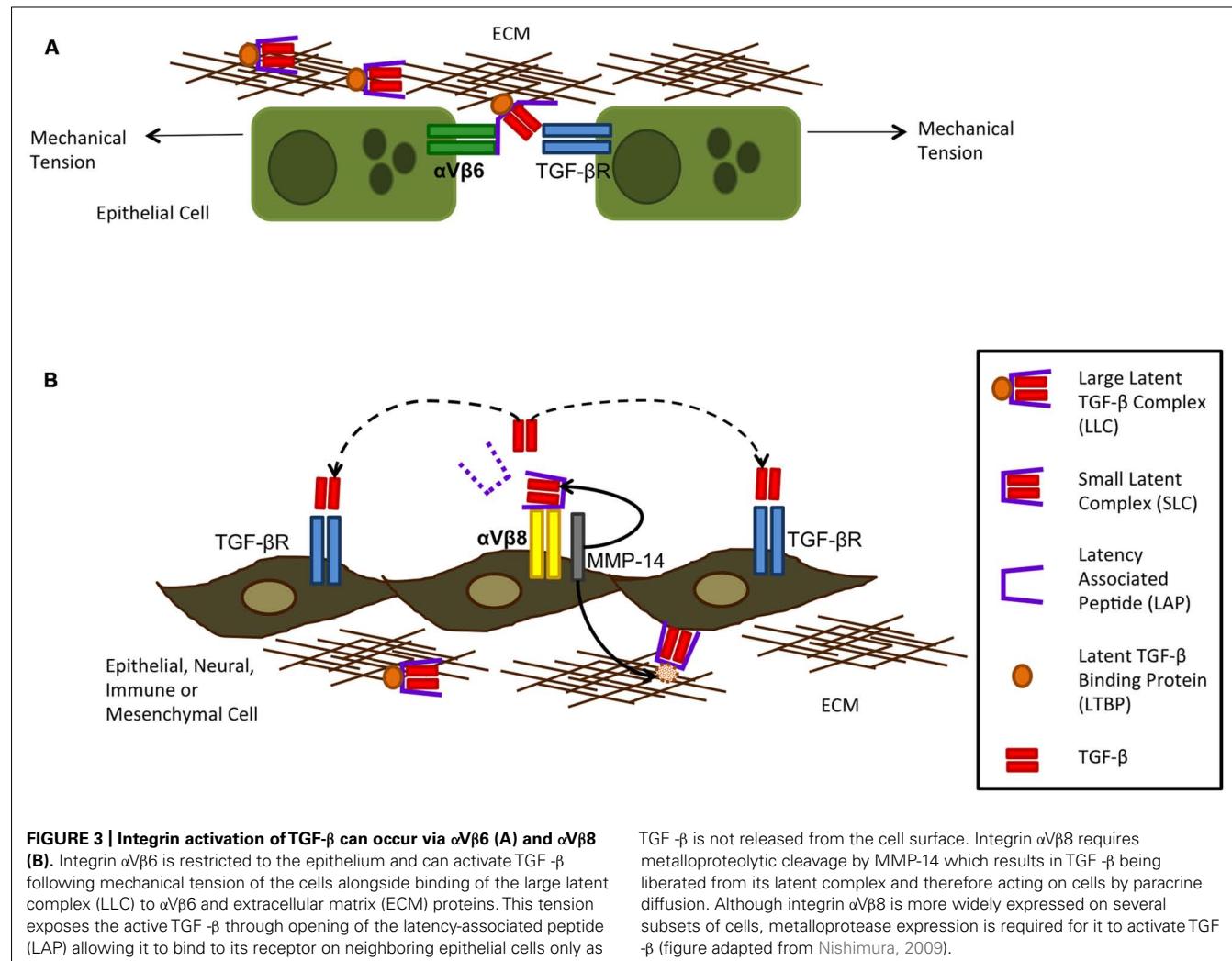
### TGF- $\beta$ AS A MOLECULAR MEDIATOR OF IPF

TGF- $\beta$  is a member of the TGF- $\beta$  superfamily and exists in three different forms in mammals which are expressed throughout the body (TGF- $\beta$ 1, TGF- $\beta$ 2, and TGF- $\beta$ 3) (Moustakas and Heldin, 2009). TGF- $\beta$  signaling is important for morphogenesis during embryonic development and in adulthood, for tissue homeostasis, however, in injured tissue, TGF- $\beta$  acts as a major pro-fibrotic cytokine, inducing fibroblast recruitment, proliferation and differentiation of myofibroblasts, and deposition of ECM

(Sheppard, 2006). The role of TGF- $\beta$  is therefore well described in IPF; it is overexpressed in both patients (Coker et al., 2001) and animal models of lung fibrosis and blocking TGF- $\beta$  signaling improves pulmonary fibrosis in mouse models (Bonniaud et al., 2005). TGF- $\beta$ 1 is produced as a complex of active TGF- $\beta$ 1 non-covalently associated with the latency-associated peptide (LAP) and these complexes are sequestered in the ECM. In order to exert its biological function TGF- $\beta$  must be released from these complexes. This process can be caused by physical mechanisms such as acidification, and oxidation, which could be initiated by injury, for example, from asbestos exposure (Sullivan et al., 2008). TGF- $\beta$  can also be cleaved by proteases such as MMP-2 and MMP-9 (Jenkins, 2008). More relevant to the epithelium is that epithelial cell integrins, more specifically  $\alpha$ V $\beta$ 6 can induce conformational changes in the latent TGF- $\beta$  complexes, and this process has been found to be a key event in pulmonary fibrosis (Tatler and Jenkins, 2012). The role of integrins will be further explored in the next section of this review. Once released from the latent complexes, TGF- $\beta$  can interact with its receptors on the surface of fibroblasts, and in the canonical signaling pathway it phosphorylates Smad proteins (Smad2 and Smad3) which then heterodimerise with Smad4 to form Smad2/4 and Smad3/4 complexes that translocate to the nucleus and bind to the promoter regions of pro-fibrotic genes like collagen, fibronectin, and  $\alpha$ -smooth muscle actin (Santibanez et al., 2011). In the non-canonical activation pathways, the c-Abelson tyrosine kinase (c-Abl) and mitogen-activated protein kinase (MAPKs) have been shown to be directly activated by TGF- $\beta$  (Daniels et al., 2004; Hu et al., 2006). In contrast to the effects of protecting fibroblasts from apoptosis and inducing their differentiation, TGF- $\beta$  promotes apoptosis in lung epithelial cells, and this has been shown both *in vitro* (Solovyan and Keski-Oja, 2006) and in a mouse model where protection from bleomycin-induced fibrosis was achieved in animals harboring a epithelial-specific deletion of the TGF- $\beta$  type II receptor (Li et al., 2011). This way TGF- $\beta$  is a bidirectional modulator, affecting both epithelial cells and fibroblasts in IPF.

### EPIHELIAL CELL INTEGRINS AND CHEMOKINES

Integrins are heterodimeric transmembrane receptors made up of one  $\alpha$  and one  $\beta$  subunit which bind to ECM proteins. So far 26 combinations have been identified. They function by transducing information between the ECM and the inside of the cell and have important roles in adhesion, migration, survival, differentiation, invasion, and maintenance of cell shape. *In vitro*,  $\alpha$ V $\beta$ 3,  $\alpha$ V $\beta$ 5,  $\alpha$ V $\beta$ 8, and  $\alpha$ V $\beta$ 6 can all activate TGF- $\beta$ 1 and TGF- $\beta$ 2 through binding of the arginine–glycine–aspartate (RGD) motif which is present in the LAP of the latent complex of TGF- $\beta$ , although  $\alpha$ V $\beta$ 6 and  $\alpha$ V $\beta$ 8 are higher affinity binders. *In vivo*, however, the role for  $\alpha$ V $\beta$ 6 and  $\alpha$ V $\beta$ 8 in TGF- $\beta$  activation is still under investigation, although expression of  $\alpha$ V $\beta$ 3 and  $\alpha$ V $\beta$ 5 is up-regulated in systemic sclerosis and  $\alpha$ V $\beta$ 5 is detected in the fibroblast foci of patients with IPF (Asano et al., 2005). Upon binding of LAP to  $\alpha$ V $\beta$ 6 or  $\alpha$ V $\beta$ 8, cleavage or conformational change occurs, allowing the active TGF- $\beta$  to interact with its receptor (**Figure 3**). The mechanisms by which  $\alpha$ V $\beta$ 6 and  $\alpha$ V $\beta$ 8 activate TGF- $\beta$  are different however. For  $\alpha$ V $\beta$ 6, mechanical stretching of the cells results in a



conformational change of the latent complex revealing the active TGF-β peptide and allowing it to bind to its receptor on cells in direct contact with each other. On the other hand, αVβ8 requires proteolytic cleavage with the help of MMP-14 releasing the active TGF-β peptide from the latent complex and allowing it to interact with its receptor in a paracrine fashion (Annes et al., 2003). The integrin αVβ8 has been shown to be expressed on several cell types such as epithelial, fibroblast, neural, and immune whereas αVβ6 is predominantly expressed on epithelial cells (Munger et al., 1999). Under normal conditions αVβ6 is expressed at low levels and further induced by inflammatory mediators unlike αVβ8 which it is expressed at high levels in the normal airway epithelium. This suggests αVβ6 may be more predominant at TGF-β activation during epithelial injury. During epithelial injury, damage to the basement membrane further allows αVβ6-expressing epithelial cells to interact with other cell types such as mesenchymal cells allowing TGF-β to exert fibrotic effects upon other cells.

In mice that do not express the β6 integrin subunit there is a decrease in active TGF-β in the lung (Jenkins et al., 2006) and these mice can develop MMP-12-dependent emphysema suggesting

epithelial specific activation of TGF-β *in vivo* is important. In addition blockade of αVβ6 either in knockout mice or through antibody neutralization protects mice from bleomycin induced fibrosis emphasizing its importance in fibrogenesis. Furthermore patients diagnosed with lung fibrosis also show enhanced levels of αVβ6 and currently there is an anti- αVβ6 (STX-100) from Stromedix in clinical development for organ fibrosis.

Angiogenesis occurs as part of the wound healing process and it has been described around the fibroblastic foci in both IPF patients and in the bleomycin model (Cui et al., 2001; Sterclova et al., 2009). CXC chemokines are important factors in the angiogenic–angiostatic balance as well as being powerful chemoattractants for inflammatory cells (Gerard and Rollins, 2001). These can be further divided into two subgroups: ELR + CXC (pro-angiogenic) binding to CXCR2 or ELR–CXC (anti-angiogenic) biding to CXCR3, depending on the presence or absence of a glutamate–leucine–arginine (ELR) motif, respectively (Strieter et al., 1995). To date, several CXC chemokines have been implicated in IPF (Figure 2). IL-8 (or CXCL8), a potent neutrophil chemoattractant produced by epithelial cells, endothelial cells, and macrophages has been shown elevated in

BAL fluid, lung tissue, and serum of IPF patients (Prasse and Muller-Quernheim, 2009). Recently, another epithelial-derived chemokine, CXCL6 (or GPC-2) was shown to be elevated in BAL fluid of both IPF patients and in the bleomycin model and that therapeutic blockade of this chemokine in this model significantly decreased both inflammation and some fibrosis markers (Besnard et al., 2013). In contrast, angiostatic CXC-chemokines such as CXCL10/IP-10 and CXCL11/I-TAC were found to be decreased in IPF patients (Sterclova et al., 2009). Apart from these, CC chemokines such as CCL2/MCP-1 has also been shown as important effectors in IPF pathogenesis by recruiting macrophages to the lung (Agostini and Gurrieri, 2006). Moreover CCL2 has been recently found to be expressed in airway epithelial cells (Mercer et al., 2009).

Two studies have found that IFN- $\gamma$ -1b therapy could have beneficial effects in IPF by regulating the angiogenic balance in IPF. One study found that subcutaneous IFN- $\gamma$ -1b delivery three times weekly for 6 months, increased CXCL11/I-TAC in both BAL fluid and plasma and that CXCL5 and type I collagen were significantly reduced (Strieter et al., 2004). The second study showed that several pro-angiogenic chemokines such as IL-8 and CXCL5 were up-regulated following IFN- $\gamma$ -1b treatment but no changes were seen in the levels of IP-10 or I-TAC (Antoniou et al., 2008). Moreover, they did not see any correlation with improvement in lung physiology or disease outcome. Therefore further research is needed to fully understand the potential benefits of this drug.

## SUMMARY AND FUTURE DIRECTIONS

Idiopathic pulmonary fibrosis is a complex disease that is refractory to treatment and carries a high mortality rate. The pharmaceutical and biotechnology industry have made many attempts to find effective treatments for IPF, but the disease has so far defied all attempts at therapeutic intervention. Clinical trial failures may arise for many reasons, including disease heterogeneity, lack of readily measurable clinical end points other than overall survival, and, perhaps most of all, a lack of understanding of the underlying molecular mechanisms of the progression of IPF. The anti-fibrotic drug Esbriet (pirfenidone) is the only drug marketed for IPF, however, it is not yet approved in the US. Other promising candidates in clinical development include the tyrosine kinase inhibitor nintedanib in Phase III (Boehringer Ingelheim), the monoclonal antibodies tralokinumab (MedImmune: AstraZeneca) and lebrikizumab (Genentech) targeting anti-IL-13, the monoclonal antibody STX-100 against integrin  $\alpha$ V $\beta$ 6 (Biogen Idec), the lysophosphatidic acid 1 inhibitor (Bristol-Myers Squibb) and the monoclonal antibody simtuzumab against lysyl oxidase-like 2 (Gilead) in Phase II.

Other trials targeting the immune system, using steroids as immune suppressants have had less success, and in fact have been associated with deleterious effects in IPF (The Idiopathic Pulmonary Fibrosis Clinical Research Network, 2012). However, despite current advances in technology and other current therapies in the clinic, lung transplantation is the only current available treatment for IPF that has been shown to improve survival (Adamali and Maher, 2012), but it carries the downside risks of donor availability, infection, and organ rejection.

A deeper understanding of the mechanisms that initiate the fibrotic pathway is urgently needed in order to develop more appropriate and more specific therapies. Accumulating evidence suggests that alveolar epithelial cell apoptosis may be the initial trigger of the disease and both genetic background and environmental exposure contribute to this outcome and initiate the “senescence-associated secretory pathway” that ultimately leads to full blown disease. It is imperative to find the common ancestor and disease initiator, so that patients can be identified earlier in the clinic and perhaps have more adequate treatment. Since this is a complex disease involving multiple pathways, it is possible that the future best therapies will involve combination drugs that target more than one pathway.

## AUTHOR CONTRIBUTIONS

Ana Camelo as first author led the direction and wrote a large proportion of the review, with input from both Deborah L. Clarke and Rebecca Dunmore. Matthew A. Sleeman provided direction and guidance.

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# NLRP3 inflammasome and its inhibitors: a review

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Inflammasomes are newly recognized, vital players in innate immunity. The best characterized is the NLRP3 inflammasome, so-called because the NLRP3 protein in the complex belongs to the family of nucleotide-binding and oligomerization domain-like receptors (NLRs) and is also known as “pyrin domain-containing protein 3”. The NLRP3 inflammasome is associated with onset and progression of various diseases, including metabolic disorders, multiple sclerosis, inflammatory bowel disease, cryopyrin-associated periodic fever syndrome, as well as other auto-immune and auto-inflammatory diseases. Several NLRP3 inflammasome inhibitors have been described, some of which show promise in the clinic. The present review will describe the structure and mechanisms of activation of the NLRP3 inflammasome, its association with various auto-immune and auto-inflammatory diseases, and the state of research into NLRP3 inflammasome inhibitors.

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## INTRODUCTION

The mammalian immune system defends against internal and external threats using innate immunity and adaptive immunity (Neill et al., 2010). The innate immune response relies on pattern-recognition receptors (PRRs) to target pathogenic microbes and other endogenous or exogenous pathogens. PRRs are expressed mainly in immune and inflammatory cells such as monocytes, macrophages, neutrophils, and dendritic cells (DCs) (Schroder and Tschopp, 2010; Fullard and O'Reilly, 2015). They present antigens to the adaptive immune system to generate long-lasting protection (Alexandre et al., 2014). Pathogen-associated molecular patterns (PAMPs), which are antigens common to a given group of pathogens (Medzhitov, 2009; Abderrazak et al., 2015b), are normally recognized by at least three PRRs: Toll-like receptors (TLRs), C-type lectins (CTLs), and Galectins (Bourgeois and Kuchler, 2012; Dzopalic et al., 2012). The innate immune system is evolutionarily conserved across vertebrates and invertebrates, which means that both human and animal studies can provide valuable insights into innate immunity (Dai et al., 2015).

A newly identified PRR, first described in detail in 2002, is the inflammasome (Martinon et al., 2002; Gentile et al., 2015; Jorgensen and Miao, 2015; Sanders et al., 2015). Numerous inflammasomes have been identified, including NLRP1, NLRP2, NLRP3, double-stranded DNA (dsDNA) sensors absent in melanoma 2 (AIM2) and NLRC4 (Ozaki et al., 2015). The best characterized is the NLRP3 inflammasome, so named because the NLRP3 protein in the complex belongs to the family of nucleotide-binding and oligomerization domain-like receptors (NLRs) and is also known as “pyrin domain-containing protein 3” (Inoue and Shinohara, 2013b; Eigenbrod and Dalpke, 2015). In addition to the NLRP3 protein, the NLRP3 inflammasome also contains adapter protein apoptosis-associated speck-like protein (ASC) and procaspase-1

(Inoue and Shinohara, 2013a; Ito et al., 2015). Interactions among these three proteins tightly regulate inflammasome function in order to ensure immune activity only when appropriate.

In the absence of immune activators, an internal interaction occurs between the NACHT domain and leucine-rich repeats (LRRs), suppressing the interaction between NLRP3 and ASC, thus preventing assembly of the inflammasome (Inoue and Shinohara, 2013a). In the presence of immune activators such as PAMPs, danger-associated molecular patterns (DAMPs), other exogenous invaders or environmental stress, NLRP3 opens up and allows interaction between the pyrin domains (PYDs) in NLRP3 and ASC. Subsequently the caspase recruitment domain (CARD) of ASC binds to the CARD domain on procaspase-1, giving rise to the NLRP3 inflammasome. Formation of this complex triggers procaspase-1 self-cleavage, generating the active caspase-1 p10/p20 tetramer and inducing the conversion of proinflammatory cytokines interleukin (IL)-1 $\beta$  and IL-18 from their immature “pro” forms to active forms that are secreted. Formation of the inflammasome also triggers a process of inflammation-related cell death termed pyroptosis (Willingham et al., 2009; Schroder and Tschoopp, 2010; Zhong et al., 2013a; Jorgensen and Miao, 2015).

## ACTIVATION OF THE NLRP3 INFLAMMASOME

### Models of NLRP3 Inflammasome Activation

The NLRP3 inflammasome is present primarily in immune and inflammatory cells following activation by inflammatory stimuli; these cells include macrophages, monocytes, DCs, and splenic neutrophils (Guarda et al., 2011b; Zhong et al., 2013a). Activation of the NLRP3 inflammasome appears to occur in two steps (Zhong et al., 2013a; Sutterwala et al., 2014; Ozaki et al., 2015; **Figure 1**). The first step involves a priming or initiating signal in which many PAMPs or DAMPs are recognized by TLRs, leading to activation of nuclear factor kappa B (NF- $\kappa$ B)-mediated signaling, which in turn up-regulates transcription of inflammasome-related components, including inactive NLRP3, proIL-1 $\beta$ , and proIL-18 (Bauernfeind et al., 2009; Franchi et al., 2012, 2014). This priming step is often studied *in vitro* using lipopolysaccharide (LPS; Park et al., 2015). The second step of inflammasome activation is the oligomerization of NLRP3 and subsequent assembly of NLRP3, ASC, and procaspase-1 into a complex. This triggers the transformation of procaspase-1 to caspase-1, as well as the production and secretion of mature IL-1 $\beta$  and IL-18 (Kim et al., 2015; Ozaki et al., 2015; Rabeony et al., 2015).

Three models have been proposed to describe the second step of inflammasome activation, as described in detail by Schroder and Tschoopp (2010) (shown in **Figure 1**). Briefly, all models assume that NLRP3 does not directly interact with exogenous activators, consistent with its ability to sense various pathogens.

In the first model, extracellular adenosine triphosphate (ATP), which acts as an NLRP3 agonist, induces K $^{+}$  efflux through a purogenic P2X7-dependent pore consisting of a pannexin-1 hemichannel. This process leads to NLRP3 inflammasome activation and assembly. Consistent with this model, K $^{+}$  efflux is a major activator of the NLRP3 inflammasome, while extracellular ATP and pore-forming toxins are the major triggers of IL-1 $\beta$  secretion by the inflammasome (Hari et al., 2014; Liu et al., 2014; Ketelut-Carneiro et al., 2015; Schmid-Burgk et al., 2015). Fluxes of intracellular and endoplasmic reticulum (ER)-related Ca $^{2+}$  may also activate the NLRP3 inflammasome (Hussen et al., 2012; Zhong et al., 2013b; Shenderov et al., 2014).

In the second model, all known PAMPs and DAMPs, including the activators mentioned above, trigger the generation of reactive oxygen species (ROS), which in turn induce assembly of the NLRP3 inflammasome. For example, damage to NADPH oxidase and other oxidative systems by mitochondrial ROS can activate the inflammasome (van Bruggen et al., 2010; Crane et al., 2014; Lawlor and Vince, 2014; Rajanbabu et al., 2015).

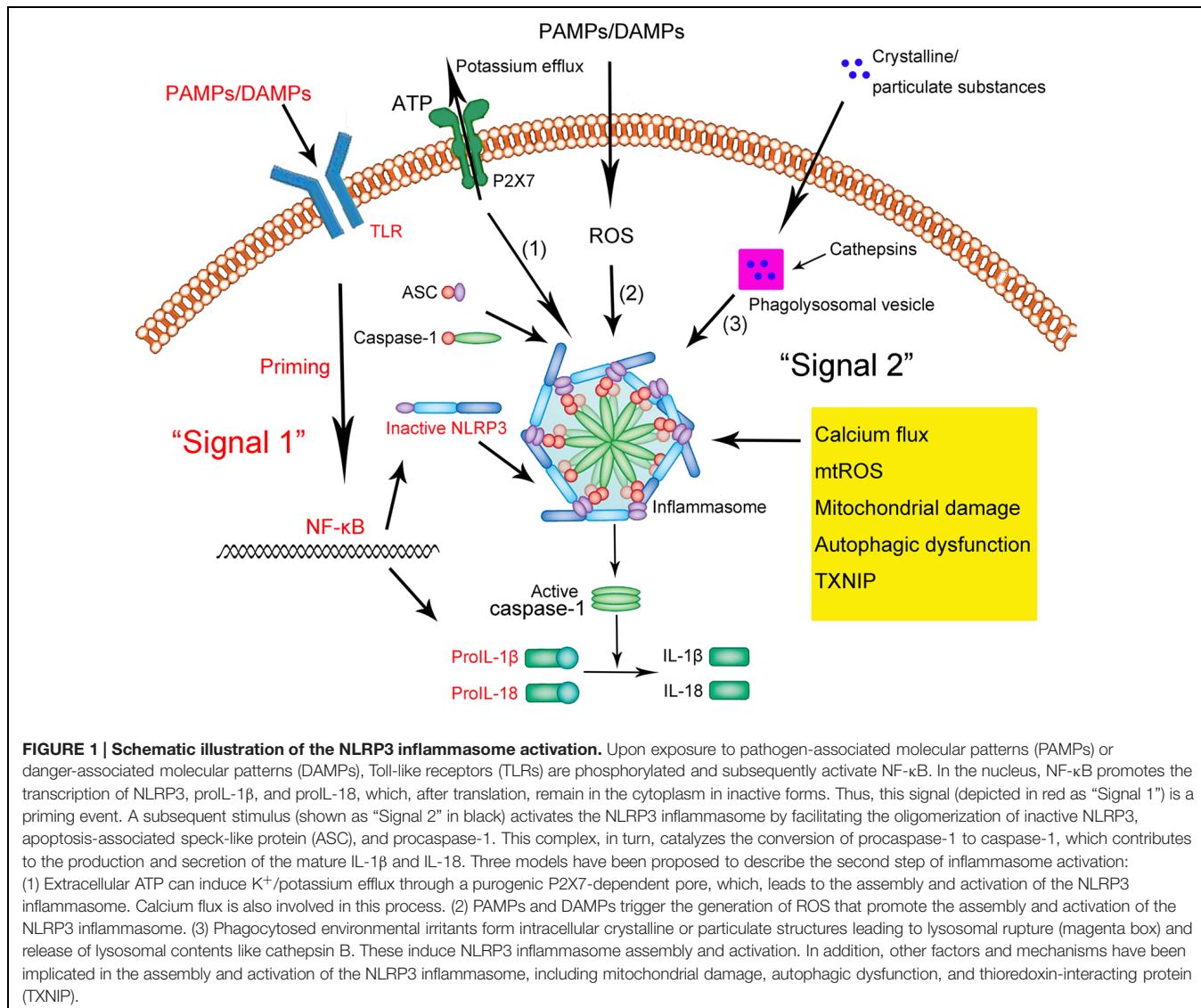
In the third model, assembly and activation of the NLRP3 inflammasome is thought to be triggered by environmental irritants (such as silica, asbestos, amyloid- $\beta$ , and alum) which form crystalline or particulate structures when engulfed by phagocytes. These aggregates cause lysosomal rupture and release of lysosomal contents via a mechanism mediated by cathepsin B. Consistent with this model, crystalline stimuli such as silica are major triggers of IL-1 $\beta$  secretion by the inflammasome.

Other factors can also activate the NLRP3 inflammasome. These include mitochondrial damage or dysfunction caused by mitochondrial Ca $^{2+}$  overload (Iyer et al., 2013; Miao et al., 2014; Zhuang et al., 2015), lysosomal disruption (Hornung et al., 2008; Sheedy et al., 2013; Tseng et al., 2013), autophagic dysfunction (Cho et al., 2014; Shao et al., 2014; Jabir et al., 2015) and the activity of thioredoxin-interacting protein (TXNIP; Li et al., 2015; Liu et al., 2015).

### The NLRP3 Inflammasome in Disease

While the innate immune response to insults can efficiently protect against disease and death, inappropriate activation of the NLRP3 inflammasome can contribute to the onset and progression of various diseases, particularly age-related diseases such as metabolic disorders and metabolic syndrome (Franceschi et al., 2000; Goldberg and Dixit, 2015). Increased production of IL-1 $\beta$  and IL-18 by the NLRP3 inflammasome contributes to atherosclerotic plaque progression and instability in atherosclerotic patients and animal models (Altaf et al., 2015; Patel et al., 2015; Peng et al., 2015). For example, Patel et al. (2015) showed that genetic ablation of the NLRP3 inflammasome suppressor known as the inhibitor of  $\kappa$ B kinase epsilon (IKBKE) enhanced the acute phase response and down-regulated cholesterol metabolism in cultured macrophages and hypercholesterolemic mice. Atherosclerosis and other inflammatory diseases were more severe in animals with the ablation.

Studies in macrophages and animal models have shown that oxidized low-density lipoprotein and cholesterol crystals trigger NLRP3 inflammasome activation (Duewell et al., 2010; Liu et al.,



2014). In macrophage and animal models of type II diabetes, hyperglycemia, and free fatty acids trigger inflammasome activation, which harms glucose metabolism and strengthens insulin resistance (Honda et al., 2014; Legrand-Poels et al., 2014; Ruscitti et al., 2015). In macrophage and animal models of uric acid accumulation, monosodium urate crystals activate the NLRP3 inflammasome, causing gout (Hari et al., 2014; Wang et al., 2014; Clephas et al., 2015). Taken together, these findings suggest that during the progression of many metabolic diseases, the accumulation of abnormal metabolic products activates the NLRP3 inflammasome. Studies in animal models suggest a similar picture in Alzheimer's disease (Vajjhala et al., 2012; Schnaars et al., 2013; Cho et al., 2014) and obesity induced by a high-fat diet (Haneklaus and O'Neill, 2015; Zhang et al., 2015).

In macrophages and in animal models, studies have also defined a role for the NLRP3 inflammasome in the initiation and development of cerebral and myocardial ischemic diseases,

including cerebral ischemia/stroke and myocardial ischemia (Sandanger et al., 2013; Marchetti et al., 2014; Hecker et al., 2015; Ito et al., 2015). Inflammasome activation appears to contribute to post-ischemic inflammation after stroke. For example, Ito et al. (2015) showed that using ibrutinib to inhibit Bruton's tyrosine kinase (BTK), an essential component of the NLRP3 inflammasome, reduced infarct volume, and neurological damage in a mouse model of cerebral ischemia/reperfusion injury. In addition, it is reported by Hecker et al. (2015) that activation of nicotinic acetylcholine receptors containing subunits  $\alpha$ 7,  $\alpha$ 9, and/or  $\alpha$ 10 inhibited ATP-mediated IL-1 $\beta$  release by human and rat monocytes, helping protect them from collateral damage. NLRP3 inflammasome-related proteins are up-regulated in myocardial fibroblasts following infarction, and this up-regulation may contribute to infarct size in ischemia-reperfusion injury (Sandanger et al., 2013). Consistent with this idea, inhibiting the NLRP3 inflammasome reduces myocardial injury after ischemia-reperfusion in mice (Marchetti et al., 2014).

NLRP3 inflammasome activation has also been linked to various auto-immune and auto-inflammatory diseases. Work from our laboratory and others has demonstrated that NLRP3 inflammasome activation contributes to progression of multiple sclerosis in humans and experimental autoimmune encephalomyelitis (EAE) in animal models (Ming et al., 2002; Jha et al., 2010; Lalor et al., 2011; Inoue et al., 2012a,b; Shao et al., 2014). Severity of multiple sclerosis in patients correlates closely with levels of IL-1 $\beta$ , IL-18, and caspase-1 (Ming et al., 2002; Jha et al., 2010; Lalor et al., 2011); the serum levels of both ILs and of active caspase-1 (p20) are elevated in mice with EAE (Inoue et al., 2012a,b). Studies in macrophages and mouse models of colitis have linked abnormal NLRP3 inflammasome activation with inflammatory bowel disease, including ulcerative colitis and Crohn's disease (Cheng et al., 2015; Guo et al., 2015; Sun et al., 2015). Polymorphism in the *NLRP3* gene is linked to colitis severity and progression in patients (Villani et al., 2009; Lewis et al., 2011), and gain-of-function mutations in the *NLRP3* gene that increase production and secretion of IL-1 $\beta$  and IL-18 are associated with cryopyrin-associated periodic fever syndrome (CAPS; Bozkurt et al., 2015; Carta et al., 2015; Zhou et al., 2015). This syndrome comprises several rare hereditary auto-inflammatory diseases in humans and animal models, including familial cold auto-inflammatory syndrome and Muckle-Wells syndrome. Inhibiting IL-1 using specific blocking agents effectively reduces systemic inflammation in CAPS patients (Kuemmerle-Deschner, 2015; Yadlapati and Efthimiou, 2015).

## PHARMACOLOGICAL USE OF NLRP3 INFLAMMASOME INHIBITORS

The extensive involvement of the NLRP3 inflammasome in such a range of diseases makes it a highly desirable drug target. Fortunately numerous promising inhibitors of NLRP3 inflammasome activation have been described, several of which are briefly described below together with their pharmacological mechanisms (shown in Table 1).

### MCC950 and $\beta$ -Hydroxybutyrate

Two small-molecule inhibitors of the NLRP3 inflammasome were described in groundbreaking reports in *Nature Medicine* this year (Coll et al., 2015; Youm et al., 2015). Coll et al. (2015) discovered that MCC950, a diarylsulfonylurea-containing compound known to inhibit caspase-1-dependent processing of IL-1 $\beta$  (Perregaux et al., 2001), also inhibits both canonical and non-canonical activation of the NLRP3 inflammasome. MCC950 inhibits secretion of IL-1 $\beta$  and NLRP3-induced ASC oligomerization in mouse and human macrophages. It reduces secretion of IL-1 $\beta$  and IL-18, alleviating the severity of EAE and CAPS in mouse models. Coll et al. (2015) further showed that MCC950 acts specifically on the NLRP3 inflammasome; it does not inhibit the activation of NLRP1, AIM2, or NLRC4 inflammasomes. Baker et al. (2015) have shown that MCC950 inhibits LPS-induced production of IL-1 $\beta$  via a mechanism

**TABLE 1 | Potential mechanisms of several NLRP3 inflammasome inhibitors.**

NLRP3 inflammasome inhibitor	Potential mechanisms involving NLRP3 inflammasome inhibition
Small-molecule inhibitor	MCC950 Blocking apoptosis-associated speck-like protein (ASC) oligomerization; Inhibiting of canonical and non-canonical NLRP3 inflammasome;
	BHB Blocking ASC oligomerization, Inhibiting K <sup>+</sup> /potassium efflux;
	Type I interferon (IFN) and IFN- $\beta$ Inducting phosphorylation of STAT1, transcription factor, Inducting IL-10 production;
Autophagy inducer	Resveratrol Inducing autophagy process, Suppressing mitochondrial damage;
	Arglabin Inducing autophagy process, Reducing cholesterol level;
	CB2R agonist Inducing autophagy process, Inhibiting priming step of NLRP3 inflammasome activation;
MicroRNA	MicroRNA-223 Suppressing NLRP3 protein expression.

dependent on the cytoplasmic LPS sensors caspase-4 and caspase-5. Krishnan et al. (2015) demonstrated that hypertension in mice treated with salt and deoxycorticosterone acetate can be reversed by treating them with MCC950, and this reversal depends on the inhibition of inflammasome activation and inflammasome-related IL-1 $\beta$  production.

Youm et al. (2015) discovered that the ketone metabolite  $\beta$ -hydroxybutyrate (BHB), but not acetoacetate or the short-chain fatty acids butyrate and acetate, reduced IL-1 $\beta$ , and IL-18 production by the NLRP3 inflammasome in human monocytes. Like MCC950, BHB appears to block inflammasome activation by inhibiting NLRP3-induced ASC oligomerization. Their *in vivo* experiments showed that BHB or a ketogenic diet alleviate caspase-1 activation and caspase-1-mediated IL-1 $\beta$  production and secretion, without affecting the activation of NLRC4 or AIM2 inflammasomes. BHB inhibits NLRP3 inflammasome activation independently of AMP-activated protein kinase, ROS, autophagy, or glycolytic inhibition. These studies raise interesting questions about interactions among ketone bodies, metabolic products, and innate immunity. BHB levels increase in response to starvation, caloric restriction, high-intensity exercise, or a low-carbohydrate ketogenic diet (Cotter et al., 2013). Vital organs such as the heart and brain can exploit BHB as an alternative energy source during exercise or caloric deficiency. Future studies should examine how innate immunity, particularly the inflammasome, is influenced by ketones and other alternative metabolic fuels during periods of energy deficiency (Shido et al., 1989; Johnson et al., 2007; McGettrick and O'Neill, 2013; Mercken et al., 2013; Newman and Verdin, 2014).

Although both MCC950 and BHB inhibit NLRP3 inflammasome activation, their mechanisms differ in key respects. BHB inhibits K<sup>+</sup> efflux from macrophages, while MCC950 does not. MCC950 inhibits both canonical and non-canonical inflammasome activation, while BHB affects only canonical activation. Nevertheless both inhibitors represent a significant advance toward developing therapies that target IL-1 $\beta$  and IL-18 production by the NLRP3 inflammasome in various diseases (Netea and Joosten, 2015).

### Type I Interferon (IFN) and IFN- $\beta$

In contrast to these newly described, NLRP3-specific inflammasome inhibitors, type I interferons (IFNs), including IFN- $\alpha$  and IFN- $\beta$ , have been used for some time to inhibit the NLRP3 and other inflammasomes in various auto-immune and auto-inflammatory diseases. These diseases include multiple sclerosis, systemic-onset juvenile idiopathic arthritis caused by gain-of-function *NLRP3* mutations, rheumatic diseases and familial-type Mediterranean fever (Guarda et al., 2011a; Inoue et al., 2012b; Inoue and Shinohara, 2013b; Malhotra et al., 2015; van Kempen et al., 2015). Type I IFNs are produced by specialized immune cells such as macrophages and DCs in response to extracellular stimuli such as bacteria and virus as well as various environmental irritants (Meylan et al., 2006). These IFNs are recognized by the type I IFN receptor (IFNAR), which is a member of the TLR family and is composed of the subunits IFNAR1 and IFNAR2. IFNAR activation involves several proteins, including Janus kinases, tyrosine kinase 2, and several kinds of signal transducers and activators of transcriptions (STATs). However, how type I IFNs affect NLRP3 inflammasome and its production of IL-1 $\beta$  and IL-18 remains unclear (Guarda et al., 2011a), despite numerous studies aimed to improve IFN-based treatments of NLRP3 inflammasome-related diseases. To provide an example of progress in this area, we focus below on studies of IFN therapy against multiple sclerosis in patients and EAE in mice, since type I IFN therapy has been used as a first-line or standard treatment of multiple sclerosis for 15 years (Inoue et al., 2012b).

Malhotra et al. (2015) classified 97 patients with multiple sclerosis into those who responded to IFN- $\beta$  therapy and those who did not, based on clinico-radiological criteria at 12 and 24 months of treatment. They found that expression of NLRP3 protein and levels of IL-1 $\beta$  were significantly lower among responsive patients who had relapsing-remitting multiple sclerosis than among other patients. Guarda et al. (2011a) found that IL-1 $\beta$  production by primary monocytes was lower in multiple sclerosis patients on IFN- $\beta$  treatment than in healthy subjects, supporting the value of IFN- $\beta$  therapy. Studies in mouse bone marrow-derived macrophages by Guarda et al. (2011a) suggest that IFN- $\beta$  may inhibit IL-1 $\beta$  production through at least two mechanisms. In one pathway, phosphorylation of STAT1 transcription factor leads to repression of NLRP1 and NLRP3 inflammasomes, which in turn inhibits caspase-1-dependent IL-1 $\beta$  maturation. In the second pathway, type I IFNs induce IL-10 production via a STAT-dependent mechanism, and the IL-10 works in an autocrine fashion to reduce levels of pro-IL-1 $\alpha$  and pro-IL-1 $\beta$  via a mechanism dependent on STAT3 signaling.

Type I IFN treatment is not effective for all types of multiple sclerosis, and the NLRP3 inflammasome may be a key determinant. Inoue et al. (2012b) conducted studies on mouse primary macrophage cultures as well as EAE mice and concluded that IFN- $\beta$  therapy is effective only when the NLRP3 inflammasome contributes directly to the disease process. Their studies further showed that IFNAR activation could be inhibited using the suppressor of cytokine signal 1 (SOCS1), which inhibited Rac1 activation and ROS generation, leading in turn to inhibition of NLRP3 inflammasome activity and less severe EAE.

These studies highlight the efficacy of type I IFN therapy and the need for future studies to elucidate the mechanisms of NLRP3 inflammasome inhibition. This work may improve clinical approaches to treating multiple sclerosis and other auto-immune and auto-inflammatory diseases.

### Other Kinds of NLRP3 Inflammasome Inhibitors

Several additional ways for inhibiting the NLRP3 inflammasome have opened up in recent years. Autophagy, a self-protective catabolic pathway involving lysosomes, has been shown to inhibit the NLRP3 inflammasome, leading researchers to explore the usefulness of autophagy-inducing treatments (Shao et al., 2014). Chang et al. (2015) showed that the plant polyphenolic compound resveratrol, known to induce autophagy, suppresses mitochondrial damage in macrophages and thereby inhibits NLRP3 inflammasome activation and NLRP3 inflammasome-mediated IL-1 $\beta$  secretion and pyroptosis. Abderrazak et al. (2015a) showed that arglabin inhibits the production and secretion of IL-1 $\beta$  and IL-18 by the NLRP3 inflammasome in a concentration-dependent manner in *ApoE*<sup>-/-</sup> mice on a high-fat diet. The reduced IL production translates to less severe atherosclerosis. Those authors reported that arglabin exerts its effects in macrophages by inducing autophagy as well as by reducing inflammation and cholesterol levels.

Cannabinoid receptor 2 (CB2R) is an already demonstrated therapeutic target in inflammation-related diseases (Smoum et al., 2015). Work from our own laboratory (Shao et al., 2014) has shown that autophagy induction may help explain why activation of the anti-inflammatory CB2R leads to inhibition of NLRP3 inflammasome priming and activation in mouse BV2 microglia stimulated with LPS and ATP as well as in a mouse model of EAE. Such CB2R activation reduces the severity of EAE in mice. Thus CB2R agonists similar to the HU-308 used in our work may become an effective therapy for treating NLRP3 inflammasome-related diseases by inducing autophagy.

MicroRNAs may provide another route for inhibiting inflammasomes. These endogenous non-coding RNAs are 20–23 nt long and bind to the 3' untranslated region (3' UTR) of protein-coding mRNAs to regulate their translation (Bartel, 2009; Chen and Sun, 2013). MicroRNA-223 binds to a conserved site in the 3' UTR of the NLRP3 transcript, suppressing protein expression and thereby inhibiting NLRP3 inflammasome priming and IL-1 $\beta$  production (Bauernfeind et al., 2012; Haneklaus et al., 2012; Chen and Sun, 2013). Deficiency in microRNA-223 leads to neutrophilia, spontaneous lung inflammation, and increased susceptibility to endotoxin

challenge in mice (Johnnidis et al., 2008; Haneklaus et al., 2013). Several other microRNAs have been reported to be involved in the activation of the NLRP3 inflammasome, including microRNA-155, microRNA-377, and microRNA-133a-1. Reducing the levels of these factors may be useful for treating inflammasome-related disease (Bandyopadhyay et al., 2013; Chen et al., 2015; Wang et al., 2015).

## CONCLUSION

The past decade has witnessed tremendous progress in understanding the structure and activation of the NLRP3 inflammasome, as well as its roles in the initiation and progression of various auto-immune and auto-inflammatory diseases, including metabolic disorders, multiple sclerosis, inflammatory bowel syndrome, and CAPS. Several types of NLRP3 inflammasome inhibitors have been developed and validated in cell culture studies and animal models of NLRP3 inflammasome-related diseases, and type I IFNs have become well established in the clinic. On the other hand, several agents have proven ineffective in clinical settings, and several

potential inhibitors require further development, such as autophagy-inducing and microRNA agents. This highlights the need for further research into what pathways activate the NLRP3 inflammasome and can therefore be targeted by appropriate inhibitors. There is still a long way to go toward exploiting NLRP3 inflammasome inhibitors in our fight against diseases.

## AUTHOR CONTRIBUTIONS

B-ZS and Z-QX were in charge of searching all the relative papers and writing this manuscript. B-ZH was in charge of drawing the picture. CL gave her valuable and professional suggestions and guide in organizing and drafting this manuscript. D-FS helped to revise the manuscript.

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# Adrenergic regulation of innate immunity: a review

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The sympathetic nervous system has a major role in the brain-immune cross-talk, but few information exist on the sympathoadrenergic regulation of innate immune system. The aim of this review is to summarize available knowledge regarding the sympathetic modulation of the innate immune response, providing a rational background for the possible repurposing of adrenergic drugs as immunomodulating agents. The cells of immune system express adrenoceptors (AR), which represent the target for noradrenaline and adrenaline. In human neutrophils, adrenaline and noradrenaline inhibit migration, CD11b/CD18 expression, and oxidative metabolism, possibly through  $\beta$ -AR, although the role of  $\alpha_1$ - and  $\alpha_2$ -AR requires further investigation. Natural Killer express  $\beta$ -AR, which are usually inhibitory. Monocytes express  $\beta$ -AR and their activation is usually antiinflammatory. On murine Dendritic cells (DC),  $\beta$ -AR mediate sympathetic influence on DC-T cells interactions. In human DC  $\beta_2$ -AR may affect Th1/2 differentiation of CD4+ T cells. In microglia and in astrocytes,  $\beta_2$ -AR dysregulation may contribute to neuroinflammation in autoimmune and neurodegenerative disease. In conclusion, extensive evidence supports a critical role for adrenergic mechanisms in the regulation of innate immunity, in peripheral tissues as well as in the CNS. Sympathoadrenergic pathways in the innate immune system may represent novel antiinflammatory and immunomodulating targets with significant therapeutic potential.

**Keywords:** noradrenaline, adrenaline, adrenoceptors, innate immunity, immunity of CNS

## Introduction

### Physiology and Pharmacology of Adrenergic Pathways

Adrenaline ("near the kidney," from Latin roots *ad* and *renes*; US: epinephrine, from the Greek roots *epi* and *nephros*, i.e., "on the kidney") belongs together with noradrenaline (the prefix "nor" standing for *nitrogen ohne radikal*, indicating the absence of a methyl group) to

**Abbreviations:** DC, Dendritic cells; NK, Natural killer cells;  $\gamma\delta$  T lymphocytes, Gamma Delta T lymphocytes; AR, Adrenoceptors; Th, T helper lymphocytes; LC, Locus coeruleus; cAMP, Cyclic adenosine monophosphate; CNS, Central nervous system; ILC, Innate lymphoid cells; PRR, Pattern recognition receptors; PAMP, Pathogen-associated molecular patterns; DAMP, Danger (or damage)-associated molecular patterns; TLR, Toll-like receptors; NLR, NOD-like receptors; CLR, C-type lectin receptors; RLR, RIG-I-like receptors; ALR, AIM2-like receptors; FPR, Formyl peptide receptors; gC1qR, gC1q receptor; SPLUNC1, Nasal epithelial clone 1; hBD, Human  $\beta$ -defensin; HNP, Human neutrophil peptide; MAO, Monoamine oxidase; VMAT, Vesicular monoamine transporter; EPO, Eosinophil peroxidase; TNF, Tumor necrosis factor; SCF, Stem cell factor; MIP, Macrophage inflammatory protein; LPS, Lipopolysaccharide; IL, Interleukine; IE, Immediate-early; MMP, Matrix metalloproteinases; PKC, Protein kinase C; MHC, Major histocompatibility complex; PLC, Phospholipase C; PKA, Protein kinase A; CpG-C ODN, Type-C CpG oligodeoxynucleotides; COX, Cyclooxygenase; THP-1, Human microglia-like cells; MDSC, Myeloid-derived suppressor cells.

catecholamines, a group of chemicals containing a catechol or 3,4-dihydroxyphenyl group and an amine function. The first step in the synthesis of Noradrenaline is the transformation of the aminoacid tyrosine in Levodopa through the enzyme tyrosine hydroxylase that is the key rate-limiting enzyme in the biosynthetic pathway of Noradrenaline. Levodopa is decarboxylated into dopamine and finally noradrenaline is synthesized from dopamine by dopamine  $\beta$ -hydroxylase and is converted to adrenaline by phenylethanolamine N-methyltransferase (Figure 1). Adrenaline was isolated as pure crystalline base in 1900 by Jokichi Takamine in New Jersey and was the first hormone to be isolated in a pure state, while noradrenaline was proved in 1949 by Ulf von Euler in Stockholm to be the main sympathomimetic neurotransmitter in humans.

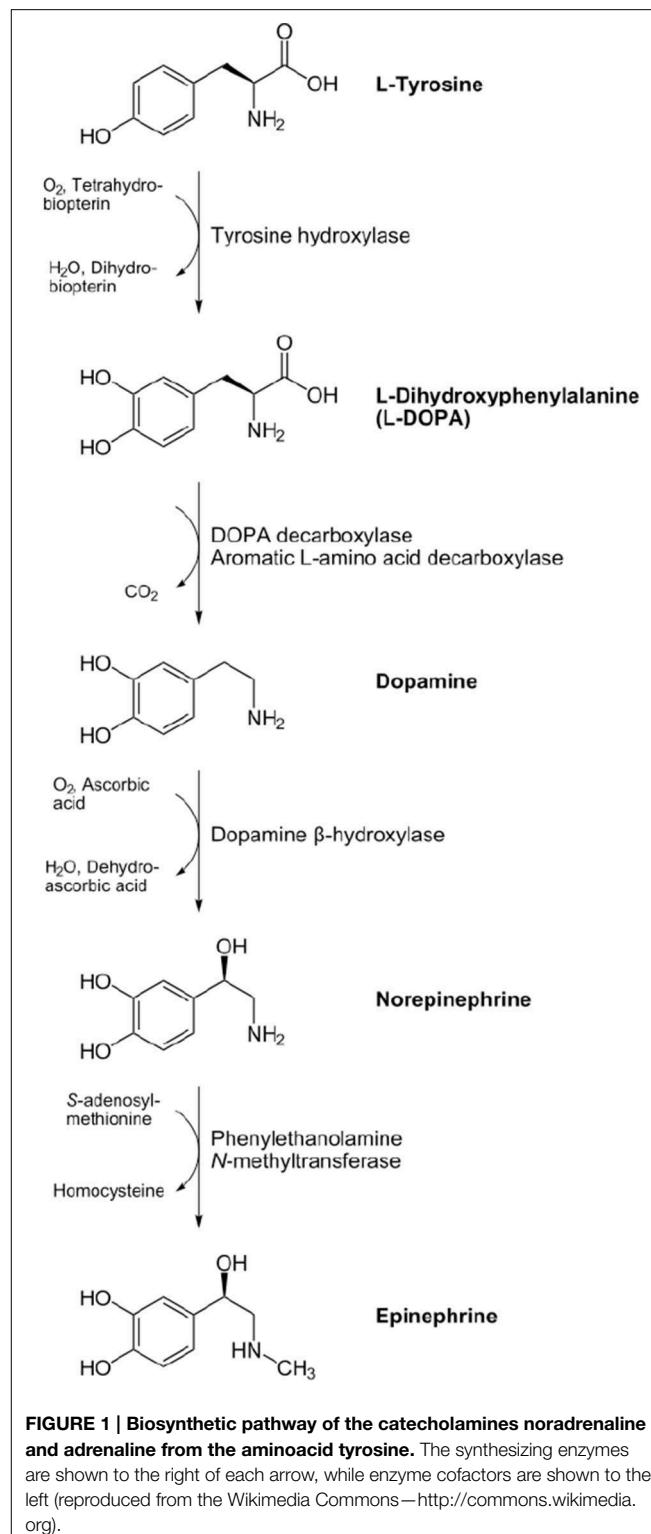
Noradrenaline act as neurotransmitter in the central and peripheral nervous systems. The sympathetic nervous system, through its preganglionic fibers, stimulates chromaffin cells in the adrenal glands to release into the bloodstream adrenaline ( $\sim 80\%$  in humans) and noradrenaline ( $\sim 20\%$ ). In the brain, noradrenergic neurons are located mainly in the *locus coeruleus* (LC), and their axons project to hippocampus, septum, hypothalamus and thalamus, cortex and amygdala, to cerebellum, as well as to spinal cord. Brain adrenergic pathways control attention, arousal and vigilance, and regulate hunger and feeding behavior. Some central nervous system (CNS) neurons, mainly located in the medullary reticular formation, utilize adrenaline as the main neurotransmitter, possibly contributing to the modulation of eating behavior and to blood pressure regulation. In the periphery, noradrenaline is the main transmitter of sympathetic postganglionic fibers. Main direct effects of noradrenaline and adrenaline on peripheral tissues include: smooth muscle contraction in blood vessels supplying skin, kidney, and mucous membranes, stimulation of exocrine glands, smooth muscle relaxation in the gut wall, bronchi, and blood vessels supplying skeletal muscle, increases of heart rate and force of contraction, increased glycogenolysis in liver and muscle, lipolysis in adipose tissue, thermogenesis in the brown adipose tissue, modulation of the secretion of insulin and rennin (Feldman et al., 1997).

## Adrenergic Receptors

The effects of noradrenaline and adrenaline are mediated by 7-transmembrane, G-protein coupled receptors called “adrenergic receptors” or “adrenoceptors” (AR) and classified in three major types— $\alpha_1$ ,  $\alpha_2$ , and  $\beta$ —each further divided into three subtypes, which are widely expressed throughout the CNS and in virtually all peripheral tissues (Ahlquist, 1948; Bylund et al., 1994).

The order of potency in the activation of these receptors by physiological ligands is noradrenaline > adrenaline for the  $\alpha_1$ - and  $\alpha_2$ -AR and adrenaline > noradrenaline for the  $\beta$ -AR.

Activation of  $\alpha_1$ -AR by adrenergic agonists induces the stimulation of a Gq and consequent phospholipase C (PLC) activation that promotes hydrolysis of phosphatidylinositol bisphosphate producing inositol trisphosphate and diacylglycerol. The results of this activation is the release of  $\text{Ca}^{++}$  as second messenger from non-mitochondrial pools or protein kinase C (PKC) and mediating intracellular release



**FIGURE 1 | Biosynthetic pathway of the catecholamines noradrenaline and adrenaline from the aminoacid tyrosine.** The synthesizing enzymes are shown to the right of each arrow, while enzyme cofactors are shown to the left (reproduced from the Wikimedia Commons—<http://commons.wikimedia.org>).

(Bylund et al., 1994). The  $\alpha_2$ -AR are considered inhibitory receptors; their activation induces the stimulation of a Gi resulting in adenylate cyclase inhibition and reduction of cyclic adenosine monophosphate (Bylund et al., 1994). Presynaptic autoreceptors mediating inhibition of neurotransmitter

release are mainly  $\alpha_2$ -AR, while postsynaptic AR include all subtypes.

$\beta$ -AR are coupled to a stimulatory Gs that leads to activation of adenylate cyclase and accumulation of the second messenger cAMP. In some situations, the  $\beta_3$ -AR, can be coupled to Gi as well as to Gs (Gauthier et al., 1996). Receptors stimulation induces the protein kinase A (PKA) activation and phosphorylation of L-type  $\text{Ca}^{++}$  channels and  $\text{Ca}^{++}$  entry (Guimarães and Moura, 2001). The  $\beta_1$ -AR is the most important receptor that mediates cardiovascular responses to noradrenaline released from sympathetic nerve terminals and to circulating adrenaline.  $\beta_2$ -AR are primarily localized on airway smooth muscle cells and are known to be involved in bronchial muscle relaxation.

AR ligands are used as drug therapeutics in different cardiovascular diseases such as hypertension, angina pectoris, congestive heart failure, or other diseases affecting million of individuals such as asthma, depression, benign prostatic hypertrophy, and glaucoma, as well as in shock, premature labor, opioid withdrawal, and as adjunct medications in general anesthesia (Bylund et al., 1994; Perez et al.)<sup>1</sup>. Agonists of  $\beta_2$ -AR are employed in therapy as first line-treatment of asthma and chronic obstructive pulmonary disease. The  $\beta_3$ -AR is known to be located primarily on adipocytes (Harms et al., 1974), but at present no ligands of this receptors subtype are employed in therapy. The physiopharmacology of AR is summarized in Table 1.

## Adrenergic Regulation of the Immune Response: an Overview

Together with the hypothalamic-pituitary-adrenal axis, the sympathetic nervous system represents the major pathway involved in the cross-talk between the brain and the immune system. Sympathoadrenergic fibers innervate both primary (bone marrow and thymus) and secondary (spleen and lymph nodes) lymphoid organs, where noradrenaline and adrenaline are released from nerve varicosities and or diffuse from the bloodstream to act on AR expressed on immune cells (Elenkov et al., 2000; Straub, 2004; Marino and Cosentino, 2013).

## The Innate Immune System

The innate immune system is usually considered as the first line of defense against invading microorganisms, however its contribution is increasingly emerging in several noninfectious diseases, including atherosclerosis (Chávez-Sánchez et al., 2014) and its ischemic complications (Courties et al., 2014), inflammatory bowel disease (Levine and Segal, 2013), systemic sclerosis (O'Reilly, 2014), multiple sclerosis, and other demyelinating disease (Mayo et al., 2012), neurodegenerative disease (Boutajangout and Wisniewski, 2013), and only as example, obesity (Lumeng, 2013) and diabetes (Lee, 2014) or that innate immunity play a role in tumor recognition (Marcus et al., 2014) and as a barrier to organ transplantation (Farrar et al.,

2013), and even in psychiatric disorders (Jones and Thomsen, 2013).

The innate immune system consists of effector molecules such as complement and antibacterial peptides as well as of effector cells. **Complement** is a proteolytic cascade system comprising around 35 different soluble and membrane-bound proteins, which is crucial for defense from microbial infections and for clearance of immune complexes and injured cells (Noris and Remuzzi, 2013). **Antibacterial peptides** are cationic peptides (i.e., with positive net charge) containing 15–45 aminoacid residues, which are both potent antibiotics usually targeting bacterial membranes as well as effective modulators of the immune response (Boman, 2003). **Innate immune cells** include: granulocytes (neutrophils, eosinophils, basophils, mast cells), monocytes/macrophages, dendritic cells (DC), natural killer (NK) cells,  $\gamma\delta$  T lymphocytes, as well as the recently described innate lymphoid cells (ILC). Immune responses in the CNS are mediated by resident microglia and astrocytes, which are innate immune cells without direct counterparts in the periphery.

Noxious stimuli alert the innate immune system through **pattern recognition receptors (PRR)**, which can be activated by both exogenous pathogen-associated molecular patterns (PAMP) and endogenous danger (or damage)-associated molecular patterns (DAMP). PRR includes families of toll-like receptors (TLR, “toll” being the German word for “amazing” or “great”), NOD-like receptors (NLR, NOD standing for nucleotide-binding oligomerization domain), C-type lectin receptors (CLR, including mannose receptors and asialoglycoprotein receptors), RIG-I-like receptors (RLR, where RIG-I stands for retinoic acid-inducible gene 1), and AIM2-like receptors (ALR, AIM-2 or “absent in melanoma 2” being part of the inflammasome and contributing to the defense against microbial DNA). Formyl peptide receptors (FPR, binding N-formyl peptides derived from the degradation of either bacterial, or host cells) and scavenger receptors (binding oxidized or acetylated low-density lipoprotein) could be also included among PRR (Kawai and Akira, 2010; Saxena and Yeretssian, 2014).

## Adrenergic Modulation of the Innate Immune System

Several excellent reviews deal with the role of sympathoadrenergic pathways in the communication between the nervous system and the immune system (Elenkov et al., 2000; Kohm and Sanders, 2001; Marino and Cosentino, 2013; Kenney and Ganta, 2014). However, most information regards adaptive immunity, and adrenergic regulation of the innate immune response is a relatively recent issue (Elenkov et al., 2000; Wrona, 2006; Flierl et al., 2008a; Marino and Cosentino, 2013). We will hereafter revise the most recent literature concerning humoral and cellular arms of the innate immune response, with specific attention to data obtained in humans and to their clinical implications.

### Complement

Although AR-mediated modulation of complement-induced innate immune response has been characterized at least in

<sup>1</sup> Adrenoceptors. Last modified on 13/06/2014. Accessed on 02/09/2014., IUPHAR database (IUPHAR-DB). Available online at: <http://www.iuphar-db.org/DATABASE/FamilyMenuForward?familyId=4>.

**TABLE 1 | Classification of AR (Perez et al.).**

<b>Main transduction mechanisms</b>	<b>Human tissue distribution</b>	<b>Physiological functions</b>	<b>Therapeutic drugs (indications)</b>
$\alpha_1A$	G <sub>q</sub> /G <sub>11</sub> (phospholipase C stimulation, calcium channel)	Cerebral cortex, cerebellum, heart, liver, predominant subtype in prostate and urethra, lymphocytes	Contraction of urethral smooth muscle, contraction of skeletal muscle resistance arteries, contraction of human subcutaneous arteries
$\alpha_1B$		Spleen and kidney, somatic arteries and veins, endothelial cells, lymphocytes, osteoblasts	Contraction of arteries and veins, osteoblast proliferation
$\alpha_1D$		Cerebral cortex, aorta, blood vessels of prostate, human bladder, lymphocytes	Contraction of arteries, ureteral contraction
$\alpha_2A$	G <sub>i</sub> /G <sub>o</sub> (adenylate cyclase inhibition, potassium channel, calcium channel, phospholipase A2 stimulation)	Brain > spleen > kidney > aorta = lung = skeletal muscle > heart = liver	Presynaptic inhibition of noradrenaline release, hypotension, sedation, analgesia, hypothermia
$\alpha_2B$		Kidney >> liver > brain = lung = heart = skeletal muscle (also reported in aorta and spleen)	Vasoconstriction
$\alpha_2C$		Brain = kidney (also reported in spleen, aorta, heart, liver, lung, skeletal muscle)	Presynaptic inhibition of noradrenaline release
$\beta_1$	G <sub>s</sub> (adenylate cyclase stimulation)	Brain, lung, spleen, heart, kidney, liver, muscle	Increase of cardiac output (heart rate, contractility, automaticity, conduction), renin release from juxtaglomerular cells, lipolysis in adipose tissue
$\beta_2$		Brain, lung, lymphocytes, skin, liver, heart	Smooth muscle relaxation, striated muscle tremor, glycogenolysis, increased mass and contraction speed, increase of cardiac output, increase of aqueous humor production in eye, dilatation of arteries, glycogenolysis and gluconeogenesis in liver, insulin secretion, broncodilation
$\beta_3$		Adipose tissue, gall bladder > small intestine > stomach, prostate > left atrium > bladder (also reported in brown adipose tissue and endothelium of coronary microarteries)	Lipolysis, thermogenesis, relaxation of miometrium and colonic smooth muscle cells, vasodilatation of coronary arteries, negative cardiac inotropic effect

animal models (Flierl et al., 2008a), nearly no information is available regarding any direct effect of adrenergic pathways on complement activity.

*In vitro*, adrenaline and noradrenaline may enhance in monocytes the synthesis of several complement components (such as C2, C3, C4, C5, factor B, properdin, beta 1H, and C3b inactivator), possibly through the activation of  $\alpha_1$ -AR

(Lappin and Whaley, 1982). Adrenaline has also been reported to inhibit C5-convertase generation from C3-convertase stabilized by nickel ions (Kozlov and Lebedeva, 1998), and to inhibit the covalent binding of the nascent C4b fragment of the human complement component to IgG (Kozlov et al., 2000), however the physiological relevance of such effects was not investigated. In COS-7 transfected cells, it was shown that

gC1qR (i.e., gC1q receptor, gC1q binding protein, p32, p33), a multifunctional cellular protein that interacts with components of the complement, kinin, and coagulation cascades as well as with select microbial pathogens, may bind with the carboxyl-terminal cytoplasmic domain of the  $\alpha_{1B}$ -AR (Xu et al., 1999).

Early studies in rodents suggested that  $\alpha$ -AR stimulation might result in increased secretion with saliva of a potent kallikrein-like anticomplementary factor (Wallace et al., 1976). In rats evidence has also been provided that the  $\beta$ -AR agonist isoprenaline reduces the clearance function of complement receptors on Kupffer cells, an effect which is sensitive to  $\beta$ -AR antagonism (Loegering and Commins, 1988), and that adrenaline may affect complement activity, both acutely (reduction followed by increase and then return to normal values in 2 h) and in the long term (increase 24 h after administration and peak after 2–3 days), however the specific contribution of AR subtypes remains to be established (Vasin and Kuznetsova, 1995). Nonetheless, another study in rats reported no effect of adrenaline (infused together with corticosterone and glucagon) on liver synthesis of albumin, complement component C3, and alpha 1-acid glycoprotein (Pedersen et al., 1989). Remarkably, it has been recently shown that the complement anaphylatoxin C5a may induce cell apoptosis in adrenal medulla following cecal ligation and puncture-induced sepsis in rats, as well as apoptosis of pheochromocytoma PC12 cells *in vitro*, resulting in impaired production of noradrenaline and dopamine (Flierl et al., 2008b), revealing a novel interaction between adrenergic pathways and the complement system.

Complement proteins may also affect brain adrenergic pathways. Indeed, early reports suggest that C5a might activate  $\alpha$ -AR in the hypothalamus, possibly explaining the neuropsychiatric symptoms sometimes associated with immune complex diseases affecting the CNS (Williams et al., 1985). Actually, C5a in the hypothalamus may act presynaptically, resulting in noradrenaline release through the activation of a specific C5a/C5ai receptor (Schupf et al., 1989).

## Antibacterial Peptides

Evidence regarding an interplay between adrenergic pathways and antibacterial peptides is recent and still fragmentary, nonetheless the few available information supports its relevance. For instance, it has been reported that the  $\beta$ -AR agonists albuterol and formoterol may increase the antimicrobial protein short palate, lung, and nasal epithelial clone 1 (SPLUNC1) (but not  $\beta$ -defensin-2/hBD-2) in normal and asthma airway epithelial cells, resulting in reduced *Mycoplasma pneumoniae* infection, and interleukin (IL)-13 attenuates such effects, decreasing SPLUNC1 and  $\beta$ -AR expression (Gross et al., 2010). A  $\beta$ -AR-dependent mechanism seems to be involved also in the psychological stress-induced decline in both cathelicidin and human  $\beta$ -defensin (hBD)-3 expression in the skin of mice. The antimicrobial peptide catestatin increased after short-term stress, but then began to decline with more sustained stress. In cultured keratinocytes, glucocorticoids downregulated catestatin expression, but  $\beta$ -AR blockade increased catestatin, as well as cathelicidin and hBD3 (Martin-Ezquerro et al., 2011). Human neutrophil peptides (HNP) 1–3 are expressed also in the synovial

lining and adjacent sublining area (but not in deeper layers of synovial tissue) in both rheumatoid arthritis and osteoarthritis subjects, and their expression is concentration-dependently inhibited by noradrenaline (Riepl et al., 2010). Adrenergic agents may therefore enhance or inhibit the expression of antibacterial peptides, depending on the specific circumstances, however the factors contributing to the specific effects have been not yet clarified.

Antibacterial peptides may also affect the response of tissues to adrenergic stimulation. Recently, it has been reported that  $\alpha$ -defensin-1/HNP1 has relaxing effects on adrenergic contractions of rat detrusor muscles, possibly through NF- $\kappa$ B pathways (Lee et al., 2011).

Finally, adrenaline and noradrenaline have been shown to downregulate *S. Typhimurium* virulence gene expression, increasing its sensitivity to the antimicrobial peptide LL-37 (Spencer et al., 2010).

## Cells

### Granulocytes

Granulocytes (also known as polymorphonuclear leukocytes) are named after the presence of intracellular granules. Granulocytes are currently divided into different types, based on their staining characteristics: neutrophils (the most abundant, representing 40–80% of total leukocytes in normal conditions), eosinophils, basophils, and mast cells. **Neutrophils** are the major cellular arm of the innate immune system. They are phagocytic cells which are recruited to sites of infection to kill pathogens, however it is increasingly evident that they also play a key role in progression of non-infectious disease as well as in conditions characterized by chronic inflammation (Mócsai, 2013; Mayadas et al., 2014). **Eosinophils** have a prominent role in allergic diseases and inflammatory responses against helminthic parasites, and are involved in diseases involving mucosal surfaces, (e.g., allergic asthma, atopic dermatitis, and gastrointestinal disorders) (Kita, 2013). **Basophils** are similar to tissue-resident mast cells and their main function is the protection against infections with parasites, including ticks, and helminths (Karasuyama and Yamanishi, 2014). Finally, **mast cells** are tissue-resident granulocytes mainly located at the interface with the external environment. They contain granules rich in histamine and heparin, and their main roles are in allergy, wound healing, defense against pathogens, as well as in antitumor immunity (da Silva et al., 2014).

Adrenergic modulation of granulocytes has been examined mainly in neutrophil, nonetheless available evidence suggests that adrenergic agents may profoundly affect all granulocyte subtypes.

### Neutrophils

The  $\beta$ -AR agonist isoprenaline inhibits the respiratory burst in human neutrophils (Nielson, 1987), an effect which has been confirmed in later studies and attributed to  $\beta_2$ -AR (Brunskole Hummel et al., 2013). IL-8 production however seems to be not sensitive to adrenaline, which only slightly reduces the expression of the adhesion molecules CD15, CD44, and CD54, and only at very high concentrations (1 mM) (Wahle et al., 2005). Recently however the *in vitro* adhesion of neutrophils to endothelial cells

was shown to be inhibited by adrenaline, an effect likely due to  $\beta$ -AR activation (Trabold et al., 2010). Desensitization of  $\beta$ -AR may occur after activation of the neutrophil respiratory burst (Vago et al., 1990), and neutrophils from elderly subjects may have decreased  $\beta$ -AR responsiveness (Cotter and O'Malley, 1983).

Ligand binding studies suggest that on average 1700–2200  $\beta$ -AR are expressed on neutrophil membranes (Pohl et al., 1991; Schwab et al., 1993), while excluding the existence of  $\alpha_2$ -AR (Musgrave and Seifert, 1994). We have recently shown that human neutrophils express mRNA for all  $\alpha$ - (with the only exception of  $\alpha_{2B}$ -) and  $\beta$ -AR, in the following order:  $\beta_3 > \beta_2 > \alpha_{1A} > \alpha_{1B} \sim \alpha_{2A} \sim \beta_1 = \alpha_{1D} = \alpha_{2C}$  and that exposure of cells to IL-8, a potent proinflammatory CXC-chemokine that promotes neutrophil chemotaxis and degranulation, increases mRNA levels of all AR, and that adrenaline, probably through the involvement of  $\beta$ -AR, profoundly affects neutrophil function (Scanzano et al., 2015).

Conditions reported to be associated with decreased  $\beta$ -AR expression on circulating neutrophils include: essential hypertension (Corradi et al., 1981), juvenile type I diabetes mellitus (Schwab et al., 1993), as well as strenuous physical exercise (Ratge et al., 1988; Fragala et al., 2011). Increased  $\beta$ -AR expression has been reported in post-traumatic stress disorder (Gurguis et al., 1999).

Circumstantial evidence suggests that neutrophils may also synthesize and release catecholamines. Noradrenaline and adrenaline (as well as dopamine and their major metabolites) have been identified in human neutrophils (Cosentino et al., 1999), which may also contain some catecholamine-degrading enzymes, such as monoamine oxidase (MAO), possibly of the B type (Balsa et al., 1989). Indeed, the MAO inhibitor pargyline may lead at least *in vitro* to increased catecholamine levels in human neutrophils (Cosentino et al., 1999). Interestingly, exposure of rodent cells to lipopolysaccharide (LPS) results in catecholamine release together with induction of catecholamine-generating and degrading enzymes, and blockade of  $\alpha_2$ -AR or pharmacological inhibition of catecholamine synthesis may suppress (while  $\alpha_2$ -AR agonism or inhibition of catecholamine-degrading enzymes enhances) lung inflammation in rodent models of acute lung injury. Adrenalectomized animals show even enhanced catecholamine release from phagocytes as well as enhanced expression of catecholamine-synthesizing enzymes in these cells. Such results have been explained suggesting that in rodent phagocytes noradrenaline and adrenaline possibly activate NF $\kappa$ B resulting in enhanced release of tumor necrosis factor (TNF)- $\alpha$ , IL-1 $\beta$  and IL-6, and in the subsequent amplification of acute inflammation via  $\alpha_2$ -AR (Flierl et al., 2007, 2009). We recently identified in human neutrophils the presence mRNA for tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of catecholamines, as well as for the vesicular monoamine transporter (VMAT) 2 (but not 1) (Scanzano et al., 2015).

### Eosinophils

The role of adrenergic pathways in eosinophils has been studied mainly in relation to their role in allergic diseases and inflammation, with particular regard to the respiratory tract. Several studies indeed deal with the effects on eosinophils exerted

by  $\beta_2$ -AR agonists used as bronchodilators, however most of those studies do not provide clear evidence that any effects are actually due to AR-operated pathways (e.g., due to the absence of experiments with AR antagonists). This is a relevant issue, since many effects exerted by  $\beta_2$ -AR agonists may actually occur through  $\beta$ -AR-independent mechanisms (Tachibana et al., 2002).

Early studies in humans examined the response of circulating eosinophils to noradrenaline, adrenaline, and emotional stress (Humphreys and Raab, 1950). In healthy subjects, the fall in circulating eosinophils following adrenaline injection, as well as the rise induced by the administration of  $\beta$ -AR antagonists is well characterized (Koch-Weser, 1968). Several decades later however  $\beta$ -AR detected on eosinophils, as well as on mast cells, macrophages, and neutrophils, were still considered of limited functional importance, due to rapid tachyphylaxis (Barnes, 1993).

Binding and functional data support the existence in eosinophils from patients with eosinophilia and from the peritoneal cavity of guinea pigs of  $\beta$ -AR possibly of the  $\beta_2$  subtype, coupled to adenylate cyclase, but apparently not involved in oxidative metabolism or degranulation (Yukawa et al., 1990). A few years later however another study showed that  $\beta_2$ -AR may inhibit stimulated leukotriene C4 secretion and eosinophil peroxidase (EPO, an eosinophil granule basic protein) release in purified human peripheral blood eosinophils (Munoz et al., 1994). Further evidence for the functional relevance of  $\beta$ -AR in eosinophils is provided by results obtained in a model of airway inflammation induced in anesthetized rats by injecting substance P or bradykinin intravenously, where activation of  $\beta_2$ -AR by the  $\beta_2$ -AR agonist formoterol was shown to reduce the amount of plasma leakage and also the number of neutrophils and eosinophils that adhered to the vascular endothelium at sites of inflammation, an effect which could be antagonized by the  $\beta_2$ -AR antagonist propranolol (Bowden Sulakvelidze and McDonald, 1994). Finally, EPO has been shown to decrease  $\beta$ -AR density on guinea pig lung membranes (Motojima et al., 1992). No information is available regarding the existence and the functional relevance on eosinophils of  $\alpha$ -AR as well as of  $\beta_1$ - or  $\beta_3$ -AR.

### Basophils

Clonidine, an  $\alpha_2$ -AR agonist used as antihypertensive, reduced stimulated secretion of histamine in human basophils, however the effect was inhibited by histamine receptor H2 blockers and not by  $\alpha_1$ -AR or  $\alpha_2$ -AR antagonists, thus a contribution of  $\alpha_2$ -AR could be ruled out (Miadonna et al., 1989). Recently, the antigenic activation of basophils isolated from the blood of atopic donors was shown to be decreased by adrenaline, an effect which was reduced by the  $\beta$ -AR antagonist propranolol (Mannaioni et al., 2010).

### Mast cells

Both ligand-binding studies and functional data suggest that IgE-mediated histamine release from mast cells may be inhibited by  $\beta$ -AR (Masini et al., 1982). Desensitization of  $\beta$ -AR on human lung mast cells may occur after prolonged exposure to  $\beta$ -AR

agonists like isoprenaline, an effect which is prevented by  $\beta$ -AR antagonists (Chong et al., 1995). Pharmacological evidence suggests that  $\beta$ -AR mediating inhibition of histamine release from human lung mast cells as well as from mast cells cultured from human peripheral blood are  $\beta_2$ -AR (Chong et al., 2002; Wang and Lau, 2006). The  $\beta_2$ -AR are also responsible in mast cells isolated from human intestinal mucosa for the inhibition exerted by adrenaline, noradrenaline, and salbutamol on IgE receptor-dependent release of histamine, lipid mediators, and TNF- $\alpha$ , as well as on proliferation, migration and adhesion to fibronectin and human endothelial cells (Gebhardt et al., 2005). Stem cell factor (SCF), the key human mast cell growth factor which primes mast cells for mediator release and markedly increases in asthmatic airways, profoundly reduces  $\beta_2$ -AR-mediated inhibition of histamine release from human lung mast cells, possibly through SCF-dependent phosphorylation of Tyr350 on the  $\beta_2$ -AR with immediate uncoupling of the receptor followed by receptor internalization (Cruse et al., 2010). At least one review exists about  $\beta_2$ -AR in human lung mast cells, including the influence of genetic polymorphisms in the  $\beta_2$ -AR gene (Kay and Peachell, 2005). A report also exists suggesting the existence of  $\alpha_1$ -AR in mast cells (Schulze and Fu, 1996) while recently Prey and coworkers shows that human mast cells are positive for the staining for both  $\beta_1$ -AR and  $\beta_2$ -AR while were negative for  $\beta_3$ -AR (Prey et al., 2014).

### Monocytes/macrophages

Between myeloid cells, monocyte/macrophages show different phenotypes, homeostatic turnover and functions in different tissues (Geissmann et al., 2010). Monocytes have long been considered as a developmental intermediate between bone marrow precursors and tissue macrophages. Monocytes and macrophages, together with DC, constitute the mononuclear phagocyte system which plays a key role maintaining tissue integrity during development and its restoration after injury, as well as the initiation and resolution of innate and adaptive immunity. Originally defined as bone marrow-derived myeloid cells circulating in the blood as monocytes and populating tissues as macrophages in the steady state and during inflammation, they have different phenotype, homeostatic turnover, and function in different tissues (Geissmann et al., 2010). Monocytes carry out specific effectors' functions during inflammation (De Kleer et al., 2014) and are usually classified in CD14++CCCD16- classical human monocytes or intermediates CD14++CCCD16+ cells.

**Monocytes** are endowed with chemokine receptors and PRR that modulate their migration from blood to tissues, where they produce proinflammatory cytokines and phagocytose cells and toxic molecules. Differentiation into DC or macrophages occurs during inflammation, and possibly in the steady state, depending on the inflammatory and PAMP/DAMP microenvironment (Serbina et al., 2008; Auffray et al., 2009).

**Macrophages** are resident phagocytic cells contributing to tissue homeostasis through clearance of apoptotic cells and production of growth factors. Macrophages are equipped with a broad range of pathogen recognition receptors that make them efficient at phagocytosis and induce production of inflammatory cytokines. Different subsets of macrophages occur in the various

tissues (including liver Kupffer cells, lung alveolar, splenic and peritoneal macrophages, dermal macrophages). Microglial cells are resident macrophages in the CNS. The specific origins and functions of all these subsets however still await thorough investigation (Yona et al., 2013). Macrophages are also major players in major disease such as cardiovascular disease (Swirski and Nahrendorf, 2013), cancer (Biswas and Mantovani, 2010), diabetes (Cnop et al., 2005).

The expression of  $\beta$ -AR on human monocytes has been documented by both ligand binding and flow cytometric studies, which also suggested that their density may be affected by physical exercise (Ratge et al., 1988; Fragala et al., 2011). Receptor desensitization has been reported in human monocytes at least *in vitro* after prolonged  $\beta_2$ -AR stimulation, possibly through up-regulation of cAMP phosphodiesterase activity (Manning et al., 1996).

The functional consequences of  $\beta$ -AR activation on human monocytes is usually antiinflammatory and immunosuppressive and includes: inhibition of oxygen radicals production (Schopf and Lemmel, 1983) upregulation of TNF receptors and inhibition of TNF (Guirao et al., 1997), reduction of *C. albicans* phagocytosis (Borda et al., 1998), inhibition of LPS-induced macrophage inflammatory protein-1  $\alpha$  (MIP-1  $\alpha$ ) (Li et al., 2003), as well as of LPS-induced IL-18 and IL-12 production (Mizuno et al., 2005). Thus, while noradrenaline and adrenaline may have antiinflammatory effects on human monocytes, reversal of their effects may result proinflammatory. As a consequence, the use of  $\beta_2$ -AR agonists has been suggested to be possibly beneficial in the treatment of sepsis through inhibiting LPS-elicited IL-18 (Mizuno et al., 2005), while recently the  $\beta_2$ -AR antagonist propranolol has been shown to reduce circulating immunosuppressive M2b monocytes in severely burned children, suggesting a role for this drug in severely burned patients to reduce their susceptibility to opportunistic infections (Kobayashi et al., 2011). Adrenergic modulation of monocytes may also contribute to explain the increased risk of viral infections following highly stressful events (e.g., herpes simplex virus type-1 and varicella zoster virus), due to activation of the sympathetic nervous system. For instance, catecholamines directly stimulate the human cytomegalovirus immediate-early (IE) enhancer/promoter in monocytic cells via  $\beta_2$ -AR, possibly leading to the development of an active human cytomegalovirus infection in latently infected patients (Prösch et al., 2000).

Adrenergic pathways in human monocytes may nonetheless result also in proinflammatory effects. Adrenaline may indeed increase monocyte attachment to laminin as well as oxidized-low density lipoprotein phagocytosis, two effects which both may be proinflammatory and atherogenic (Sarigianni et al., 2011). In particular, activation of  $\beta$ -AR have been shown, under certain conditions (e.g., in unstimulated cells), to lead to proinflammatory responses in monocytes, including: increased production of IL-18 (Takahashi et al., 2003), upregulation of IL-4-induced CD23 (low affinity IgE receptor/Fc epsilon RII) expression (Mencia-Huerta et al., 1991), and potentiated IgE/anti-IgE-induced production of IL-6 (Paul-Eugène et al., 1992, 1994), IgE (Paul-Eugène et al., 1993), increased generation of superoxide anion, nitric oxide, and TXB2 (Paul-Eugène et al.,

1994). LPS- or IL-1-stimulated human monocytes exposed to  $\beta_2$ -AR agonists may produce more antiinflammatory IL-10 as well as pro-inflammatory IL-8 (Kavelaars et al., 1997). Adrenaline may upregulate the surface expression of L-selectin (Rainer et al., 1999), and noradrenaline and adrenaline may increase matrix metalloproteinases (MMP)-1 in both circulating monocytes and monocyte-derived macrophages (Speidl et al., 2004). The  $\beta$ -AR agonist isoprenaline may decrease the response to LPS but *per se* result in increased phorbol ester-induced production of TNF- $\alpha$ , IL-12, and nitric oxide (Szelenyi et al., 2006). Isoprenaline may also increase LPS-induced production of IL-1 $\beta$ , possibly through the activation of  $\beta_1$ -AR, which were directly identified by immunoblot techniques as well as by radioligand binding studies in the monocytic cell line THP-1 (Grisanti et al., 2010). Recently,  $\beta_1$ -AR autoantibodies isolated from the sera of heart failure patients were shown to cause (TNF- $\alpha$ ) secretion from the murine macrophage-like cell line RAW264.7 (Du et al., 2012).

Early pharmacological evidence suggested the occurrence of  $\alpha$ -AR in human monocytes enhancing the synthesis of complement components (Lappin and Whaley, 1982). Culturing human circulating monocytes with dexamethasone or the  $\beta_2$ -AR agonist terbutaline may indeed trigger the expression of  $\alpha_{1B}$ - and  $\alpha_{1D}$ -AR mRNA (Rouppé van der Voort et al., 1999). LPS may result in a similar effect, possibly through the activation of ERK-2 (Rouppé van der Voort et al., 2000). The proinflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$  respectively upregulate and reduce  $\alpha_{1B}$ - and  $\alpha_{1D}$ -AR mRNA in the human THP-1 monocytic cell line (while IL-6 and IL-8 seem to be ineffective) (Heijnen et al., 2002). Recently, on monocytes a homogenous  $\alpha_{1B}$ -AR subtype population was identified, which changed to a heterogeneous receptor subtype expression pattern when differentiated to macrophages. The agonist phenylephrine synergistically increased LPS-induced IL-1 $\beta$  production and this effect was blocked in the presence of a selective  $\alpha_1$ -AR antagonist as well as of inhibitors of PKC, suggesting the occurrence on human monocytes of  $\alpha_1$ -AR mediating proinflammatory responses (Grisanti et al., 2011). Differentiation of human monocytes into macrophages may result, at least *in vitro*, in loss of  $\beta$ -AR responsiveness despite a functional adenylyl cyclase system (Baker and Fuller, 1995). Expression of  $\beta$ -AR on human macrophages is actually regulated upon activation in a stimulus-dependent manner, thus that changes in receptor number may occur in different states of cell maturation and function (Radojcic et al., 1991). Remarkably, as human monocytes adhere to surfaces and begin differentiation into macrophages, they may lose their surface  $\beta_2$ -AR and hence become insensitive to the inhibitory effects of  $\beta_2$ -AR agonists on LPS-induced TNF- $\alpha$  production, an observation which has been related to the lack of anti-inflammatory effect of  $\beta_2$ -AR agonists on alveolar macrophages or in clinical asthma (Ezeamuzie et al., 2011). Activation of  $\beta_2$ -AR may inhibit the production of TNF- $\alpha$  and of IL-6 and increase the production of IL-10 in PMA-differentiated U937 human macrophages (Izeboud et al., 1999), however care should be exerted when using these cells to study the physiopharmacology of  $\beta$ -AR in human monocytes/macrophages, since  $\beta_2$ -AR, which are the main subtype of  $\beta$ -AR expressed by these cells, exhibit lower expression on undifferentiated (monocytes) than

in PMA-differentiated U937 (macrophages) (Izeboud et al., 1999). Quite interestingly, it has been proposed that in severe sepsis priming via gut-derived noradrenaline may contribute to increased release in pro-inflammatory cytokines from Kupffer cells, ultimately leading to organ dysfunction. Such an effect, which has been tentatively defined “sympathetic excitotoxicity in sepsis,” could be mediated  $\alpha_2$ -AR on Kupffer cells (Miksa et al., 2005). Recently, in a porcine model of wound healing, both macrophage infiltration and angiogenesis were initially decreased, whereas dermal fibroblast function was impaired after treatment with  $\beta_2$ -AR agonists, suggesting the potential of these drugs to improve skin scarring (Le Provost and Pollar, 2015). Interestingly, human monocyte-derived macrophages of stressed subjects displayed decreased superoxide anion-responses after stress, in direct correlation with higher plasma noradrenaline, and noradrenaline-treated cells showed reduced superoxide anion-production, an effect blocked by prior incubation with the  $\alpha$ -AR antagonist phentolamine (Kuebler et al., 2013). Similarly, evidence about a negative effect of stress was recently shown in models of tumor in which increased levels of catecholamines contributes to the recruitment of macrophages and to the increased activation of these cells in the tumor environment (Armaiz-Pena et al., 2015). In addition negative effects were elucidated by the sympathetic activation on cancer progression. For example, metastasis in breast cancer, in mouse, was more pronounced after stress and this effects were mimicked by  $\beta$ -AR stimulation (Sloan et al., 2010).

Finally, it has been reported that, at least *in vitro*,  $\alpha_2$ -AR stimulation of type A (macrophage-like) and B (fibroblast-like) synoviocytes produced an increase and a decrease in the respective cell number, probably through Gi-coupled PLC activation and the resulting stimulation of the PKC betaII/MAP kinase (Mishima et al., 2001), providing preliminary evidence for a role of  $\alpha_2$ -AR regulating local innate immunity in synovial tissues.

In summary, consistent evidence supports the occurrence of  $\beta$ -AR on human monocytes/macrophages:  $\beta_2$ -AR are usually regarded as mainly antiinflammatory, although under certain conditions they can result in proinflammatory effects, while recent evidence suggests also the occurrence of  $\beta$ -AR (possibly  $\beta_1$ -AR)-mediated proinflammatory responses (Grisanti et al., 2010). Alpha-AR can also occur upon appropriate stimulation and may mediate both pro- and antiinflammatory responses, but defining their functional role still requires careful investigation.

According to evidence obtained in rodent cells, monocytes/macrophages may also produce and utilize noradrenaline and adrenaline as local transmitters. Indeed, Spengler et al. (1994) showed in mouse peritoneal macrophages stimulated with LPS that the  $\beta$ -AR selective antagonist propranolol increased (and the  $\alpha_2$ -AR selective antagonist idazoxan decreased) TNF- $\alpha$  production. In the same study, the presence of intracellular noradrenaline was also reported and interpreted as an evidence supporting the existence of an adrenergic autocrine loop, possibly even more pronounced in macrophages obtained from rats with streptococcal-cell-wall-induced arthritis (Chou et al., 1998). Further support to the possibility that macrophages may produce endogenous

catecholamines has been provided by Nguyen et al. (2011), who showed that exposure of mice to cold temperature rapidly promoted alternative activation of adipose tissue macrophages, which secreted catecholamines to induce thermogenic gene expression in brown adipose tissue and lipolysis in white adipose tissue. Absence of alternatively activated macrophages impaired metabolic adaptations to cold, whereas administration of IL-4 increased thermogenic gene expression, fatty acid mobilization, and energy expenditure, all in a macrophage-dependent manner (Nguyen et al., 2011). Finally, it should be mentioned that MAO type A is expressed in human monocytes in particular after incubation with IL-4, and upregulation of its expression may contribute in switching naive monocytes into a resolving phenotype, indirectly highlighting another potential role for endogenous adrenergic pathways in these cells (Chaitidis et al., 2004, 2005).

### Dendritic Cells

DC are specialized antigen-processing and presenting cells, with high phagocytic activity as immature cells and high cytokine producing capacity as mature cells. DC circulate in blood and migrate from tissues to lymphoid organs, regulating T cell responses both in the steady-state and during infection (Mellman and Steinman, 2001; De Kleer et al., 2014). Remarkably, DC found in the epidermis are the Langerhans cells, which derive from the bone marrow, under steady-state conditions are maintained locally, but during skin inflammation may be replaced by blood-borne progenitors (Merad et al., 2002).

Few information exists regarding adrenergic pathways in human DC. In CD40-stimulated human DC, activation of  $\beta_2$ -AR increases intracellular cAMP and inhibits IL-12 production, resulting in inhibition of Th1 and promotion of Th2 differentiation (Panina-Bordignon et al., 1997). In human DC obtained from cord blood CD34+ precursor cells, noradrenaline acting through  $\beta_2$ -AR and increased cAMP inhibits LPS-stimulated production of IL-23, IL-12 p40, TNF- $\alpha$ , and IL-6 without affecting IL-10 (Goyarts et al., 2008). This response is similar to that obtained in mouse skin DC (Maestroni, 2005, 2006), thus suggesting that noradrenaline may regulate human skin DC function resulting in decreased Th1 differentiation of CD4+ T cells. Indirect evidence for adrenergic regulation of human DC comes also from a study showing in 18 professional athletes a correlation between the increase of peripheral blood DC after intensive physical activity and serum adrenaline and noradrenaline levels (as well as with the extent of heart rate elevation during exercise) (Suchánek et al., 2010). Recently, adrenaline was shown to inhibit migration of human DC through  $\beta_1$ -AR signaling through arrestin2-PI3K-MMP9/CCR7 (Yang et al., 2013) and that DC functions are strongly affected by catecholamines (probably  $\beta_2$ -AR mediated) that induces a profound suppression of the production of proinflammatory cytokines (Nijhuis et al., 2014).

Most of the information so far available regarding adrenergic pathways in DC has been obtained in murine DC, where AR mediate sympathetic nervous system influences on DC-T cells interactions contributing to the shaping of the appropriate adaptive immune response (reviewed by Maestroni, 2005, 2006). Both  $\alpha_1$ - and  $\beta_2$ -AR are expressed on murine DC:  $\alpha_1$ -AR

stimulate DC migration, which on the contrary is inhibited by  $\beta_2$ -AR. Noradrenaline decreases IL-12 and increases IL-10 production in both skin and bone marrow-derived DC stimulated with bacterial TLR agonists and, as a consequence, DC-induced T helper (Th) 1 priming is impaired. Such observations may explain how reduced noradrenaline activity in the skin may promote contact sensitizers-induced Th1 responses (Maestroni, 2004). Noradrenaline also activates  $\beta_2$ -AR-mediated cAMP-PKA pathways to enhance DC production of IL-33, resulting in direct Th2 differentiation and possibly contributing to the stress-related progression of Th2-associated disorders (Yanagawa et al., 2011). Sympathoadrenergic modulation of the skin innate and adaptive immune response occurring after stimulation with TLR2 (but not TLR4) agonists may promote a Th1 adaptive response possibly relevant to Th1-sustained autoimmune inflammatory skin diseases (Manni and Maestroni, 2008). In agreement with these findings, the  $\beta_2$ -AR agonists salbutamol may bias DC preexposed to TLR-2 and NOD2 agonists toward increasing the Th17/Th1 cell ratio finally resulting in an IL-17 immune response, which may be relevant in defense against extracellular bacteria, in the pathogenesis of inflammatory diseases and for the antitumor response (Manni et al., 2011). Adrenaline however was also shown to lead bone marrow-derived murine DC to generate a dominant Th2/Th17 phenotype, possibly through the activation of  $\beta_2$ -AR (Kim and Jones, 2010). Nonetheless, recently it was shown that  $\beta_2$ -AR agonist-exposed mature murine DC displayed a reduced ability to cross-present protein antigens while retaining their exogenous peptide presentation capability, an effect which could be mediated through a nonclassical inhibitory G (G $\alpha$ i/0) protein. Inhibition of cross-presentation was neither due to reduced costimulatory molecule expression nor antigen uptake, but rather to impaired phagosomal antigen degradation. A crosstalk between the TLR4 and  $\beta_2$ -AR transduction pathways at the NF- $\kappa$ B level was also described, and *in vivo* treatment with a  $\beta_2$ -AR agonist resulted in inhibition of antigen protein cross-presentation to CD8+ T cells, however with preservation of their exogenous major histocompatibility complex (MHC) class I peptide presentation capability (Hervé et al., 2013).

Recently, pharmacological evidence was also provided for the occurrence on murine DC of  $\alpha_2$ -AR, which may mediate enhancement of antigen capture, possibly contributing to explain immune enhancement following acute stress (Yanagawa et al., 2010). Dexmedetomidine, a highly-selective  $\alpha_2$ -AR agonist, has been recently shown to affect murine bone marrow-derived DC, delaying the intracellular proteolytic degradation of ovalbumin, decreasing the expression of the surface molecules I-A(b) and CD86, and suppressing Th-cell proliferation. Dexmedetomidine also suppressed DC migration, and vaccination of animals with dexmedetomidine-treated DC significantly suppressed the contact hypersensitivity reaction *in vivo* (Ueshima et al., 2013).

AR-dependent modulation of DC may be relevant also to cancer vaccine strategies. Botta and Maestroni (2008) found that  $\beta_2$ -AR antagonism along with TLR2 activation at the site of intradermal cancer vaccination may either enhance the resulting antitumor response or be tolerogenic in dependence of the maturation state of the transferred DC. Manipulation of  $\beta_2$ -AR

expressed in the site of DC inoculation may thus profoundly influence the efficacy of the subsequent antitumor response.

### Natural Killer Cells

NK cells, like other innate immune cells, were described as nonspecific in their interactions with tumors or virus-infected cells, however it is now well-defined that they express a repertoire of inhibitory receptors (some specific for MHC class I, others binding non-MHC ligands) that regulate their activation. NK cells also express activating receptors, and their complex interplay with inhibitory receptors is a matter of intense investigation. NK cells circulate through the blood, lymphatics and tissues, patrolling the body for the presence of transformed or pathogen-infected cells (Yokoyama, 2005; Lanier, 2008; Chijioka and Münz, 2013).

Human NK cells express high levels of  $\beta$ -AR. The highest number of  $\beta$ -AR was found in CD16+CD56+ NK cells, and it was even increased after physical exercise (Maisel et al., 1990). Noradrenaline and adrenaline decrease NK cell cytotoxicity through the activation of  $\beta$ -AR (likely  $\beta_2$ -AR) (Whalen and Bankhurst, 1990; Takamoto et al., 1991), however adrenaline may also stimulate NK cell cytotoxicity at lower (submicromolar-picomolar) concentrations (Hellstrand et al., 1985). *In vitro*,  $\beta_2$ -AR activation on NK cells reduces cell adhesion to endothelial cells (Benschop et al., 1994, 1997), and in human subjects administration of both adrenaline and noradrenaline modulates the migratory capacity of human NK cells via spleen-independent  $\beta_2$ -AR mechanism (Schedlowski et al., 1996; Benschop et al., 1997). Nonetheless, evidence for  $\beta$ -AR-dependent increase of NK cytotoxicity has been obtained in rats treated with amphetamine (Glac et al., 2006) and more recently it has been shown that also repeated social disruption in mice "primes" NK cells in the spleen and lung to be more proficient in their cytolytic and anti-viral/tumor effector functions through  $\beta$ -AR activation (Tarr et al., 2012). Also in light of such evidence, the possibility that the previously reported  $\beta$ -AR-dependent decreased NK cells function may arise from methodological issues has been the subject of an interesting commentary (Ben-Eliyahu, 2012). Nonetheless, a recent *in vitro* study which screened 1200 in-use or previously approved drugs for their biological effect on freshly isolated human peripheral blood-derived NK cells included  $\beta_2$ -AR agonists among the confirmed inhibitors (Theorell et al., 2014) and that in general acute administration of catecholamines (mimicking a stress condition) *in vivo*, through the interaction with  $\beta$ -AR, suppress NK activity (Rosenne et al., 2014).

Human NK cells also express  $\alpha$ -AR. In CD16+ lymphocytes,  $\beta_2$ -,  $\alpha_1$ -,  $\alpha_2$ -AR (but not  $\beta_1$ -AR) were identified, and infusion of adrenaline (but not noradrenaline) significantly decreased all AR on NK cells (Jetschmann et al., 1997). Recently, rat NK cells were shown to express both  $\alpha_1$ - and  $\alpha_2$ -AR. Activation of either subtypes of  $\alpha$ -AR augmented NK cytotoxicity,  $\alpha_1$ -AR possibly signaling through PLC, while  $\alpha_2$ -AR effect through PKA (Xiao et al., 2010).

Decreased NK activity induced by activation of  $\beta$ -AR is presently considered among the main mechanisms responsible for cancer progression associated with stressful conditions resulting in activation of the sympathetic nervous system

(Shakhar and Ben-Eliyahu, 1998; Ben-Eliyahu et al., 2000; Page and Ben-Eliyahu, 2000). Noradrenaline however may also inhibit the generation of specific antitumor cytotoxic T lymphocytes (Kalinichenko et al., 1999), and even chemical denervation may lead to tumor growth (Brenner et al., 1992), thus suggesting a complex role of the sympathetic nervous system in the regulation of antitumor immunity.

In animal models, activation of the sympathoadrenergic system through either stressful events or direct stimulation of  $\beta$ -AR usually leads to compromised resistance to tumor development and metastasis (Stefanski and Ben-Eliyahu, 1996; Shakhar and Ben-Eliyahu, 1998). In a mouse model of restraint stress, plasma adrenaline significantly rose immediately after the release from restraint, while NK cells were decreased in the lungs and blood but not in the spleen. Decreased number of NK cells in the lungs and blood was reversed by the  $\beta$ -AR antagonist propranolol, suggesting that acute stress reduces the number of intraparenchymal lung NK cells via activation of  $\beta$ -AR receptors (Kanemi et al., 2005). Impairment of NK activity and reduced antitumor resistance due to stress and  $\beta$ -AR stimulation is affected by age (Page and Ben-Eliyahu, 2000) and by gender (Page et al., 2008). Administration of type-C CpG oligodeoxynucleotides (CpG-C ODN) was shown to improve NK activity and immunocompetence, potentially reducing metastatic dissemination after enhanced sympathetic stress responses (Goldfarb et al., 2009), and it was proposed to limit postoperative immunosuppression and metastatic progression in association with pharmacological blockade of  $\beta$ -AR and cyclooxygenase (COX) inhibition (Goldfarb et al., 2011). Blockade of  $\beta$ -AR in association with COX inhibitors have been recently proposed even in patients with hematological malignancies, based on results obtained in animals and showing that endogenous adrenaline together with prostaglandins may mediate the promoting effects of stress on leukemia progression through suppression of NK activity (Inbar et al., 2011).

Epidemiological studies support the hypothesis that exposure to  $\beta_2$ -AR antagonists may indeed reduce cancer progression and mortality, e.g., in melanoma (De Giorgi et al., 2011) and in breast cancer (Powe et al., 2010), although conflicting results have also been reported (Shah et al., 2011; Choi et al., 2014). Whether such effects are related to  $\beta$ -AR-induced suppression of NK antitumor activity and/or to other effects of adrenaline and noradrenaline on antitumor immunity and on tumor biology is still a matter of debate. In any case, well-designed randomized clinical trials are needed for several cancer types to establish the potential of AR manipulation as antitumor therapy.

### $\gamma\delta$ T Lymphocytes

Gamma delta ( $\gamma\delta$ ) T lymphocytes are unconventional T cells that, like NK cells, functionally and phenotypically belong to both the innate and the adaptive immune system and represent a connection between the two. They represent about 1–10% of circulating T cells (and even 50% at some mucosal sites), and are involved in the defense against infectious diseases as well as in the inhibition of tumor development and progression (Carding and Egan, 2002). Recently, one study compared lymphocytosis in response to an acute speech stress task, high

and low intensity concentric exercise, and isoproterenol infusion at two different doses, showing that  $\gamma\delta$  T lymphocytes were mobilized in response to all three tasks in a dose-dependent manner, and that their mobilization was greater than that of CD8+ T lymphocytes and less than NK cells. The authors suggest that mobilization of  $\gamma\delta$  T lymphocytes may provide protection in the context of situations in which antigen exposure is more likely to occur (Anane et al., 2009). Another study in healthy subjects using infusion of adrenaline at physiological concentrations confirmed such results, showing that  $\gamma\delta$  T cells, together with CCR7–CD45RA+CD8+ effector T cells, CD3+CD56+ NKT-like cells, CD16+CD56dim cytotoxic NK cells, and CD14dimCD16+ proinflammatory monocytes, show a rapid and transient increase after adrenaline. The proposed mechanism is adrenaline-induced attenuation of cell attachment to endothelium and subsequent demargination and release into the circulation to provide immediate protection from invading pathogens (Dimitrov et al., 2010).

### **Microglia**

The first definition of the role of microglia in CNS was provided by Pio del Rio-Hortega in 1932 who in the work entitled “Cytology and cellular pathology of the nervous system” described the role and effects of these cell population into the brain (Del Rio-Hortega, 1932). In the CNS, microglia are resident mononuclear phagocytes involved mainly in immune responses and inflammatory diseases, which originate during embryogenesis from the yolk sac and enter the CNS quite early in the life of embryos. In a more recent paper, the complex physiology of these cells was widely clarified (Kettenmann et al., 2011) and in this review we can find informations about the presence of A-DR and several other receptors. Microglial cells play an important role in managing neuronal cell death, neurogenesis, and synaptic interactions, and they contribute to T-cell activation within the CNS (Katsumoto et al., 2014).

Adrenergic pathways have never been investigated in human microglia, therefore available evidence so far regards murine models. In murine microglia, by means of microarray and immunohistochemistry,  $\beta_2$ -AR and possibly  $\beta_1$ -AR and  $\alpha_2$ -AR have been identified (Hertz et al., 2010).  $\beta$ -AR activation increases the production of IL-1 $\beta$ , TNF- $\alpha$ , and IL-6 through cAMP and cAMP-dependent protein kinase (Tomozawa et al., 1995) as well as ERK1/2 and P38 MAPK (Wang et al., 2010). Nonetheless, noradrenaline acting on  $\beta$ -AR may also induce IL-1ra and IL-1 type II receptor expression in enriched cultures of murine microglia, thus protecting cortical neurons against IL-1 $\beta$ -induced neurotoxicity (McNamee et al., 2010), and exposure to both  $\beta_1$ - and  $\beta_2$ -AR agonists decreased TNF- $\alpha$ , IL-6 and monocyte chemoattractant protein-1 production, prevented microglia activation, reduced inflammation and exerted neuroprotective effects in LPS-treated murine hippocampal slices (Markus et al., 2010). Both noradrenaline and isoprenaline promote amyloid  $\beta$  peptide uptake and degradation by murine microglial cells through activation of  $\beta_2$ -AR, thus providing a potential link between central noradrenergic neurotransmission and neuroinflammatory mechanisms in Alzheimer's disease (Kong

et al., 2010). Preliminary evidence obtained in human microglia-like (THP-1) cells seems to confirm the antiinflammatory and neuroprotective role of noradrenaline in Alzheimer's disease pathology. In these cells, noradrenaline suppressed A $\beta$ 1-42-mediated cytotoxicity and MCP-1 secretion, while enhancing A $\beta$ -mediated IL-1 $\beta$  secretion through action at  $\beta_2$ -AR, and activation of cAMP/PKA pathway and CREB (Yang et al., 2012). As regards  $\alpha$ -AR, no information exists with the exception of a recent study in a rat model of monoarthritis, where it was shown that spinal glia, as well as dorsal root ganglion primary afferent neurons, express  $\alpha_2$ -AR and that the  $\alpha_2$ -AR agonist dexmedetomidine exerted analgesic effects involving the blockade of spinal glial activation (Xu et al., 2010).

### **Astrocytes**

The term astrocytes includes at least two main categories of cells that can be divided according to their morphology and anatomical localization: protoplasmic cells and fibrous cells (Sofroniew and Vinters, 2010). The first type are present in the gray matter and were the first type of astrocytes identified by means of silver impregnation. Fibrous astrocytes are localized into the white matter and are quite different in the morphology. At present we know that astrocytes are the most abundant and heterogeneous neuroglial cells, their functions including participating in the formation of the blood-brain barrier and regulation of blood flow (by releasing several molecular mediators such as prostaglandins, nitric oxide, and arachidonic acid), maintaining the ion, pH, and transmitter homeostasis of the synaptic interstitial fluid, sensing transmitter release at the synaptic cleft and possibly releasing gliotransmitters, defending the CNS from all types of insults and disease through reactive gliosis. Astrocytes may also contribute to neuroinflammation upon severe challenges by releasing pro-inflammatory molecules (e.g., TNF- $\alpha$ , IL-1, IL-6) and possibly by contributing to antigen presentation under autoimmune response, although this latter function needs further investigation (Kimmelberg and Nedergaard, 2010; Endo et al., 2015).

The main AR expressed by human astrocytes is the  $\beta_2$ -AR, which regulates glycogen metabolism, immune responses, release of neurotrophic factors, as well as astrogliosis in response to neuronal injury. Astrocytic  $\beta_2$ -AR are potent regulators of astrocytic TNF- $\alpha$ -activated genes, including IL-6, CXCL2, CXCL3, VCAM1, and ICAM1 expression, and in rats co-administration of the  $\beta_2$ -AR agonist clenbuterol and TNF- $\alpha$  skewed the T cell population toward a double negative phenotype and induced a shift in the myeloid brain cell population toward a neutrophilic predominance, suggesting that astrocytic  $\beta_2$ -AR and their downstream signaling pathway may serve as potential targets to modulate neuroinflammatory responses (Laureys et al., 2014). Nonetheless,  $\beta$ -AR stimulation together with TNF-receptor triggering may also induce synergistic IL-6 expression in astrocytes, which may contribute to neurodegeneration and glioma development (Spooren et al., 2011). Downregulation of the astrocytic  $\beta_2$ -AR-pathway has been proposed to contribute to several neurological conditions such as multiple sclerosis, Alzheimer's disease, human immunodeficiency virus encephalitis, stroke, and hepatic

encephalopathy (Laureys et al., 2010). In particular, regarding multiple sclerosis, available evidence indicates that  $\beta_2$ -AR are decreased in astrocytes of patients, both in normal-appearing white matter as well as in chronic active and inactive plaques (De Keyser et al., 1999; Zeinstra et al., 2000), and it has been proposed that in this disease astrocytes may serve as primary (facultative) antigen-presenting cells due to a failure of  $\beta_2$ -AR-mediated suppression of MHC II molecules (De Keyser et al., 2003). Astrocyte  $\beta_2$ -AR dysregulation however may contribute to pathogenesis and progression of multiple sclerosis also through deficient inhibition of nitric oxide and proinflammatory cytokine production and glutamate uptake, as well as through deficient glycogenolysis and production of trophic factors (De Keyser et al., 2004), and reduced perfusion of normal-appearing white matter (De Keyser et al., 2008). Astrocytes as therapeutic targets in multiple sclerosis were challenged in a proof of concept clinical study by use of fluoxetine, which activates PKA in astrocytes. PKA is physiologically activated by  $\beta_2$ -AR-mediated cAMP increase and in turn suppresses coactivator class II transactivator, which regulates MHC class II molecule transcription (De Keyser et al., 2010). Direct activation of PKA could in principle bypass the functional deficiency of astrocytes, however preliminary results need to be confirmed and extended in larger, randomized studies.

No information exists regarding  $\alpha$ -AR, with the exception of a study showing the occurrence of  $\alpha_1$ -AR in astrocytes from human optic nerves (Mantyh et al., 1995). Interestingly, the human U373 MG astrocytoma cell line express  $\alpha_{1B}$ -AR coupled to phosphoinositide hydrolysis and calcium mobilization, which mediate a mitogenic response to  $\alpha_1$ -AR-agonists (Arias-Montañó et al., 1999).

## Conclusions and Perspectives

Although adrenergic pathways represent the main channel of communication between the nervous system and the immune system, their role has received more attention as regards modulation of adaptive immunity (Elenkov et al., 2000; Cosentino and Marino, 2013; Marino and Cosentino, 2013), in comparison to innate immunity.

Consistent evidence however indicates that adrenergic mechanisms play a significant role even in immune cells. In particular, in human neutrophils migration, CD11b/CD18 expression, and oxidative metabolism are inhibited possibly through  $\beta$ -AR, although a contribution by  $\alpha_1$ - and  $\alpha_2$ -AR cannot be discarded. Inhibitory  $\beta$ -AR may occur also on NK cells, which also express  $\alpha$ -AR with undefined functional role. Monocytes express  $\beta$ -AR which are usually antiinflammatory, even if in certain conditions proinflammatory responses may arise. Murine DC express  $\beta$ -AR which modulate DC-T cells interactions, while in human DC  $\beta_2$ -AR may affect Th1/2 differentiation of CD4+ T cells.  $\beta_2$ -AR dysregulation in microglia and astrocytes

may contribute to neuroinflammation in autoimmune and neurodegenerative disease. As a whole, the main AR expressed on innate immune cells are  $\beta$  (possibly  $\beta_2$ )-AR, although  $\alpha$ -AR may occur on selected cell types and under specific conditions. Further studies however are needed to define the functional significance of  $\alpha$ -AR-mediated influence on the innate immune response.

On these basis, evaluation of  $\beta$ -AR agonists as potential antiinflammatory drugs is strongly warranted. Agonists of  $\beta_2$ -AR are currently used as bronchodilating agents in asthma, however the relative contribution of any eventual immunomodulating activity of these drugs to their overall therapeutic effects remains to be established. In addition, their usefulness in different inflammatory conditions such as atherosclerosis, where neutrophils are emerging key players (Marino et al., 2015), should be carefully considered. On the other side,  $\beta$ -AR antagonists might enhance the innate immune response, and therefore their usefulness could be evaluated e.g., in the potentiation of antitumor immunity. The possible immune effects of  $\alpha$ -AR ligands require additional investigation.

Issues awaiting clarification include AR expression and function in the various innate immune cells subtypes, as well as their effects on the humoral innate immune system (complement, antibacterial peptides). In particular, no information is yet available on innate immune cells which have been recently discovered and characterized, such as ILC (Spits et al., 2013) and myeloid-derived suppressor cells (MDSC) (Gantt et al., 2014).

Circumstantial evidence also suggests the opportunity to apply a pharmacogenetic approach to better understand adrenergic modulation of the immune response and in particular of innate immunity. For instance, individuals who were homozygous for  $\beta_2$ -AR Arg16 had higher levels of specific IgE to *Ascaris lumbricoides*, higher *A. lumbricoides* egg counts, and larger wheal sizes following skin-prick testing with *A. lumbricoides* allergen (Ramsay et al., 1999), and inhibition of IgE-mediated release of histamine from human lung mast cells is more resistant to desensitization when the  $\beta_2$ -AR bears mutant (gly16 and glu27) forms compared to wild-type (arg16 and gln27) forms (Chong et al., 2000).

Nonetheless, the present knowledge about the adrenergic modulation of innate immunity already supports relevant therapeutic applications, such as the use of  $\alpha_2$ -AR antagonists in acute lung injury (Flierl et al., 2007, 2009), as well as of  $\beta$ -AR antagonists to reduce the risk of opportunistic infections in severely burned patients (Kobayashi et al., 2011). Moreover, as adrenergic pathways also provide a link between stressful events and chronic inflammatory disease such as atherosclerosis (Heidt et al., 2014), investigating neuroimmune adrenergic mechanisms will likely provide several opportunities to repurpose the wide array of sympathoadrenergic agents currently used in medical therapy for various non-immune indications for novel and previously unanticipated indications.

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# Pharmacological Intervention in Hepatic Stellate Cell Activation and Hepatic Fibrosis

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The activation and transdifferentiation of hepatic stellate cells (HSCs) into contractile, matrix-producing myofibroblasts (MFBs) are central events in hepatic fibrogenesis. These processes are driven by autocrine- and paracrine-acting soluble factors (i.e., cytokines and chemokines). Proof-of-concept studies of the last decades have shown that both the deactivation and removal of hepatic MFBs as well as antagonizing profibrogenic factors are in principle suitable to attenuate ongoing hepatic fibrosis. Although several drugs show potent antifibrotic activities in experimental models of hepatic fibrosis, there is presently no effective pharmaceutical intervention specifically approved for the treatment of liver fibrosis. Pharmaceutical interventions are generally hampered by insufficient supply of drugs to the diseased liver tissue and/or by adverse effects as a result of affecting non-target cells. Therefore, targeted delivery systems that bind specifically to receptors solely expressed on activated HSCs or transdifferentiated MFBs and delivery systems that can improve drug distribution to the liver in general are urgently needed. In this review, we summarize current strategies for targeted delivery of drugs to the liver and in particular to pro-fibrogenic liver cells. The applicability and efficacy of sequestering molecules, selective protein carriers, lipid-based drug vehicles, viral vectors, transcriptional targeting approaches, therapeutic liver- and HSC-specific nanoparticles, and miRNA-based strategies are discussed. Some of these delivery systems that had already been successfully tested in experimental animal models of ongoing hepatic fibrogenesis are expected to translate into clinically useful therapeutics specifically targeting HSCs.

**Keywords:** liver disease, drug targeting, gene therapy, differential expression, biomedicine, antifibrotic, fibrogenesis, translational medicine

## CHARACTERIZATION AND SIGNIFICANCE OF LIVER FIBROSIS

Hepatic fibrosis is a naturally occurring wound healing reaction, driven by inflammation, which in turn is caused by persistent parenchymal injury. The main causes of hepatic damage are chronic hepatitis B and hepatitis C virus infections, alcohol abuse, biliary obstruction, autoimmune disease, hereditary haemochromatosis, and NAFLD (Ramachandran and Iredale, 2012). Untreated liver fibrosis ultimately progresses to cirrhosis and increases the risk of developing HCC. Both hepatic fibrosis and cirrhosis are crucial causes of global morbidity and mortality (Iredale, 2007) and the incidence of advanced fibrosis in a cohort of 7000 persons was roughly estimated to be 2.8% (Pynard et al., 2010). Because there are no effective antifibrotic treatments available, there is an urgent need to develop such interventions, particularly given the progressive character and high prevalence of this disease.

The liver is made up of parenchymal and non-parenchymal cells (PCs). Hepatocytes are the only PC type, whereas KCs, LSECs, HSCs, also referred to as Ito or fat storing cells, and biliary epithelial cells belong to the group of non-PCs (Kmiec, 2001; Chen and Sun, 2011). In addition, the liver is enriched with a large variety of immune cells, including circulating intrahepatic lymphocytes and liver resident dendritic cells (Racanelli and Rehermann, 2006). In regard to hepatic fibrosis, HSCs are of particular importance, because this cell type is the source of the majority of MFBs (Povero et al., 2010). In their quiescent state, HSCs store vitamin A and retinoids, but upon tissue damage they undergo a transition to MFBs, a process that is commonly known as transdifferentiation. This process is induced by soluble mediators, including TGF- $\beta$ 1, PDGF, and TNF (Schon and Weiskirchen, 2014). After injury, these factors are released by hepatocytes, by activated KCs (Cubero and Nieto, 2006) and by inflammatory monocyte-derived macrophages (Zimmermann et al., 2010). The activated MFBs are highly proliferative, have the ability to migrate, and synthesize components of the ECM

**Abbreviations:**  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin; Ad, adenovirus; BDNF, brain-derived growth factor; BiPPB, bicyclic PDGF $\beta$ R-binding peptide; BSA, bovine serum albumin; CAR, coxsackievirus-adenovirus receptor; CCl<sub>4</sub>, carbon tetrachloride; CREBBP, CREB binding protein; CTGF, connective tissue growth factor; DMN, dimethylnitrosamine; DOX, doxorubicin; ECM, extracellular matrix; EMT, epithelial-to-mesenchymal transition; GFP, green fluorescent protein; HCC, hepatocellular carcinoma; HGF, hepatocyte growth factor; HSA, human serum albumin; HSC(s), hepatic stellate cell(s); IFN- $\gamma$ , interferon- $\gamma$ ; IL10, interleukin-10; KC(s), Kupffer cell(s); LAP, latency-associated peptide; LOXL2, lysyl oxidase like 2; LSEC(s), liver sinusoidal endothelial cell(s); M6P, mannose 6-phosphate; M6P/IGFII, mannose 6-phosphate/insulin-like growth factor II; MFB(s), myofibroblast(s); mim  $\gamma$ , IFN- $\gamma$  peptidomimetic; miR, miRNA(s); MMP, matrix metalloproteinase; MPA, mycophenolic acid; NGF, nerve growth factor; MSCs, mesenchymal stem cells; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NT, neurotrophin; OM, Oxymatrine; p75NTR, p75 neurotrophin receptor; PDGF, platelet-derived growth factor; PDGF $\beta$ R, PDGF receptor type  $\beta$ ; PEG, polyethylene glycol; PLGA, poly(lactic-co-glycolic acid); PPAR $\gamma$ , peroxisome proliferator-activated receptor  $\gamma$ ; PPB, PDGF $\beta$ R-recognition cyclic peptide; PTEN, phosphatase and tensin homolog; PTX, pentoxifylline; RBP, retinol-binding protein; SSLs, sterically stable liposomes; TFO(s), triplex forming oligonucleotide(s); TGF, transforming growth factor; TIMP, tissue inhibitor of metalloproteinase; TNF, tumor necrosis factor; Trk, tropomyosin-related kinase; TSP-1, thrombospondin-1.

including collagen type I and type III, the latter being central to hepatic fibrogenesis. In this scarring process, the altered non-functional connective tissue replaces functional liver tissue. In addition to their activation by cytokines, HSCs maintain and even potentiate liver fibrosis through the effect of numerous autocrine feedback loops, thereby substantially contributing to the progression of the disease (Poelstra et al., 2012).

Similarly, portal fibroblasts that are located in portal areas are able to acquire an MFB-like phenotype and play a particularly important role in biliary fibrosis (Povero et al., 2010). Furthermore, bone marrow-derived stem cells, such as MSCs and circulating fibrocytes, can differentiate into MFBs. The cellular fraction of profibrogenic cells is further increased by hepatocytes and cholangiocytes that both have the capacity to lose their epithelial phenotype and gene expression characteristics and change into mesenchymal cells with the ability to form ECM compounds. This concept of EMT has implicated PCs as direct cellular sources contributing to matrix synthesis and fibrogenesis (Povero et al., 2010). Although EMT of cholangiocytes has been confirmed in the context of biliary fibrosis, the actual quantity of EMT of PCs in the development of liver fibrosis is still a subject of debate (Povero et al., 2010; Scholten and Weiskirchen, 2011). These somewhat contradictory observations show that activated fibroblasts in the liver are a heterogenous pool of cells (Zeisberg and Kalluri, 2013). As such, it is not surprising that the course of fibrotic disease can be so varied not only within the liver but also differ from that in other organs such as the lung, kidney, and heart, despite the obvious macroscopic and microscopic commonalities that they share. Indeed, several organ-specific features of hepatic fibrosis have been noted. While viral infections trigger hepatic fibrogenesis, infections are not a common cause for fibrosis in kidney, heart, and lung. Likewise, compared to all other organs, the liver has by far the best regenerative capacity to repair acute and chronic insults. In addition, the final outcome of fibrosis in different organs may differ dramatically. The liver is also unique with regards to the fact that advanced liver fibrosis can progress to cirrhosis and HCC (Gressner and Weiskirchen, 2006). In addition, there are several good serum markers for measurement of hepatic fibrosis, whereas biochemical blood tests that correspond for example with heart fibrosis are not available (Zeisberg and Kalluri, 2013).

Fibrosis research in hepatology employs animal models in order to detect relevant disease-promoting mechanisms and to further translate these findings into the development of antifibrotic drugs. Currently, models using rodents are the gold standard, because these models can reproduce or mimic any human liver disease independent of its cause (Liedtke et al., 2013).

At present, two models are most frequently used in experimental fibrosis (Iredale, 2007). The first model employs repetitive toxic damage such as that induced by CCl<sub>4</sub>. The application of this hepatotoxin creates a model of postnecrotic fibrosis (Brenner, 2009). In contrast bile duct ligation, the second model, simulates secondary biliary fibrosis (Brenner, 2009). Additional commonly used models include the use of special diets such as the methionine choline deficient diet which causes a rapid micro- and macro-vesicular steatosis resulting in liver

injury similar to human NASH as well as the administration of heterologous serum, which mimics autoimmune hepatitis (Brenner, 2009). These models have been used to highlight the action of cytokine signaling pathways in liver fibrosis.

Due to their crucial role in the activation of HSCs and progression of hepatic fibrosis, cytokines, cytokine receptors, and cytokine-driven pathways offer numerous attractive therapeutic targets. Principally, there are three possibilities to target the fibrotic activities of cytokines. Firstly, a cytokine with a known antifibrotic activity can be delivered to specific target cells. Secondly, the pro-fibrogenic effect of a cytokine can be blocked by a potent inhibitor or antagonist at the site of fibrosis, thus developing an antifibrotic effect. And thirdly, cytokine receptors on specific target cells can be used as docking sites for targeted drug delivery, thereby ameliorating efficacy and reducing adverse drug reactions due to lower off-target effects. In the following, several examples that illustrate these three possibilities are given.

## CELL-SPECIFIC DELIVERY OF CYTOKINES WITH AN ANTIFIBROTIC ACTIVITY

Interleukin-10 is the prototype of an anti-inflammatory cytokine that suppresses inflammatory reactions and can attenuate excessive tissue scarring (Hammerich and Tacke, 2014). To circumvent comprehensive effects in other body regions and to evade its speedy clearance by the kidney, one research group linked IL10 to M6P-groups, which specifically bind to the M6P/insulin-like growth factor II (M6P/IGFII) receptor (Rachmawati et al., 2007). This receptor is predominantly expressed on the surface of activated HSCs during the course of liver fibrosis (de Blaser et al., 1995). The study demonstrated that the M6P-IL10 compound retained the anti-inflammatory and antifibrotic action typical of IL10, the latter indicated by a reduction of collagen type I deposition in a CCl<sub>4</sub>-induced rat model of experimental fibrosis. As expected, M6P-IL10 accumulated in rat livers. However, due to its negative charge, M6P does not only bind to HSCs but also to the scavenger receptor type A, which is present in KCs and sinusoidal endothelial cells (LSECs). Therefore, the modification of IL10 via M6P should not be considered an HSC-specific delivery, but rather a means to improve liver accumulation and pharmacokinetics, thus creating a potential candidate for therapeutic application.

Besides IL10, the cytokine IFN- $\gamma$  also exhibits antifibrotic effects. IFN- $\gamma$  evidently blocks different steps during the activation of HSCs as well as the synthesis of ECM in fibroblasts. Furthermore, it can even reduce fibrosis in certain patients (Rockey, 2008). Since IFN- $\gamma$  has extensive pro-inflammatory properties, major problems arise in systemic therapy including adverse reactions, such as flu-like symptoms, generalized activation of immune cells, hyperlipidemia, and provocation of autoimmune reactions, and toxicity to the bone marrow and induction of depression (Bansal et al., 2011). These side effects can occur due to the fact that IFN- $\gamma$  receptors are present on

virtually every cell type in the body, which is likely why its antifibrotic activity is not as profound *in vivo* as it is *in vitro*. To ameliorate the benefits whilst at the same time minimizing adverse effects, it was proposed to directly deliver IFN- $\gamma$  to activated HSCs. Since the platelet-derived growth factor beta receptor (PDGF $\beta$ R) is abundantly found on the cell surface of activated HSCs, first a cyclic peptide specifically binding to this receptor was constructed, displaying the amino acid sequence \*CSRNLIDC\* as a structural element (Beljaars et al., 2003; Bansal et al., 2011). Next, this PDGF $\beta$ R-recognizing cyclic peptide (PPB) was linked to IFN- $\gamma$  either directly or *via* a PEG bridge. Then, the effect of both compounds on HSCs and on fibroblasts was determined *in vitro*. Finally, an *in vivo* test followed employing mice with CCl<sub>4</sub>-induced acute and chronic stages of liver damage. Consistently, the variant containing the PEG linker (IFN- $\gamma$ -PEG-PPB) generated the most remarkable antifibrotic activity: The compound blocked both angiogenesis and hepatic inflammation and even caused fibrolysis in the advanced stage of hepatic fibrosis, while it also led to a decline of IFN- $\gamma$ -associated adverse reactions (Bansal et al., 2011).

Bansal et al. (2014a,b) and coworkers further refined this approach by developing a synthetic compound consisting of the signaling portion of IFN- $\gamma$  and lacking the binding site for the IFN- $\gamma$  receptor, termed mim  $\gamma$ , and a BiPPB, both linked *via* heterobifunctional PEG adapter units. The resulting chimeric structure (mim  $\gamma$ -BiPPB) could solely bind to the PDGF $\beta$ R on activated HSCs and considerably blocked CCl<sub>4</sub>-induced acute and chronic stages of hepatic fibrosis in mice as indicated by a reduction of  $\alpha$ -SMA, desmin, and collagen type I mRNA and protein expression, while off-target effects were not observed (Bansal et al., 2014b).

## TARGETED BLOCKING OF THE PRO-FIBROGENIC EFFECT OF CYTOKINES

Apart from PDGF, TGF- $\beta$ 1 is indeed the major pro-fibrogenic cytokine involved in hepatic fibrosis, as it regulates the production and deposition of ECM (Qi et al., 1999; Breitkopf et al., 2005). Generally, there are different ways for interfering with TGF- $\beta$  signaling: Firstly, TGF- $\beta$  expression can be down-regulated by applying anti-sense oligonucleotide mRNA, secondly, a targeted blocking of a specific isoform of TGF- $\beta$  by means of monoclonal antibodies is feasible, thirdly, the activation of TGF- $\beta$  receptors can be inhibited by the use of specific inhibitors, thereby halting downstream signaling, and fourthly, the local activation of TGF- $\beta$  induced by  $\alpha_5\beta_6$  integrin and by TSP-1 can be prevented (Hayashi and Sakai, 2012).

## TISSUE-SPECIFIC BLOCKING OF THE LOCAL ACTIVATION OF TGF- $\beta$

In an early study, it was established that the amino acid sequence Leu-Ser-Lys-Leu (LSKL) naturally occurs in the region of the amino terminus of the LAP and that it can hamper the activation

of latent TGF- $\beta$  by TSP-1 through competitive inhibition (Ribeiro et al., 1999). The LAP, which forms a dimer with mature TGF- $\beta$ , is necessary for the transfer of TGF- $\beta$  through the cell membrane and hampers its receptor binding before activation (Hayashi and Sakai, 2012).

To determine the influence of LSKL peptides on hepatic fibrogenesis, rats were treated with DMN for 4 weeks (Kondou et al., 2003). DMN leads to liver atrophy and fibrosis, but a concomitant daily administration of LSKL peptides significantly reduced the amount of changes compared to that observed in animals of control groups. Furthermore, the quantity of both active TGF- $\beta$ 1 and phosphorylated Smad2 was lower in the LSKL-treated group, indicating that LSKL blocks the activation of TGF- $\beta$ 1 and as a consequence the entire signaling cascade, thus avoiding the further progress of hepatic fibrosis (Kondou et al., 2003).

Later it was demonstrated that a loss of TSP-1 gives rise to a decrease of TGF- $\beta$ 1/Smad signaling and an increase in the proliferation of hepatocytes (Hayashi et al., 2012). Since TGF- $\beta$ 1 strongly blocks the proliferation of hepatocytes *in vivo* and also triggers their apoptosis, antagonizing TSP-1 down-regulates local activation of TGF- $\beta$ 1, up-regulates the proliferation of hepatocytes and thereby promotes liver regeneration (Hayashi et al., 2012). In contrast to its antifibrotic and regenerative effects in the liver, the lack of TSP-1 does not impact the activation of TGF- $\beta$ 1 during fibrogenesis in other tissues. In thrombopoietin-induced myelofibrosis as well as in bleomycin-induced pulmonary fibrosis a lack of TSP-1 does not impact bioavailability of TGF- $\beta$  and does not protect from fibrosis (Evrard et al., 2011; Ezzie et al., 2011). Therefore, the local activation of TGF- $\beta$  by TSP-1 seems to be tissue-specific (Hayashi and Sakai, 2012), and blocking of TSP-1, for example with LSKL peptides, and its resulting antifibrotic effect might be exclusively relevant to the liver.

## ADENOVIRUS-MEDIATED TRANSDUCTION OF HSCs

In order to investigate the effect of overexpression of PPAR $\gamma$  on liver fibrosis in mice, one research group employed an adenovirus expressing PPAR $\gamma$  for transduction (Yu et al., 2010). PPAR $\gamma$  is a nuclear hormone receptor primarily occurring in the liver and in adipose tissue, which is known to regulate a multitude of genes in adipocytes (Sugii and Evans, 2011; Zhang et al., 2013). Beside endogenous fatty acids also thiazolidinediones (i.e., the glitazones), which are synthetic therapeutic agents for the treatment of type 2 diabetes, are known ligands of PPAR $\gamma$  (Zhang et al., 2013). With respect to hepatic fibrosis, PPAR $\gamma$  plays a central role in the activation of HSCs, as PPAR $\gamma$  expression alone is enough to restore the quiescent phenotype in activated HSCs (Hazra et al., 2004) and both activation and proliferation of HSCs correspond to exhausted PPAR $\gamma$  expression (Zhang et al., 2013). In mice, 2 weeks after adenoviral transduction the expression of fibrosis-associated genes markedly declined, and liver fibrosis resolved (Yu et al., 2010). Also the shift of activated HSCs to the quiescent state induced by PPAR $\gamma$  expression was accompanied

by a sharp decline of proliferation and induction of cell cycle arrest in the G<sub>0</sub>/G<sub>1</sub> phase as well as a rise in cell death *in vitro*, altogether leading to the conclusion that it is possible to reverse fibrosis by overexpression of PPAR $\gamma$  (Yu et al., 2010). In line, rosiglitazone (Avandia, GlaxoSmithKline) – a thiazolidinedione – could both reduce hepatic fibrosis in mice by downregulation of the protein expression of  $\alpha$ -SMA, TGF- $\beta$ 1, and CTGF and re-establish the expression of PPAR $\gamma$  (Nan et al., 2009). The researchers concluded that PPAR $\gamma$ , induced by rosiglitazone, blocks the activation of HSCs and eliminates the expression of TGF- $\beta$ 1 and CTGF, thus improving liver fibrosis (Nan et al., 2009). In addition, M6P-HSA-based delivery of rosiglitazone inhibited HSC activation and diminished fibrosis in a rat model of CCl<sub>4</sub>-based chronic liver injury (Patel et al., 2012). However, in a large clinical trial in patients with NASH, administration of pioglitazone for 96 weeks did not significantly ameliorate hepatic fibrosis compared to placebo or a control treatment with high-dose vitamin E (Sanyal et al., 2010). Possibly, clinical data from this so called Pioglitazone vs Vitamin E vs Placebo for Treatment of Non-Diabetic Patients with Non-alcoholic Steatohepatitis (PIVENS)-trial highlight the importance of improved targeting for PPAR $\gamma$  agonists.

## LIPOSOME-MEDIATED DELIVERY OF IFN- $\alpha$ 1b TO ACTIVATED HSCs

A study published by Du et al. (2007) was aimed at designing an improved drug carrier that specifically targets HSCs. In preparation for the planned *in vitro* and *in vivo* experiments, they changed a cyclic peptide already characterized earlier (Marcelino and McDevitt, 1995) that had been shown to specifically recognize and bind to a non-integrin collagen type VI receptor expressed on HSCs and upregulated on activated HSCs (Beljaars et al., 2000). The amino acid sequence of the original cyclic peptide used by Beljaars et al. (2000) was Cys-Gly-Arg-Gly-Asp-Ser-Pro-Cys or C\*GRGDSPC\*, where C\* indicates the cysteine residues linked *via* a disulfo bond, thus forming the ring. The modification introduced by Du and his group was the substitution of cysteine with lysine, resulting in the sequence C\*GRGDSPK\*. This cyclic peptide, which displayed a higher stability than the original one, was combined with SSLs by means of the sulfhydryl group of the cysteine residue. SSLs are liposomes chemically modified by attaching lipid derivatives of PEG, thus sterically stabilizing them (Allen et al., 1995). PEG also served as a spacer for the linkage of the cyclic peptide with the Arg-Gly-Asp motif (cRGD). The SSLs reached a diameter of around 100 nm and were long-circulating, an important basis for drug delivery *in vivo*. The experiments assessed the binding affinity of the cRGD peptide to the collagen type VI receptor on HSCs as well as determined the efficiency of delivery of IFN- $\alpha$ 1b in rats subjected to surgical ligation of the bile duct (BDL), a common model for cholestatic fibrosis. It was shown that the cRGD peptide tended to bind to activated HSCs instead of binding to hepatocytes, that the amount of cRGD peptide-labeled liposomes (cRGD-SSLs) in HSCs extracted from BDL rats was increased by 10-fold

compared to the quantity of unlabeled SSLs, and that IFN- $\alpha$ 1b delivered in cRGD-SSLs caused a significant reduction of hepatic fibrosis in BDL rats in comparison to BDL rats of the control group or to the group of BDL rats injected with IFN- $\alpha$ 1b delivered in unlabeled SSLs (Du et al., 2007). Blocking the proliferation of activated HSCs by boosting apoptosis, cutting ECM synthesis, and shutting down the release of TGF- $\beta$  are counted among the pharmacological effects of IFN- $\alpha$ , responsible for the observed improvement of liver fibrosis in BDL rats (Du et al., 2007).

Taken together, this study was the first successful delivery of a drug to activated HSCs using liposomes as a carrier. cRGD-SSLs specifically bind to activated HSCs and are incorporated via receptor-mediated endocytosis, followed by the release of the drug. Due to their binding specificity they might qualify for targeted drug delivery to activated HSCs to improve antifibrotic therapy (Du et al., 2007). It should be noted that non-functionalized liposomes, however, do not target HSCs. In mouse models of hepatic fibrosis, dexamethasone-loaded liposomes have antifibrotic effects, but they primarily target hepatic macrophages and T-lymphocytes (Bartneck et al., 2015).

## LIPOSOME-MEDIATED DELIVERY OF OXYMATRINE TO HSCs

Chai et al. (2012) also applied RGD peptide-labeled liposomes for the specific delivery of OM in order to examine whether this herbal medicinal product would have a beneficial effect on CCl<sub>4</sub>-induced liver fibrosis in rats. OM is a natural quinolizidine alkaloid obtained from the roots of the *Sophora alopecuroides* L. and other *Sophora* plants (Figure 1), which exhibits various

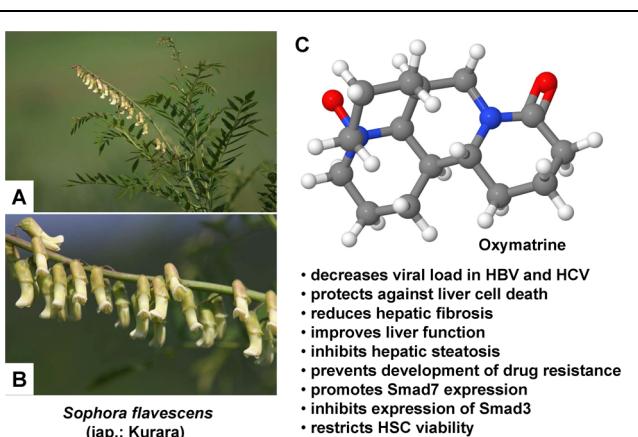
pharmacological properties: It blocks the replication of the hepatitis B virus (Chen et al., 2001; Wang et al., 2011) and also stops liver fibrosis (Chen et al., 2001). Besides this antiviral effect, OM additionally shows apoptosis-inducing activity, predominantly observed in several cancer cell lines, for example in human pancreatic cancer cells (Ling et al., 2011) as well as in human hepatoma SMMC-7721 (Song et al., 2006; Liu et al., 2015) and Hep-G2 cells (Liu et al., 2015). An antifibrotic effect has been studied in rats with CCl<sub>4</sub>-induced liver fibrosis, since OM potently limits the production as well as deposition of collagen, probably by upregulation of *SMAD7* and downregulation of *SMAD3* and *CREBBP* (CREB binding protein) gene expression, thus interfering with the canonical TGF- $\beta$ 1 signaling pathway (Wu et al., 2008). Before conducting their experiments, the researchers formed the OM-liposomes using lipids and the OM-containing aqueous solution and afterward coupled the RGD peptides and the OM-liposomes. Then OM-RGD-liposomes, OM-liposomes, and empty liposomes were allocated to three different groups of rats, while a fourth group of rats remained untreated. Liver fibrosis improved upon administration of OM, as assessed by reduced deposition of collagen and reduced expression of genes co-occurring with liver fibrosis, such as MMP-2, TIMP-1, and type I procollagen (Chai et al., 2012). *In vitro*, apoptosis of HSCs was induced by OM-RGD-liposomes and gene expression of *MMP2*, *TIMP1*, and *COL1A2* was inhibited. Furthermore, RGD labeling improved binding to HSCs (Chai et al., 2012).

In sum, OM improved CCl<sub>4</sub>-induced liver fibrosis in rats by restricting the viability of HSCs and by inducing their apoptosis. Additionally, labeling of liposomes with RGD peptides intensified binding to HSCs, thereby boosting the therapeutic benefit (Chai et al., 2012).

## LIPOSOME-MEDIATED TRANSDUCTION OF HSCs

While most groups attempted to target HSCs and to deliver antifibrotic agents in pursuit of the goal of impairing TGF- $\beta$ 1-induced collagen deposition or of eliminating activated HSCs (Adrian et al., 2007a; Sato et al., 2008; Chai et al., 2012), Narmada and coworkers, instead, focused on deploying the capacities inherent in HSCs in order to trigger liver regeneration. To that end the research group examined the antifibrotic effects of HGF production induced in HSCs (Narmada et al., 2013).

Throughout the development and differentiation of the liver in rats, HGF is mainly produced in HSCs and LSECs (Hu et al., 1993; Nakamura et al., 2011; Mogler et al., 2015). Among the various other physiological functions of HGF, suppression of apoptosis might be the most notable feature: In mice, HGF was shown to block apoptosis of hepatocytes, thus circumventing hepatic failure (Kosai et al., 1999). Contrary to this observed boost of hepatocyte survival, HGF inhibited proliferation and furthered apoptosis of  $\alpha$ -SMA-expressing portal MFBs and activated HSCs, accompanied by resolution of cirrhosis, which was attributed to the expression of the c-Met receptor in both liver cell types (Kim et al., 2005). Furthermore, HGF countered TGF- $\beta$ 1-mediated



**FIGURE 1 | Therapeutic potential of Oxymatrine in hepatic injury. (A,B)** Oxymatrine can be extracted from the roots of *Sophora* plants. *Sophora flavescens* is an evergreen, slow growing, nitrogen fixing shrub that requires a sunny site for growing. The flowers of this plant grow in simple inflorescences that vary in color from white, yellow, orange, red, or purple. **(C)** Oxymatrine is a heterocyclic quinolizidine alkaloid that has a variety of therapeutic activities in the liver. Several of these activities that were identified *in vitro* or in animal models are listed. The photos in **(A,B)** were kindly provided by Hiroshi Moriyama (<http://wildplantsshimane.jp>).

production of collagen type III and  $\alpha$ -SMA in fibroblasts (Jiang et al., 2008) and also decreased TGF- $\beta$ 1-induced expression of CTGF in fibroblasts in the kidney, thereby blocking synthesis of  $\alpha$ 1(I) procollagen (Inoue et al., 2003). Besides antagonizing TGF- $\beta$ 1-induced collagen deposition, an additional antifibrotic effect arises from its ability to trigger the expression of proteases which participate in the degradation of the ECM, including MMP-9 (Nakamura et al., 2011), MMP-3, and MMP-13 (Kanemura et al., 2008). And in addition to boosting proliferation of hepatocytes (Nakamura, 1994), HGF strengthened proliferation as well as migration of different cell types, including endothelial (Morimoto et al., 1991) and mesothelial cells (Warn et al., 2001), but in multiple cancer cell lines, such as in hepatoma HepG2 cells, HGF exerted a potent antiproliferative effect, thus obviously displaying bidirectional impacts (Tajima et al., 1991).

To target HSCs, Narmada et al. used vitamin A-coupled liposomes surrounding the HGF transgene. To that end, the researchers had to produce a plasmid containing the HGF gene, referred to as the pDsRed2-HGF plasmid. After coating of the liposomes with vitamin A, the plasmid DNA was inserted into the vitamin A-liposomes, resulting in vectors with the size of approximately 600 nm (Narmada et al., 2013). The use of vitamin A-coated liposomes as carriers addressed the ability of HSCs to take up and store vitamin A, which is why HSCs are also termed vitamin A-storing cells (Senoo et al., 2007) and the coating procedure was analogous to the one described earlier (Sato et al., 2008). It is hypothesized that vitamin A-uptake by HSCs depends on RBP and that, after forming of the vitamin A-RBP-complex, specific receptors occurring on HSCs then bind the RBP-subunit and incorporate the complex *via* receptor-mediated endocytosis (Senoo et al., 2007). But there is also evidence that RBP may not be essential for the uptake of vitamin A by HSCs: It was shown that hepatic vitamin A levels in RBP-deficient and in wild-type mice did not differ significantly, leading to the conclusion that a lack of RBP does not affect the uptake of vitamin A in the liver (Quadro et al., 1999). Moreover, vitamin A storage is not involved in profibrogenic functions of HSC *per se* (Kluwe et al., 2011). In their study Sato and colleagues could verify that vitamin A-coupled liposomes specifically target HSCs and stated that uptake *via* specific receptors for RBP would be most likely (Sato et al., 2008).

To prove the antifibrotic effects of vitamin-A-liposomes comprising the pDsRed2-HGF plasmid, Narmada et al. (2013) conducted *in vitro* investigations using a cell culture of the rat stellate cell line HSC-T6 as well as a co-culture of HSC-T6 and hepatocytes, and additionally administered the liposome formulation to rats suffering from DMN-induced hepatic fibrosis. *In vitro* experiments showed that the modified liposomes were capable of down-regulating fibrotic factors (Narmada et al., 2013). Fibrotic rats only once treated with a plasmid-containing liposomes by means of retrograde intrabiliary infusion exhibited a rise of HGF gene expression and a simultaneous reduction of the fibrotic markers  $\alpha$ -SMA, TGF- $\beta$ 1, and collagen type I, resulting in regression of hepatic fibrosis (Narmada et al., 2013).

Taken together, targeting pDsRed2-HGF plasmid-containing vitamin A-liposomes to HSCs entailed raised HGF gene

expression and a decline in fibrosis-specific marker proteins and could possibly be optimized to cure liver fibrosis with the help of gene therapy.

## **THERAPEUTIC NANOPARTICLES**

Strictly taken, nanoparticles are sized from one up to 10 nm, however, the definition is frequently being extended to the sub-micron range up to 500 nm (Zhang et al., 2008). Principally, one can distinguish organic and inorganic nano-sized particulates. Gold nanoparticles are probably the most frequently used inorganic nanoparticles and are popular due to the ease of tailoring size, shape, and functionalization (Bartneck et al., 2010). Metal-based nanotherapeutics offer optical and magnetic properties, which allow their usage in imaging techniques such as computerized tomography (Bartneck et al., 2012). On the downside, inorganic carriers accumulate in the body since they are not biodegradable (Bartneck et al., 2012). Organic nanoparticles such as lipid or polymer-based constructs exhibit the advantage that many of them are biodegradable and therefore should be considered as clinically relevant nanocarriers.

Irrespective of the particle nature, its size and functionalization determine the distribution in different organs. Generally, particles or constructs sizing above 10 nm translocate to liver and spleen and only minor amounts enter the kidney, whereas those below 10 nm were shown to also enter kidney, testis, and brain (Nikoobakht et al., 2002). Nanotechnological modifications offer particle engulfment with compounds that also affect the physicochemical properties of the construct. PEG is among the most popular modifications to modify particle properties (Bartneck et al., 2010). The PEG-induced reduction of unspecific interactions with proteins also reduced uptake by liver phagocytes due to the resulting neutral particle charge (Bilzer et al., 2006).

## **TARGETED DELIVERY OF THERAPEUTIC AGENTS WITH THE HELP OF A CYTOKINE RECEPTOR**

Similar to the chemical modification of cytokines, such as IL10 and IFNy, to enable their binding to specific receptors on predefined target cells, drugs can be linked to specific carriers, allowing them to bind to cytokine receptors on target cells. With regard to liver fibrosis, the activated HSC constitutes the target cell of choice, and several efforts were carried out to selectively deliver drugs with an antifibrotic effect to activated HSCs in experimentally induced liver fibrosis.

## **TARGETING THE M6P/IGF-II RECEPTOR ON ACTIVATED HSCs**

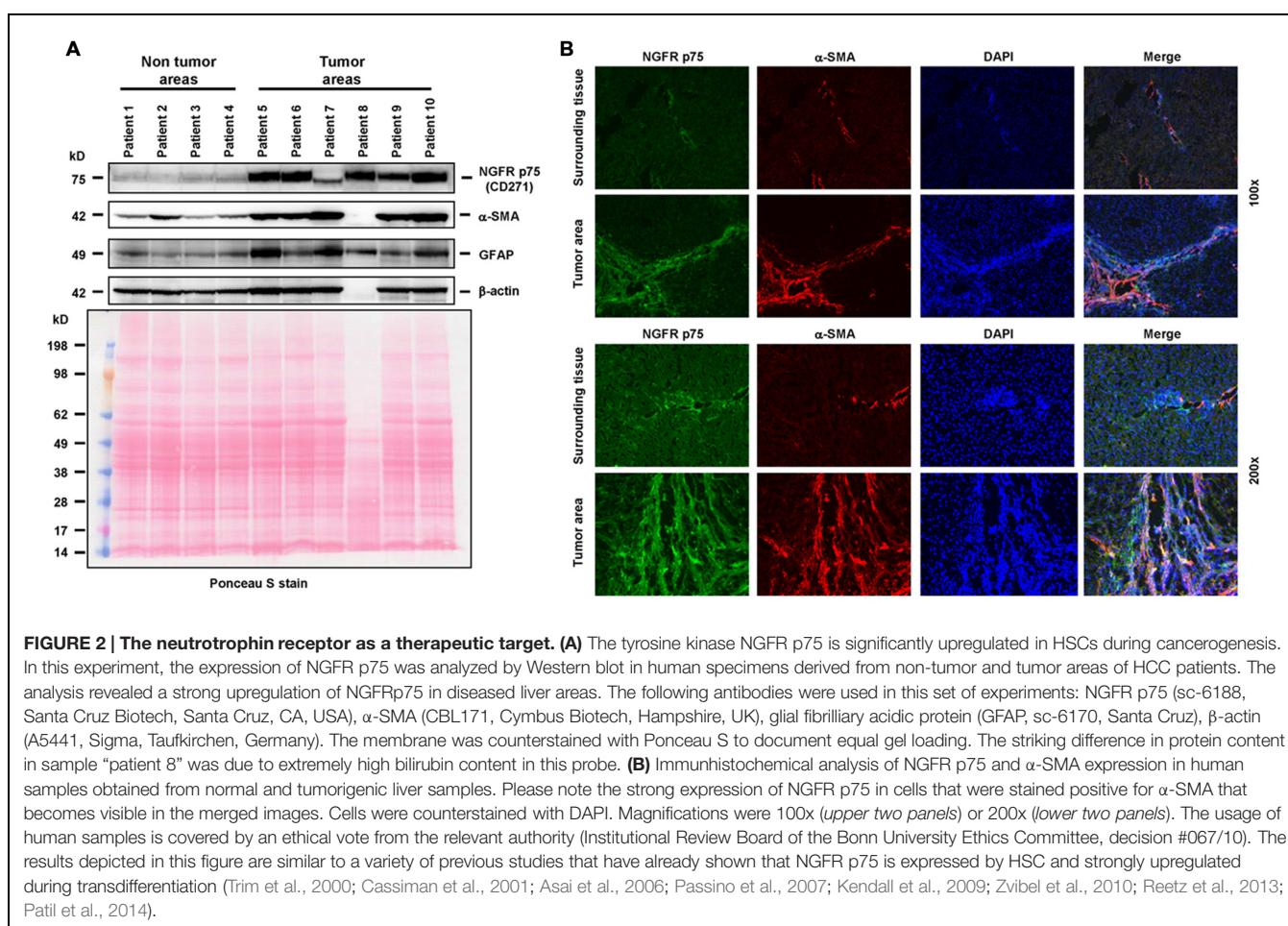
The most frequently used receptor is the M6P/IGF-II receptor, which is a transmembrane glycoprotein with a wide range of regulatory functions. Due to its structure, it can act as a

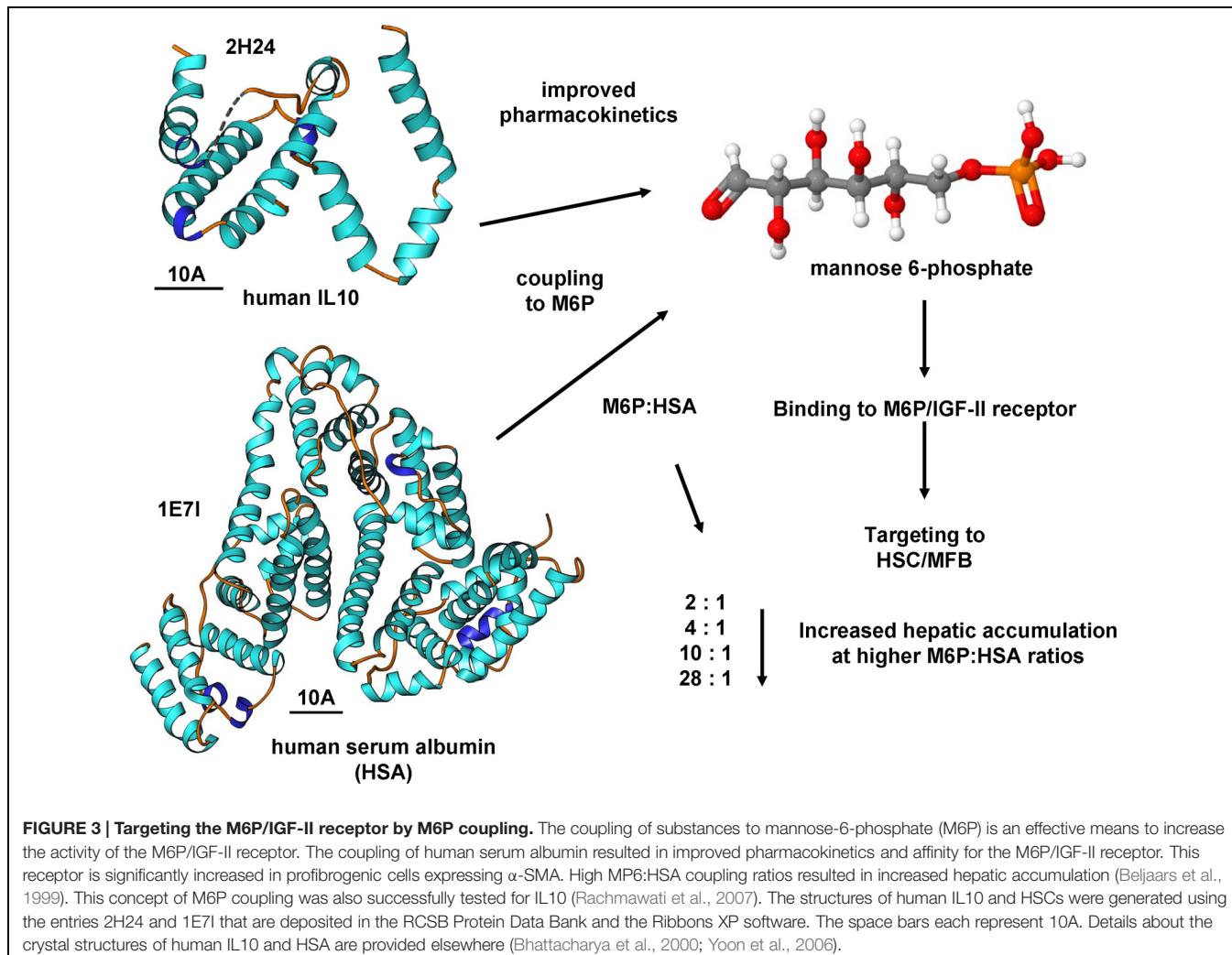
clearance receptor, leading to degradation of proteins through endocytosis and as a signaling receptor engaged in G-protein dependent signal transduction (El-Shewy and Luttrell, 2009; Heger and Schlüter, 2013). Based on the large extracellular portion of the M6P/IGF-II receptor, binding of many different ligands is possible: Both interactions with M6P-containing ligands, such as renin, latent TGF- $\beta$ 1, thyroglobulin, proliferin, leukemia inhibitory factor, and granzyme B, and recognition of M6P-free ligands, for example IGF-II, retinoic acid, urokinase-type plasminogen activator receptor, and plasminogen are well documented (Heger and Schlüter, 2013). The cytoplasmic domain does not display any catalytic activity, so after ligand binding the receptor shuttles between the cell membrane and intracellular compartments, such as lysosomes (El-Shewy and Luttrell, 2009; Heger and Schlüter, 2013).

The first step on the path to targeted delivery was made by Beljaars et al. (1999), when they developed and tested a carrier, designed to specifically bind to the M6P/IGF-II receptor on activated HSCs. This receptor is strongly activated during transdifferentiation of HSC and is closely associated with expression of  $\alpha$ -SMA and the pathophysiology of liver disease (Trim et al., 2000; Cassiman et al., 2001). For example, it is strongly upregulated in HSC during hepatic carcinogenesis (**Figure 2**).

The mentioned carrier consisted of HSA, to which M6P groups had been added: Four different constructs with an increasing amount of M6P groups, i.e., with a molar ratio of M6P:HSA amounting to 2:1, 4:1, 10:1, and 28:1, were generated (Beljaars et al., 1999). First, the accumulation of the compounds in fibrotic livers of rats was determined: the uptake rose with an increasing degree of substitution (**Figure 3**). Compounds with a molar ratio less than or equal to 10:1 accumulated only at rates of  $9\% \pm 5\%$  or less in the liver, whereas a ratio of 28:1 led to a jump to  $59\% \pm 9\%$  of the originally delivered dose (Beljaars et al., 1999). Next, the accumulation in HSCs was ascertained employing double-immunostaining techniques, i.e., for HSA and HSCs: Here, too, the uptake of M6P-HSA rose with an increasing degree of substitution and  $70\% \pm 11\%$  of the intrahepatic staining for the conjugate with a 28:1 ratio was located in HSCs (Beljaars et al., 1999). In addition, it could also be shown that BSA linked to M6P massed in human non-PCs of the liver (Beljaars et al., 1999). The authors concluded that M6P-albumins could function as selective drug carriers to HSCs.

Since then, the M6P-HSA carrier has been used more often for the delivery of antifibrotic drugs to HSCs. The immunosuppressive drug MPA, which is capable of blocking proliferation of fibroblasts, was coupled to M6P-HSA, intending to avoid immunosuppression and to improve its antifibrotic





activity (Greupink et al., 2005). Specific binding of the conjugate to HSCs and a reduction of their proliferation could be observed *in vitro*. *In vivo*, it selectively accumulates in the liver – primarily in HSCs and also in KCs and LSECs, but not in primary and secondary lymphoid tissues (Greupink et al., 2005). Furthermore, in BDL treated rats the compound minimized liver inflammation and, likewise, the mRNA expression of  $\alpha$ - $\beta$ -Crystallin, which functions as a marker for the activation of HSCs (Greupink et al., 2005). In summary, coupling of MPA to the M6P-HSA carrier and subsequent targeted delivery led to a decline of the activation of HSCs.

Using the same carrier, the antifibrotic drugs PTX, a drug that has an antiproliferative effect on HSCs and blocks their activation *in vitro* (Gonzalo et al., 2006), DOX, which also potently impairs proliferation of HSCs *in vitro* (Greupink et al., 2006), 15-deoxy- $\Delta$ 12,14-prostaglandin J2 (15dPGJ2), an apoptosis-inducing drug (Hagens et al., 2007), as well as 4-chloro-N-[4-methyl-3-(4-pyridin-3-yl-pyrimidin-2-ylamino)-phenyl]-benzamide (PAP19), a tyrosine kinase inhibitor and a derivative of imatinib (Gonzalo et al., 2007) and 18 $\beta$ -glycyrrhetic acid (18 $\beta$ -GA) also known as enoxolone (Luk et al., 2007) could

all be successfully delivered to HSCs to selectively unfold their respective activity.

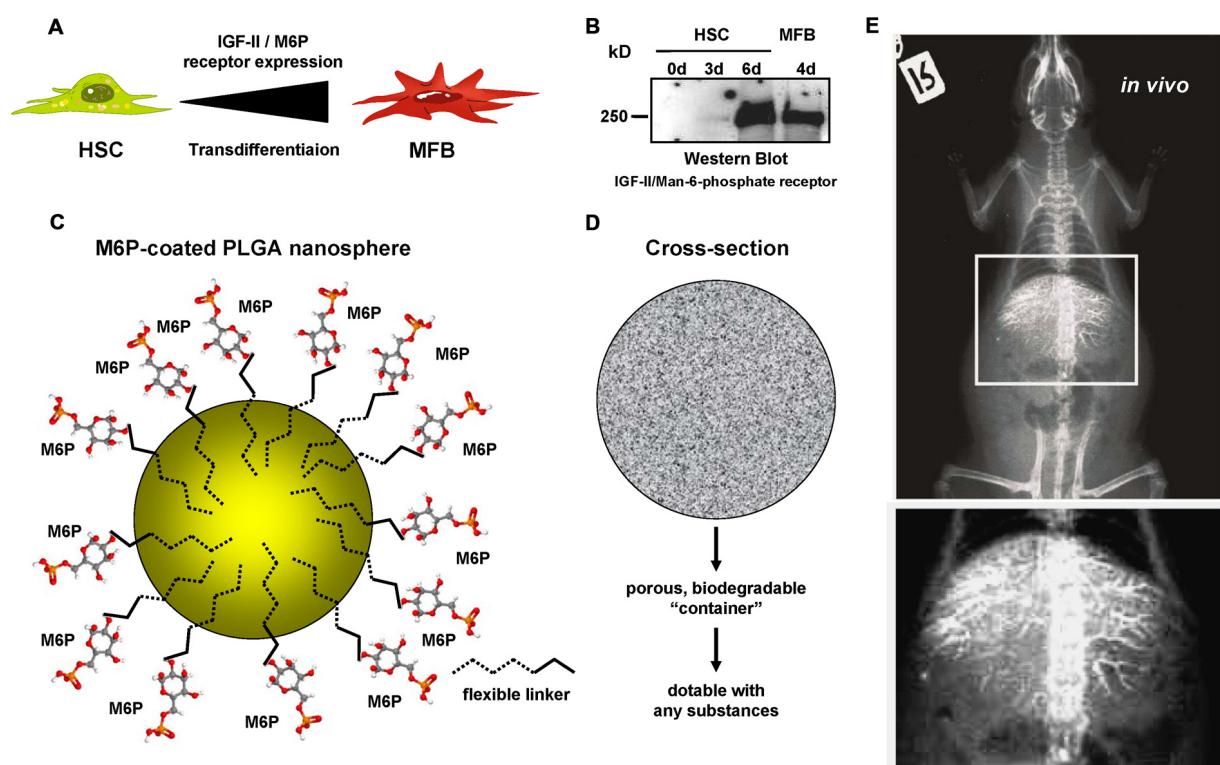
Another research group applied a M6P-BSA carrier in a slightly different form: The researchers coupled TFOs and M6P-BSA with the help of disulfide bonds (Ye et al., 2005). TFOs can be used for gene silencing through triplex formation with genomic DNA, i.e., promoter sequences, thereby inhibiting transcription of the genes concerned. To optimize the uptake of M6P-BSA-TFO by the liver, based on prior testing a conjugate with a molar ratio of M6P:BSA equal to 20:1 was chosen. It could be determined that nearly 66% of the administered conjugate had accumulated in the liver of rats 30 min following its injection, and a high amount of the delivered dose could be detected in HSCs, thus suggesting its use for the therapy of liver fibrosis (Ye et al., 2005).

Adrian et al. (2007b) described the coupling of M6P-HSA to liposomes with the aim to ascertain the pharmacokinetics of M6P-HSA-liposomes and their specificity to target HSCs. Liposomes constitute artificial globular entities with one or more phospholipid bilayers forming an envelope around an aqueous core (Giannitrapani et al., 2014; Neophytou and

Constantinou, 2015) and are capable of carrying both hydrophilic and lipophilic agents: Hydrophilic drugs are placed within the aqueous core whereas lipophilic molecules are integrated into the phospholipid bilayer. Being counted among the organic nanoparticles, liposomes feature several advantages: Beyond their solid biocompatibility they can be easily produced, exhibit only minor systemic toxicity, and are quickly eliminated from the blood and taken up by the reticuloendothelial system (Giannitrapani et al., 2014). In preparation for their study, the researchers added 29 M6P groups on average to each albumin molecule and, in a second step, about 31 M6P-HSA molecules on average were linked to one liposome. The resulting M6P-HSA-liposomes had a size of roughly 100 nm. BDL-treated rats were employed to perform the following *in vivo* experiments: Radioactively labeled M6P-HSA-liposomes was injected intravenously, and both their elimination from the blood as well as their distribution were monitored. Ten minutes following injection, 90% of the original dose of  $^3\text{H}$ -M6P-HSA-liposomes was removed from the blood and predominantly aggregated in the liver (Adrian et al., 2007b). Apart from HSCs M6P-HSA-liposomes also concentrated in KCs and LSECs,

and accumulation in the last two cell types could be blocked by previously administered polyinosinic acid, which acts as a competitive inhibitor of scavenger receptors class A, indicating that this receptor type is responsible for the recognition of M6P-HSA by KCs and LSECs and not by HSCs (Adrian et al., 2007b). Furthermore, injection of uncombined M6P-HSA prior to the administration of M6P-HSA-liposomes resulted in a decreased liver uptake of 15% accompanied by an increase of accumulation in the lungs, due to an extended presence in the blood circulation and probably binding to pulmonary intravascular macrophages within the injured lungs of BDL rats.

In previous studies (Pereira Paz, 2004; Benner, 2007), we in cooperation with other groups have established novel biodegradable M6P-coupled PLGA nanoparticles that have a porous structure allowing to be dotted with various substances (Figure 4). Based on the finding that the IGF-II/M6P receptor is found to be strongly upregulated during transdifferentiation, we tested if these vehicles can be targeted into the liver. Therefore, we dotted these nanoparticles with barium sulfate that acts as a radiopaque contrast media and found that respective



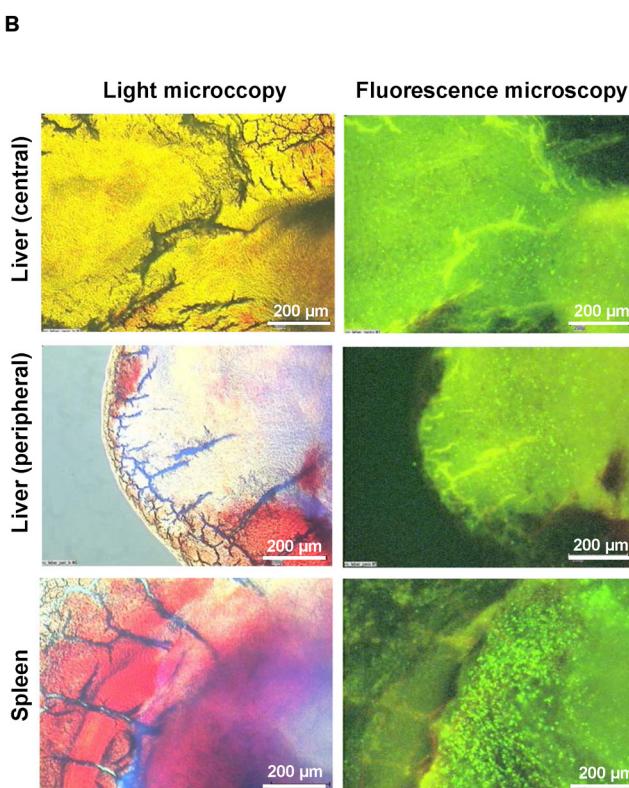
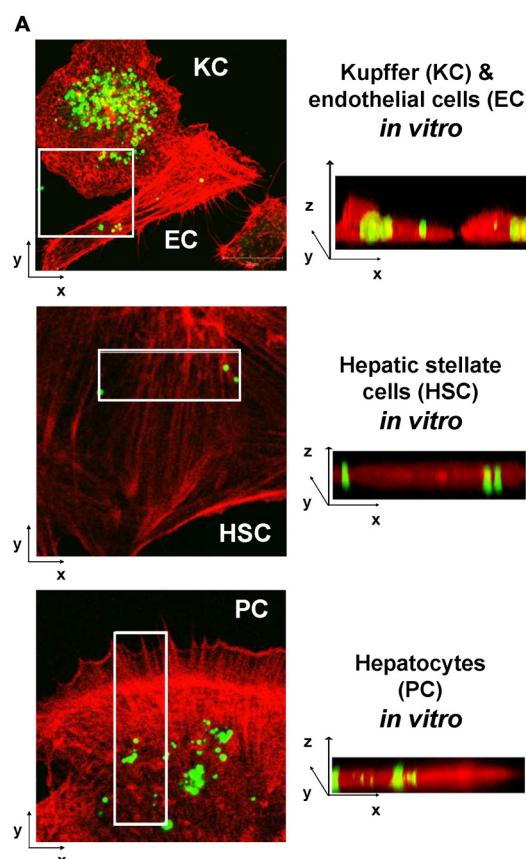
**FIGURE 4 |** Mannose-6-Phosphate coating of nanoparticles for targeted delivery of pharmacological active compounds to activated HSCs and MFBs. **(A)** Several reports have shown that the expression of the IGF-II/M6P receptor is strongly upregulated during transdifferentiation of quiescent HSCs to MFBs. **(B)** Western blot analysis of cellular protein extracts of rat HSCs and rat MFBs that were cultured for indicated time intervals. The blot was probed with a goat-anti-IGFIR (sc-14413, Santa Cruz Biotechnology, Santa Cruz, CA, USA). **(C)** In a former study, we have generated biocompatible nanoparticles that are based on PLGA and coated with M6P via flexible linkers. **(D)** These biodegradable particles have a porous container structure allowing to pack up (“dotting”) various compounds into the cavities. **(E)** Respective nanoparticles were dotted with barium sulfate and injected into the tail vein of a rat. The radiography, that was prepared approximately 2 h later, shows that these particles were enriched in the liver. More details about the production of the modified PLGA particles used in this study can be found elsewhere (Pereira Paz, 2004).

nanoparticles were enriched in the liver when applied *via* the tail vein (cf. **Figure 4**). Unfortunately, in our hands the synthesized particles caused a serious pulmonary embolism that prevented further exploration of these biodegradable vehicles. In addition, when we tested their specificity *in vitro*, we found that respective FITC-dextran-loaded particles were taken up not only by HSCs but also by ECs and hepatocytes and highly effective by KCs (**Figure 5A**). This finding was more or less independent from their surface loading with M6P (not shown), possibly indicating the exceptional scavenging ability of the cells. Likewise, the application of FITC-dextran-loaded nanospheres *in vivo* resulted in a strong uptake of the respective particles not only in the liver but also in the spleen (**Figure 5B**).

In sum, M6P-HSA-liposomes might be suitable to specifically deliver drugs to HSCs, LSECs and KCs, and both M6P/IGF-II and scavenger receptors have the ability to detect and bind the M6P-HSA subunit (Adrian et al., 2007b).

## TARGETING THE p75 NEUROTROPHIN RECEPTOR ON HSCs

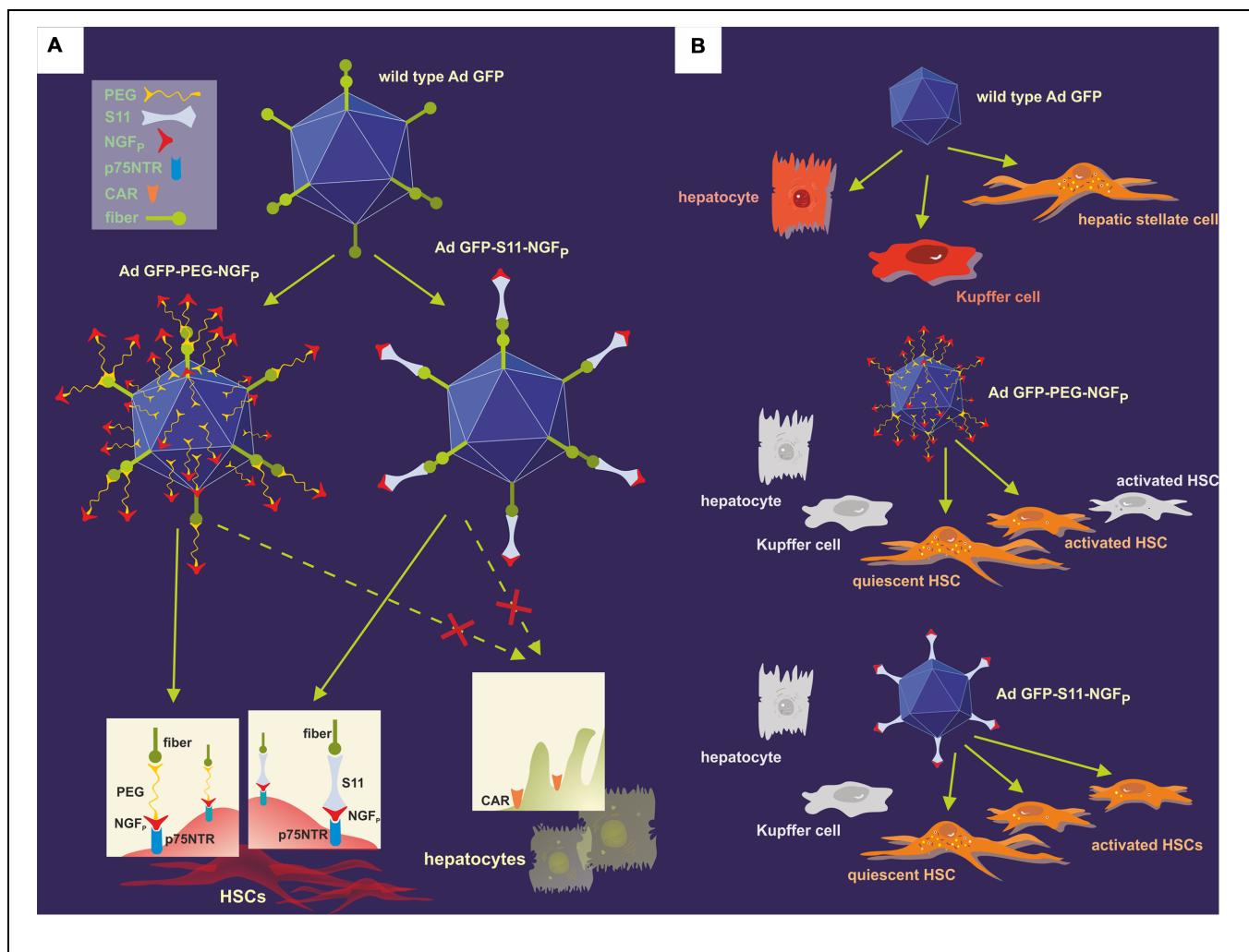
Aiming at developing an effective strategy to specifically target HSCs using a gene delivery vector, Reetz et al. (2013) modified the Ad serotype 5 envelope by two frequently used strategies for *in vivo* targeting and evaluated the transduction efficiency of both procedures. Since the wild-type Ad is capable of infecting a wide spectrum of different cell types in the liver, such as hepatocytes, KCs and HSCs, its application for cell-specific infection is challenging. This ubiquitous tropism is caused by the modality of viral entry into the cell. First, Ad fiber knobs interact with receptors on the surface of a host cell, particularly with the CAR, thereby attaching the virus to the cell surface, followed by a further interaction of the virus-CAR-complex with other co-receptors, as for instance various integrins (Sharma et al., 2009; Reetz et al., 2013). An RGD amino acid sequence within the penton base, i.e., the coat protein of the Ad, is responsible for



**FIGURE 5 | Limitation of the M6P targeting strategy. (A)** Primary isolated KC, endothelial cells (EC), HSCs, and hepatocytes (parenchymal cells, PC) were incubated with FITC-dextran-dotted M6P-coated nanospheres. After 24 h, excess nanospheres were removed by washing two times with phosphate-buffered saline. The growth medium was renewed and cells further incubated for another 24 h period. Then the cells were fixed in 4% paraformaldehyde and the cellular actin was stained with a phalloidin-rhodamine complex. After a further wash step, the cells were fixed in Moviol (Merck Biosciences, Bad Soden, Germany) and analyzed by confocal microscopy. **(B)** FITC-dextran doted nanospheres were injected via the tail vein into a stunned rat. 24 h later, the animal was sacrificed and organ specimen derived from the central or peripheral area of the liver as well as from the spleen analyzed by light microscopy (left panels) and fluorescence microscopy (right panels). Please note the high concentration of nanospheres (green beads) within the spleen. More details about this set of experiments can be found elsewhere (Benner, 2007).

binding to the vitronectin receptors  $\alpha v\beta 3$  and  $\alpha v\beta 5$ , whereupon *via* integrin-mediated signaling the internalization of the Ad is effected by means of receptor-mediated endocytosis (Wickham et al., 1995; Sharma et al., 2009). Since the virus-CAR interaction features high affinity in contrast to the rather low affinity of the virus-integrin interaction, the former is critical for the efficiency of infection and the tissue-specific occurrence of the CAR is one of the key factors which determine adenoviral tropism (Sharma et al., 2009). In consequence, to restrict the natural tropism of the Ad it is necessary to sidestep binding of the virus to the primary receptor and, instead, redirect the virion to a receptor solely expressed on the target cell, which should be genetically modified. To put this approach into practice Reetz et al. (2013) developed a peptide ( $NGF_P$ ) derived from the

$NGF$  and specifically binding to the p75 NT receptor (p75NTR) expressed on HSCs. Being part of the TNF superfamily, p75NTR is structurally characterized by a cysteine repeat motif, which acts as a ligand binding domain and a cytoplasmic portion called the death domain (Casaccia-Bonelli et al., 1999). Ligands of p75NTR are the NTs with  $NGF$  as prototype (Roux and Barker, 2002), as well as BDNF, NT-3, and NT-4, which are relevant for the development of the nervous system in vertebrates (Lee et al., 2001). Additionally, NTs can also initiate signaling by binding to Trk receptors with tyrosine kinase activity and, depending on the participating receptors, signal response can vary. If both p75NTR and Trk-A are stimulated, cell survival will be triggered, whereas exclusive interaction with p75NTR induces cellular death (Trim et al., 2000; Roux and Barker, 2002). The

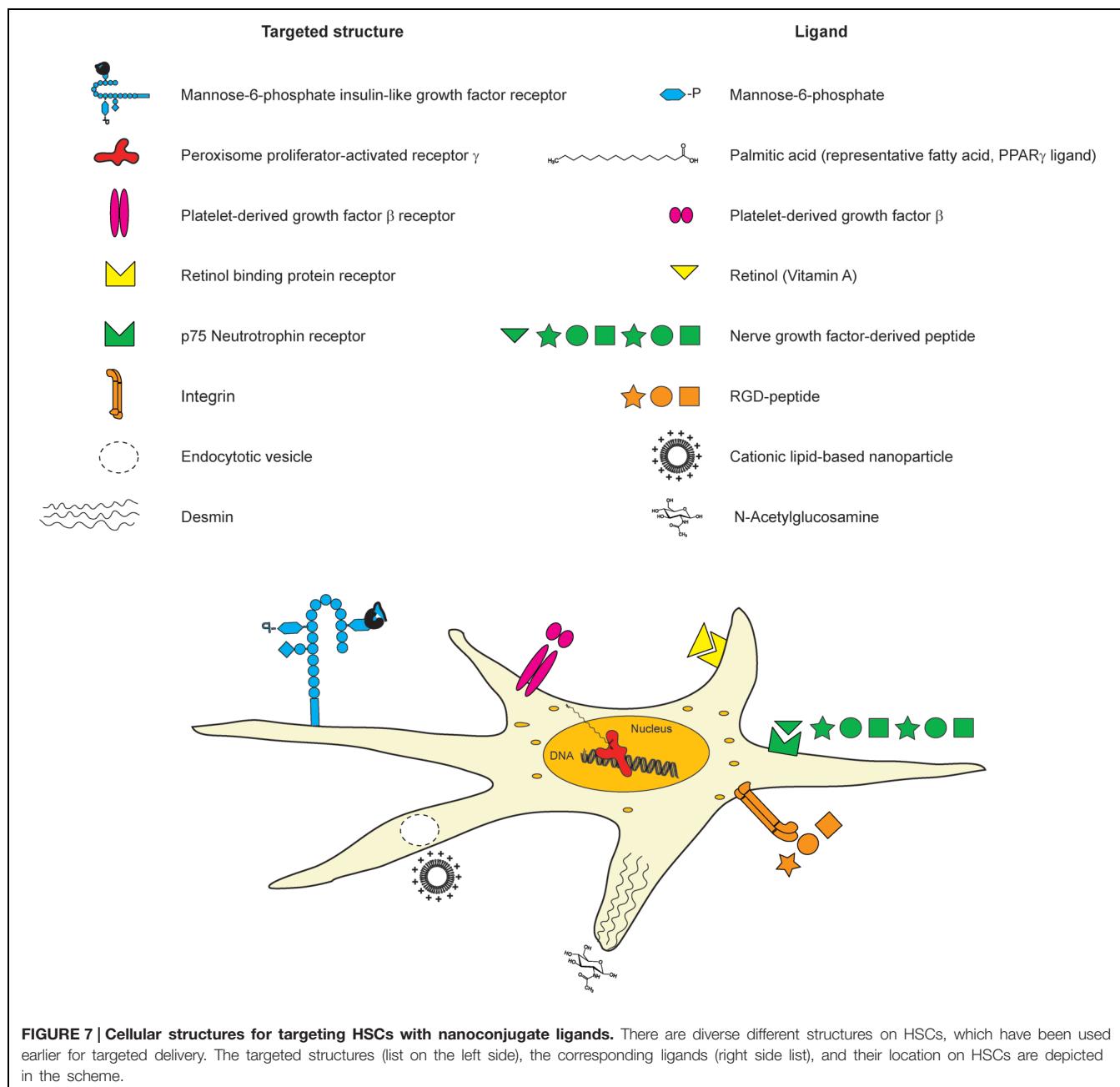


**FIGURE 6 | Modification of the natural adenoviral tropism employing two different approaches resulted in the specific targeting of the p75NTR on HSCs. (A)** The p75NTR-binding peptide  $NGF_P$  and the wild-type Ad.GFP were covalently joined using PEGylation, thus forming Ad.GFP-PEG- $NGF_P$  (left) as well as merged with the help of S11 adapter molecules, resulting in Ad.GFP-S11- $NGF_P$  (right). The single chemical bonds of PEG and viral proteins are close together, thereby concealing both fiber knobs and RGD sequences on the viral surface, whereas the S11 fragment only binds to the fiber knobs, keeping the RGD sequences free for integrin interaction. Since fiber knobs are blocked in either case, binding to CAR, for example on hepatocytes, is inhibited and exclusive interaction of  $NGF_P$  with p75NTR on the surface of HSCs can occur. **(B)** Wild-type Ad.GFP vectors can transduce hepatocytes, Kupffer cells, and HSCs in the liver, identifiable by the green fluorescence of GFP (top). As a result of the viral modifications, both Ad.GFP-PEG- $NGF_P$  (middle) and Ad.GFP-S11- $NGF_P$  (below) exclusively bind to and transfect quiescent and activated HSCs, but Ad.GFP-S11- $NGF_P$  showed an enhanced transduction efficiency concerning activated HSCs (below).

fact that p75NTR is expressed by HSCs and that NGF-binding to p75NTR induces apoptosis of activated HSCs suggested that stimulation of p75NTR could selectively target and eliminate HSCs (Trim et al., 2000).

More than a decade later, Reetz et al. (2013) employed this concept to demonstrate the adenovirus-mediated transfection of HSCs for the first time. In their experiments, the researchers employed a wild-type Ad serotype 5 (Ad.GFP) as the vector, transferring the GFP gene and retaining the adenoviral natural tropism (Figure 6). The synthetic NGF<sub>P</sub>, composed of amino acids 25–36 of the original NGF protein and exactly corresponding to the portion of NGF necessary for binding

to p75NTR, was then coupled to Ad.GFP using two different techniques in order to vary the viral tropism: A single chain antibody fragment (S11), specifically recognizing the Ad fiber knobs as well as NGF<sub>P</sub>, served as an adapter molecule to form Ad.GFP-S11-NGF<sub>P</sub>. Furthermore, NGF<sub>P</sub> and Ad.GFP were covalently joined by means of polyethylene glycol – a procedure termed PEGylation – thus building Ad.GFP-PEG-NGF<sub>P</sub>. Subsequently, both derivatives were tested *in vitro* and *in vivo*, allowing determining the efficiency of transfection based on the level of green fluorescence. Additionally, in healthy and in BDL-treated mice HSCs were detected on the basis of their ultraviolet vitamin A-fluorescence, as recorded by intravital



fluorescence microscopy. Data demonstrated that usage of both Ad.GFP-S11-NGF<sub>P</sub> and Ad.GFP-PEG-NGF<sub>P</sub> restricted liver tropism characteristic of the wild-type Ad, enlarged transfection of HSCs, and both vectors equally displayed higher transduction efficiency in fibrotic liver versus healthy organs, but – compared with Ad.GFP-PEG-NGF<sub>P</sub> – Ad.GFP-S11-NGF<sub>P</sub> showed a higher targeting efficiency of activated HSCs (Reetz et al., 2013).

In conclusion, targeting of p75NTR on HSCs with the use of a modified adenoviral carrier could constitute a practicable and powerful way of applying gene therapy to activated HSCs (Reetz et al., 2013).

## TARGETING PDGF AND THE RBP RECEPATORS ON HSCs

PDGF is the most mitogenic factor that leads to increased HSC proliferation in liver fibrosis. PDGFR $\beta$ , which is one of its two receptors, is critically upregulated on activated HSCs (Bonner, 2004). Encouraging studies revealed a sterically-stabilized liposome (SSL) equipped with the cyclic peptide “C\*SRNLIDC\* (pPB) with strong binding activity for the PDGF- $\beta$  receptor (and loaded with IFN- $\gamma$ ) enhanced anti-fibrotic effects of IFN- $\gamma$  in a murine model of TAA-based hepatic fibrosis (Li et al., 2012).

The retinol binding protein receptor which is involved in storing retinol is another putative structure for targeting HSCs. Hence, lipid-encapsulated and retinol-decorated siRNA versus heat-shock protein 47, a collagen-specific chaperone, was shown to ameliorate fibrosis in diverse experimental mouse models (Sato et al., 2008). Nanoconjugate siRNA against TGF- $\beta$ 1 equipped with an N-acetylglucosamin targeting moiety intending to reach HSCs via desmin was reported to colocalize with HSCs and to reduce fibrosis (Kim et al., 2013). Due to the cytosolic presence of desmin, the conjugates may also enter the cells via alternative routes. Recently, siRNA directed against the procollagen  $\alpha$ 1(I) gene has been deployed using cationic nanoparticles, leading to an amelioration of fibrosis. However, despite the therapeutic success in preclinical studies, the delivery route was rather unspecific and probably occurs through endocytosis since none of the known cell-targeting motifs were used (Jiménez Calvente et al., 2015) (Figure 7).

## TRANSCRIPTIONAL TARGETING APPROACHES FOR ACTIVATED HSCs/MFBs

Comparative mRNA and protein expression analyses demonstrated that HSCs have a characteristic and unique gene signature that is significantly modulated during the transdifferentiation into MFBs (Kristensen et al., 2000; Ji et al., 2012; Azimifar et al., 2014; Zhang et al., 2015). Therefore, it was assumed that several of these genes might be attractive targets for tracking, targeting and cell isolation (Zhang et al., 2015). There is a growing list of genes that are either expressed preferentially

in HSCs (within the liver) or become significantly upregulated in response to cellular activation and transdifferentiation. The group of “transdifferentiation-sensitive genes” include transcription factors, ECM proteins, cell adhesion molecules, smooth muscle specific genes, cytokine receptors, and genes encoding proteins involved in matrix remodeling, or cytoskeletal organization (Table 1).

The identification of factors and *cis*- or *trans*-acting elements within such genes that trigger preferred expression in HSCs/MFBs offer important therapeutic opportunities. Such regulatory elements could be exploited to express antifibrotic or apoptosis-associated transgenes specifically in physiologically altered HSCs, thereby inducing targeted clearance of reprogrammed, activated HSCs/MFBs or preventing hepatic expression of excessive ECM constituents. Several years ago, we have shown in culture-activated HSCs that promoter fragments of the CSRP2 and SM22 $\alpha$  genes that are expressed exclusively in the liver in HSCs are sufficient to mediate reporter gene expression (Herrmann et al., 2004). Similarly, we found that stretches taken from the transdifferentiation-sensitive TIMP-1 promoter were able to achieve transgene expression in culture-activated HSCs but not in cultured hepatocytes (Herrmann et al., 2004). In a similar targeted gene transcription approach it was shown that a 2.2-kbp fragment derived from the human GFAP gene promoter was not only capable of directing HSC-specific expression *in vitro*, but also conferred dose- and time-dependent sensitivity to TGF- $\beta$  that is the major cytokine responsible for ECM formation (Maubach et al., 2006).

In another investigation, the GFAP promoter was fused to the herpes simplex virus thymidine kinase gene. This transgene was able to render susceptibility for ganciclovir-induced cell death, both in *in vitro* and *in vivo* (Puche et al., 2013). However, the usage of the GFAP promoter as a regulatory element to drive transgene expression in activated HSCs or transdifferentiated MFBs is potentially hindered by the finding that HSCs downregulate expression of GFAP and that this gene is also significantly expressed in neuronal tissues and cholangiocytes (Yang et al., 2008). A similar finding was noticed for the vimentin promoter that is predominantly active in activated HSCs and MFBs in the liver, but is also effectively transcribed in vascular smooth muscle cells and portal fibroblasts (Troeger et al., 2012).

Unfortunately, the partial cellular selectivity of other promoters that we observed *in vitro* was not found *in vivo* emphasizing the complexity of regulatory factors that are necessary to guarantee specific expression in HSCs (Herrmann et al., 2004). In addition, the detailed analysis of  $\alpha$ -SMA and collagen I gene transcriptional patterns in primary cultures of HSCs and in an *in vivo* model of secondary biliary fibrosis by means of transgenic mice that express the red fluorescent protein and the enhanced GFP under transcriptional control of respective promoter elements revealed that these two genes were not co-expressed in all cells (Magness et al., 2004). These data indicate that HSCs are a fraction of cells that show heterogeneity during hepatic insult, thereby potentially hampering and restricting target approaches that use “HSC-specific” or promoters that become activated during hepatic insult.

**TABLE 1 | Selected genes preferentially expressed in HSCs or showing transdifferentiation dependent activation in activated HSCs/MFBS.**

Gene	Experimental finding	Reference
ABCC9 (SUR2, ATP-binding cassette, subfamily C, member 9)	Identified by proteomic functional comparative hotspot analysis of liver cells for genes associated with ECM assembly	Azimifar et al., 2014
ACTA2 ( $\alpha$ -smooth muscle actin)	$\alpha$ -smooth muscle actin increases during prolonged culturing in rat HSCs	Ramadori et al., 1990; Kristensen et al., 2000*
ACTG1 ( $\gamma$ actin)	Activated during <i>in vitro</i> and <i>in vivo</i> transdifferentiation	Kristensen et al., 2000
ADAMTSL2 (ADAMTS-like protein 2)	Identified by proteomic functional comparative hotspot analysis of liver cells for genes associated with ECM assembly	Azimifar et al., 2014
ANGPTL2 (Angiopoietin-like Protein 2)	Identified by proteomic functional comparative hotspot analysis of liver cells for genes associated with ECM assembly	Azimifar et al., 2014
ANGPTL6 (AGF, Angiopoietin-like Protein 6)	Identified by proteomic functional comparative hotspot analysis of liver cells for genes associated with ECM assembly	Azimifar et al., 2014
ARP3- $\beta$ (Actin-related protein 3- $\beta$ )	Identified by proteomic functional comparative hotspot analysis of liver cells for genes associated with ECM assembly	Azimifar et al., 2014
BAG2 (Bcl2-associated athanogene 2)	Up-regulated in rat HSCs during activation process	Ji et al., 2012*
BAG3 (Bcl2-associated athanogene 3)	Up-regulated in rat HSCs during activation process	Ji et al., 2012
BDNF (brain-derived neurotrophic factor)	HSCs show immunoreactivity for the brain-derived neurotrophin	Cassiman et al., 2001
BGN (biglycan)	The steady-state levels of the mRNA for biglycan increased threefold during primary HSC culture	Meyer et al., 1992; Jit et al., 2012
C1S (Complement component 1s, Serine protease)	Activated during <i>in vitro</i> and <i>in vivo</i> transdifferentiation	Kristensen et al., 2000
CALB3 (Calbindin D9k)	Identified by proteomic functional comparative hotspot analysis of liver cells for genes associated with ECM assembly	Azimifar et al., 2014
CALD1 ( $\eta$ -caldesmon)	Activated rat HSCs express this smooth muscle cell marker	Wirz et al., 2008
CAPN6 (Calpain 6)	Identified by proteomic functional comparative hotspot analysis of liver cells for genes associated with ECM assembly	Azimifar et al., 2014
CAPZ1 (F-actin capping protein $\alpha$ 1)	Activated during <i>in vitro</i> and <i>in vivo</i> transdifferentiation	Kristensen et al., 2000
CD47 (IAP, integrin-associated protein)	Identified by proteomic functional comparative hotspot analysis of liver cells for genes associated with ECM assembly	Azimifar et al., 2014
CD73 (NTSE, ecto-5'-nucleotidase)	CD73 is weakly expressed in quiescent HSCs and portal fibroblasts but is markedly upregulated at the transcriptional level in myofibroblastic HSCs and portal fibroblasts	Fausther et al., 2012
CD276 (B7-3, B7 homolog 3)	Up-regulated in rat HSCs during activation process	Ji et al., 2012
CNN1 (Calponin H1)	Activated rat HSCs express this smooth muscle cell marker	Wirz et al., 2008
COCH (Cochlin)	Identified by proteomic functional comparative hotspot analysis of liver cells for genes associated with ECM assembly	Azimifar et al., 2014
COL1A1 (Collagen $\alpha$ 1(I))	The rate of collagen synthesis by HSCs isolated from CCl <sub>4</sub> -treated rats is four- to sixfold higher than in HSCs isolated from untreated control animals. The gene is activated during <i>in vitro</i> and <i>in vivo</i> transdifferentiation	Shiratori et al., 1987; Kristensen et al., 2000
COL1A2 (Collagen $\alpha$ 2(I) c-terminal propeptide)	Activated during <i>in vitro</i> and <i>in vivo</i> transdifferentiation	Kristensen et al., 2000
COL3A1 (Collagen $\alpha$ 1(III))	Activated during <i>in vitro</i> and <i>in vivo</i> transdifferentiation	Azimifar et al., 2014
COLEO11 (Collectin-11)	Identified by proteomic functional comparative hotspot analysis of liver cells for genes associated with ECM assembly	Kristensen et al., 2000
COMT (Catechol-o-methyltransferase)	Activated during <i>in vitro</i> and <i>in vivo</i> transdifferentiation	Kristensen et al., 2000
CRTAP (Cartilage-associated protein)	Activated HSCs grown on plastic produce more chondroitin than HSCs grown on a basement membrane-like matrix	Friedman et al., 1989
CSPG4 (Chondroitin, Chondroitin sulphate proteoglycan)	In liver, the gene CSRP2 is exclusively expressed by stellate cells, whereas no transcripts are detectable in hepatocytes, sinusoidal endothelial cells or Kupffer cells	Weiskirchen et al., 2001
CSRP2 (cysteine and glycine-rich protein 2)	Activated during <i>in vitro</i> and <i>in vivo</i> transdifferentiation	Kristensen et al., 2000
CTSD (Cathepsin D)	STAP is dramatically induced in <i>in vivo</i> activated HSCs isolated from fibrotic liver and in HSCs undergoing <i>in vitro</i> activation during primary culture	Kawada et al., 2001; Kawada, 2015
CYGB (Cyroglobin, STAP, stellate cell activation-associated protein)	The steady-state levels of the mRNA for decorin increased fourfold during primary HSC culture	Meyer et al., 1992; Jit et al., 2012
DCN (decorin)	Identified by proteomic functional comparative hotspot analysis of liver cells for genes associated with ECM assembly	Azimifar et al., 2014
DPT (Dermatoptopin)	Desmin increases during culturing in rat HSCs	Ramadori et al., 1990

(Continued)

**TABLE 1 | Continued**

Gene	Experimental finding	Reference
<i>EDSP1</i> (Dermatan sulphate proteoglycan)	Activated HSCs grown on plastic produce more dermatan than HSCs grown on a basement membrane-like matrix	Friedman et al., 1989
<i>ENO2</i> ( $\gamma$ enolase)	Activated during <i>in vitro</i> and <i>in vivo</i> transdifferentiation	Kristensen et al., 2000
<i>FBLN5</i> (Fibulin-5)	Identified by proteomic functional comparative hotspot analysis of liver cells for genes associated with ECM assembly	Azimifar et al., 2014
<i>FDPS</i> (Farnesyl pyrophosphate synthetase)	Activated during <i>in vitro</i> and <i>in vivo</i> transdifferentiation	Kristensen et al., 2000
<i>GAL1</i> (galectin-1)	Activated during <i>in vitro</i> and <i>in vivo</i> transdifferentiation	Kristensen et al., 2000; Jit et al., 2012
<i>GFAP</i> (glial fibrillary acidic protein)	GFAP is a cell type specific marker for HSCs	Neubauer et al., 1996
<i>GKN2</i> (Gastokin-2)	Identified by proteomic functional comparative hotspot analysis of liver cells for genes associated with ECM assembly	Azimifar et al., 2014
Heparan ( <i>HS-PG2</i> )	Activated HSCs grown on plastic produce more heparan than HSCs grown on a basement membrane-like matrix	Friedman et al., 1989
<i>KLF6</i> (Krüppel-like factor 6, ZF9, zinc finger transcription factor 9)	Both the expression and biosynthesis are increased markedly in activated HSCs <i>in vivo</i> compared with quiescent HSCs	Rietz et al., 1998
<i>LOX12</i> (lysyl oxidase-like 2)	Identified by proteomic functional comparative hotspot analysis of liver cells for genes associated with ECM assembly	Azimifar et al., 2014
<i>MMP2</i> (72 kDa type IV collagenase, MMP 2)	Activated during <i>in vitro</i> and <i>in vivo</i> transdifferentiation	Kristensen et al., 2000
<i>MYH11</i> (smooth muscle myosin heavy chain 11)	Activated rat HSCs express this smooth muscle cell marker	Wirz et al., 2008
<i>MYOCD</i> (Myocardin)	Activated rat HSCs express this smooth muscle cell marker	Wirz et al., 2008
<i>NCAM1</i> (Neural cell adhesion molecule 1, CD56)	Rat HSCs specifically express N-CAM and expression is activated during <i>in vitro</i> and <i>in vivo</i> transdifferentiation	Kittel et al., 1996a; Kristensen et al., 2000
<i>NES</i> (nestin)	The neural stem cell marker nestin is induced during activation of rat hepatic stellate cells	Niki et al., 1999
<i>NGF</i> (nerve growth factor)	HSCs show immunoreactivity for the neurotrophin NGF	Cassiman et al., 2001
<i>NGFR</i> (p75(NTR), TNFRSF16, CD271, nerve growth factor receptor)	Activated HSCs express p75(NTR)	Trim et al., 2000;
<i>NTF3</i> (NT3, neurotrophin 3)	HSCs show immunoreactivity for the neurotrophin 3	Cassiman et al., 2001
<i>NTF4/5</i> (NT4/5, neurotrophin 4/5)	HSCs show immunoreactivity for the neurotrophin 4/5	Cassiman et al., 2001
<i>NTRK2</i> (neurotrophic tyrosine kinase receptor type 2, TrkB, Tyrosine kinase receptor B)	HSCs show immunoreactivity for the tyrosine kinase receptors (Trk) B	Cassiman et al., 2001
<i>NTRK3</i> (neurotrophic tyrosine kinase receptor type 3, TrkC, Tyrosine kinase receptor C)	HSCs show immunoreactivity for the tyrosine kinase receptors (Trk) C	Cassiman et al., 2001
<i>P4HA2</i> (Prolyl 4-hydroxylase $\alpha$ )	Activated during <i>in vitro</i> and <i>in vivo</i> transdifferentiation	Kristensen et al., 2000
<i>PAI1</i> (Plasminogen activator inhibitor 1)	PAI-1 production in HSCs is stimulated by TGF- $\beta$	Kittel et al., 1996b
<i>PCDH7</i> (Protocadherin 7)	Identified as a HSC-specific surface marker	Zhang et al., 2015**
<i>PDGFR<math>\alpha</math></i> (PDGFR $\alpha$ , platelet-derived growth factor receptor $\alpha$ )	PDGFR $\alpha$ is primarily expressed in HSCs, and <i>Pdgfra</i> expression increased in injured mouse livers	Hayes et al., 2014
<i>PDGFR<math>\beta</math></i> (PDGFR $\beta$ , platelet-derived growth factor receptor $\beta$ )	PDGFR $\beta$ mRNA and protein were induced in response to TGF- $\beta$ 1 in human HSCs	Pinzani et al., 1995
<i>PLA2R1</i> (PLA2R, phospholipase A2 receptor)	Identified by proteomic functional comparative hotspot analysis of liver cells for genes associated with ECM assembly	Azimifar et al., 2014
<i>PRNP</i> (Prion-related protein PrP $^C$ )	PrP expression is closely related to stellate cell activation	Ikeda et al., 1998
<i>RBPI</i> (CRBP1, cellular retinol-binding protein)	CRBP-1 expression gradually increase during culture activation of HSCs	Uchio et al., 2002; Jit et al., 2012
<i>S100A6</i> (Calcydin, S100 calcium binding protein A6)	Activated during <i>in vitro</i> and <i>in vivo</i> transdifferentiation	Kristensen et al., 2000
<i>S100A11</i> (Calgizzarin, Calcium-binding protein A11)	Activated during <i>in vitro</i> and <i>in vivo</i> transdifferentiation	Kristensen et al., 2000
<i>SLC2A13</i> (Hmit, solute carrier family 2)	Identified by proteomic functional comparative hotspot analysis of liver cells for genes associated with ECM assembly	Azimifar et al., 2014
<i>SMOC2</i> (SPARC-related modular calcium-binding protein 2)	Identified by proteomic functional comparative hotspot analysis of liver cells for genes associated with ECM assembly	Azimifar et al., 2014

(Continued)

**TABLE 1 | Continued**

Gene	Experimental finding	Reference
SPARC (Secreted protein, acidic, cysteine-rich, osteonectin, ON, BM40)	Activated during <i>in vitro</i> and <i>in vivo</i> transdifferentiation	Kristensen et al., 2000
SPP1 (Secreted phosphoprotein 1, Osteopontin)	Osteopontin is significantly increased during the progressive activation of cultured rat HSCs and induced during experimental hepatic fibrosis	Lee et al., 2004
SYP (Synaptophysin)	Synaptophysin is a marker for quiescent as well as activated human and rat HSCs	Cassiman et al., 1999
TAGLN (Transgelin, SM22α)	The SM22α promoter is sufficient to achieve strong or partially selective expression <i>in vitro</i> but is not able to direct a specific or inducible expression of transgenes in HSCs/MFBs <i>in vivo</i>	Herrmann et al., 2004
TGFA (TGF- $\alpha$ , transforming growth factor $\alpha$ )	During transdifferentiation MFBs increasingly express TGF- $\alpha$	Gressner, 1996
TGF $\beta$ 1 (TGF- $\beta$ 1, transforming growth factor- $\beta$ 1)	During transdifferentiation MFBs increasingly express TGF- $\beta$ 1	Gressner, 1996
TGF $\beta$ 3 (TGF- $\beta$ 3, transforming growth factor- $\beta$ 3)	Induced in rat HSCs during culture activation	Jit et al., 2012
TMIP1 (Tissue inhibitor of metalloproteinase 1)	TIMP-1 expression is upregulated in culture-activated rat HSCs and rat models of liver injury and fibrosis	Iredale et al., 1996
TMOD2 (Tropomodulin 2)	Identified by proteomic functional comparative hotspot analysis of liver cells for genes associated with ECM assembly	Azimifar et al., 2014
TNA (Tetranectin)	Identified by proteomic functional comparative hotspot analysis of liver cells for genes associated with ECM assembly	Azimifar et al., 2014
VIM (vimentin)	Vimentin increases during culturing in rat HSCs	Ramadori et al., 1990

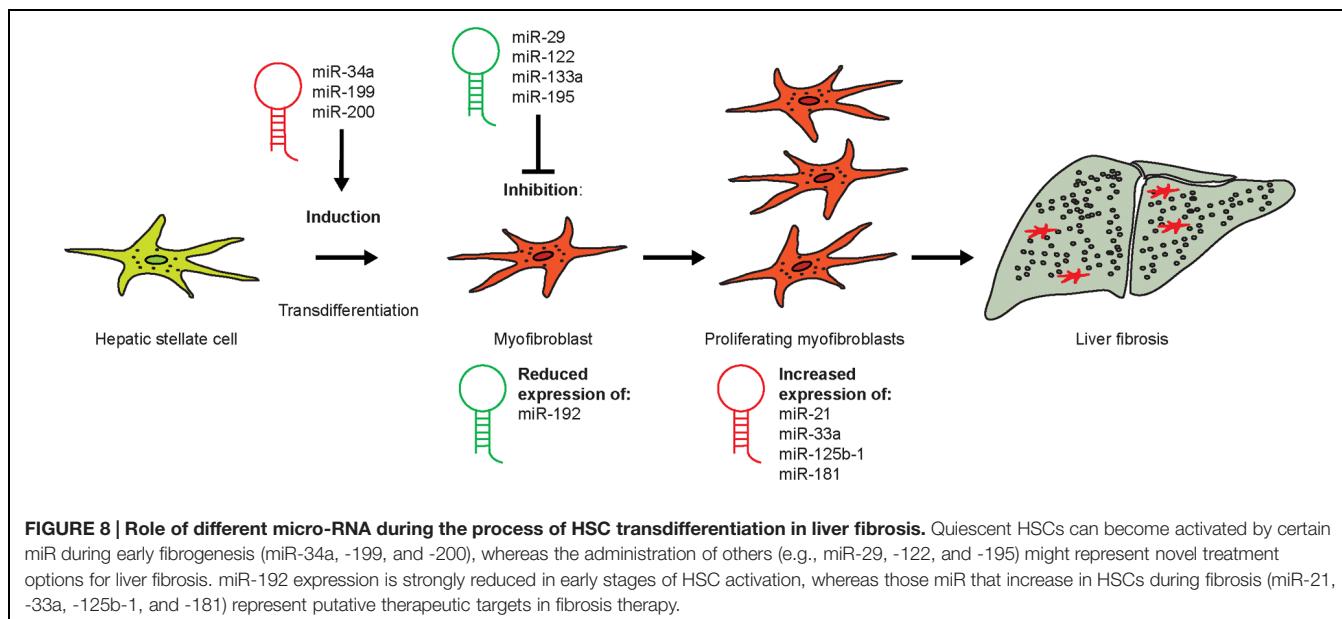
\*In this study, 21 proteins/polypeptides were identified that are transdifferentiation-dependent upregulated *in vitro* and *in vivo*, and a couple of proteins that become differentially activated during both experimental settings. \*\*This proteome study identified 319 rat proteins that were significantly upregulated during HSC activation from which 126 were already identified in a previous study (Kristensen et al., 2000). \*\*\*This study compared HSCs, immune and hepatic transcriptome profiles and defined a 122-gene HSC signature.

## miRNA-BASED STRATEGIES FOR HSC TARGETING

Micro-RNAs (miR) are small but powerful molecules, which usually size around 20 nucleotides and affect the regulation of mRNA (Lam et al., 2015). miR act by binding to the 3' untranslated region (3' UTR) of the target mRNA (Lam et al., 2015). Since the complementarity of miR-to-mRNA sequence is only partial, the miR can bind to multiple different mRNAs (Lam et al., 2015). Bioinformatics-based predictions have led to the assumption that about 60% of protein coding genes are regulated by miR (Friedman et al., 2009). Many different miR are involved in inflammatory diseases (Roy et al., 2015). Inflammation is considered the main initiating event in fibrogenesis and an important driver of fibrosis. During inflammation and fibrogenesis, HSCs undergo transdifferentiation into activated MFBs accompanied with cell proliferation. Several different miRs have specific roles during the process of HSC transdifferentiation in liver fibrosis (Figure 8).

Moreover, miR-34a and -199 play pivotal roles in the initiation of HSC transdifferentiation (Roy et al., 2015). miR-34a, which targets ASCL1, is upregulated during the initiation of liver fibrosis in a model of DMN-induced fibrosis (Li et al., 2011). The upstream target of miR-34a is the tumor suppressor p53 that can initiate cell cycle arrest, which explains the effects on HSC proliferation (Okada et al., 2014). Serum levels of miR-34a were also reported to be increased in patients with NAFLD (Cermelli et al., 2011). A hallmark study identified key miRs using miR arrays by comprehensively studying experimental fibrosis models in mice (Roderburg et al., 2011). Among these miRs, miR-29b is part of a signaling nexus involving TGF- $\beta$ - and NF- $\kappa$ B-dependent signals in HSCs (Roderburg et al., 2011), while miR-133a mediates TGF- $\beta$ -dependent derepression of collagen synthesis in HSCs (Roderburg et al., 2013). These and other studies suggested circulating miR may be potentially a novel diagnostic biomarker for liver fibrosis progression (Roderburg et al., 2012). For instance, members of the miR-199 and miR-200 family, namely miR-199a, -199a\*, -200a, and -200b, correlated with the progression of liver fibrosis both in murine models and human patients (Murakami et al., 2011).

Besides being critical initiators of HSC activation and fibrogenesis, there are also miR, which exhibit therapeutic potential due to their inhibiting effects on HSC disease progressing activation and which can therefore be designated as antifibrotic. The miR-29 was reported to down-regulate ECM synthesis in mouse models and human patients by among others targeting several collagen-associated genes (Bandyopadhyay et al., 2011). Characterized by the sole inhibition of P4HA1, the liver-specific miR-122 was found to reduce proliferation and activation of HSCs (Li et al., 2013; Shah et al., 2013). Down-regulation of cyclin E1 was shown for miR-195, and thereby, proliferation of HSCs was reduced in humans (Sekiya et al., 2011). Recently, miR-192 was identified as an important molecule in the transdifferentiation process of HSCs by Coll et al. (2015). It was strongly downregulated in cirrhotic livers compared to healthy organs as an early event during fibrosis progression and had a large number of 28 predicted target genes, based on an



analysis of human HSCs. The authors found miR-192 to be strongly downregulated (nearly abrogated) already during early CCl<sub>4</sub>-based liver fibrosis (Coll et al., 2015). These observations put miR-192 into the role of a promising biomarker or target for early fibrosis.

After having progressed into activated, proliferating HSCs, the cells were observed to express a variety of miR indicative for their activation status. These molecules represent potential molecular targets for interventions, such as miR21, which modulates ERK1 signaling in HSC activation and the EMT of hepatocytes *via* inhibiting SPRY2 and HNF4α mRNA (Zhao et al., 2014). miR-33a particularly increases during the progression of human and murine liver fibrosis in HSCs only. Toward their stimulation with TGF-β, HSCs react with upregulation of miR-33a, a regulator of lipid and cholesterol metabolism, which probably acts through Smad7 (Huang et al., 2015). The expression of miR125b-1 was described as being significantly increased in HSCs of liver fibrosis patients (Coll et al., 2015). miR-181b, which activates HSCs based on the phosphatase and tensin homolog deleted on chromosome 10 (PTEN)/Akt pathway, and further correlates with human liver disease progression (Yu et al., 2015).

## CLINICAL TRANSLATION OF PRECLINICAL FINDINGS

Despite numerous innovative anti-fibrotics in preclinical settings, the number of those entering clinical studies is limited. At present, the best option for fibrosis treatment remains in eliminating the underlying cause of liver disease. For instance, antiviral therapies in virus hepatitis, alcohol abstinence or lifestyle changes represent effective antifibrotic measures in the respective liver diseases (Tacke and Trautwein, 2015).

Many preclinical studies suffer from the drawback that they focus on single cells or molecules, which help to identify mechanisms but ignore the complexity of biological systems. For instance, IFN-α or IFN-γ act as efficient anti-fibrotics *in vitro* (Mallat et al., 1995), but were not successful in the clinics (Fernández et al., 2006; Di Bisceglie et al., 2008). Earlier attempts to neutralize TGF-β suffer from a large number of effects of the cytokine on other cell types when administered parenterally. Therefore, specificity is an important issue, which has to be addressed more properly. For this aim, nanotechnological and innovative formulations, which specifically target HSC-expressed receptors such as the Rho kinase inhibitor Y27632 coupled to mannose-6-phosphate, may point toward novel directions (van Beuge et al., 2011).

Despite the fact that the existence of fibrosis regression represents a major source of hope for the success of novel treatment options, it has to be considered further that many of the standard models of hepatic rodent fibrosis, such as CCl<sub>4</sub>-mediated liver fibrosis, is reversible, which is in big contrast to human liver cirrhosis. It is therefore advisable that rodent models with little degree of regression should be used as well. Additionally, the time frame for human fibrogenesis usually comprises years but not weeks. Nevertheless, new compounds targeting such “late events” are currently under investigation. The antibody simtuzumab antagonizes LOXL2, which is an enzyme that cross-links collagen fibers as a late event in fibrogenesis (Barry-Hamilton et al., 2010).

Another challenge is the design of proper clinical trials, including suitable endpoints for such trials. At the moment, most trials rely on serial liver biopsies. Novel biomarkers, which allow specific fibrosis staging, and other non-invasive methods are promising novel directions for monitoring the success of antifibrotic therapies (Schuppan and Pinzani, 2012). Progresses in HSC targeting with nanotechnological formulations or innovative molecules such as miR might help to finally bring

novel personalized therapeutic strategies to the clinics (Schuppan and Pinzani, 2012).

## ETHICS STATEMENT

The analysis of human samples (**Figure 2**) was approved by the Institutional Review Board of the Bonn University Ethics Committee (decision #067/10). Isolation of primary rat liver cells (**Figure 5**) and application of antifibrotic gene devices (**Figure 4**) was approved by the Bezirksregierung Köln (Cologne, Germany) and the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (Recklinghausen, Germany).

## AUTHOR CONTRIBUTIONS

H-TS, MB, TL, FT, and RW have written this review. EB-K performed the experiments depicted in **Figure 2**. JN provided the human samples and requested the necessary ethic vote for the experiments shown in **Figure 2**. All authors have read this review and gave their agreement for submission.

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# The P2X7 Receptor-Interleukin-1 Liaison

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Interleukin-1 $\beta$  (IL-1 $\beta$ ) plays a central role in stimulation of innate immune system and inflammation and in several chronic inflammatory diseases. These include rare hereditary conditions, e.g., auto-inflammatory syndromes, as well as common pathologies, such as type II diabetes, gout and atherosclerosis. A better understanding of IL-1 $\beta$  synthesis and release is particularly relevant for the design of novel anti-inflammatory drugs. One of the molecules mainly involved in IL-1 $\beta$  maturation is the P2X7 receptor (P2X7R), an ATP-gated ion channel that chiefly acts through the recruitment of the NLRP3 inflammasome-caspase-1 complex. In this review, we will summarize evidence supporting the key role of the P2X7R in IL-1 $\beta$  production, with special emphasis on the mechanism of release, a process that is still a matter of controversy. Four different models have been proposed: (i) exocytosis via secretory lysosomes, (ii) microvesicles shedding from plasma membrane, (iii) release of exosomes, and (iv) passive efflux across a leaky plasma membrane during pyroptotic cell death. All these models involve the P2X7R.

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## THE INFLAMMATORY PROCESS

Inflammation has been the object of countless studies and experimental observations since its definition more than 2000 years ago (Celso, De Medicina, 47 CE). Nevertheless, many aspects of this process are not fully understood, and therefore inflammation is still nowadays a field of extensive investigation, especially in view of its crucial role in the pathogenesis of many acute and chronic diseases. Accordingly, inflammation is a fertile ground of research for the development of novel drugs. Diverse chemical mediators with pro- or anti-inflammatory activity have been identified over the years. These range from histamine to bioactive lipids, e.g., prostaglandins and leukotriens, from free radicals, e.g., reactive oxygen species (ROS) and nitric oxide (NO), to cytokines, e.g., interleukins (ILs) and tumour necrosis factor (TNF). Among all these mediators, interleukin-1 $\beta$  (IL-1 $\beta$ ) is recognized as one of the earliest and most potent pro-inflammatory agents synthesized and released in response to infectious agents and injuries, and therefore central to both septic and sterile inflammation (Gabay et al., 2010; Dinarello, 2011).

## AN OVERVIEW ON INTERLEUKIN-1 (IL-1)

The term Interleukin-1 (IL-1), also known as leukocyte endogenous mediator, hematopoietin 1, endogenous pyrogen, catabolin and osteoclast activating factor, was used in the past to indicate a factor mediating many different pro-inflammatory and catabolic effects.

The history of IL-1 begins with studies on the endogenous factor produced by activated leukocytes that causes fever. As such, IL-1 was originally described in Menkin (1943), who reported the isolation of a pyrogenic euglobulin from inflammatory exudate named “pyrexin” or “endogenous pyrogen.”

These initial studies were followed by the groundbreaking contributions of Beeson (1948) who confirmed Menkin's observation and further reported that an endotoxin-free, protein-containing material, released from rabbit peritoneal leukocytes, caused the rapid onset of fever after injection into rabbits. This was the first time in which the mechanism behind fever, in the absence of infection, was described. After Beeson's paper, there was a surge of studies on the links between infection/inflammation and fever, that culminated in the demonstration by Bodel and Atkins (1967) that human blood monocytes produced a pyrogen, similar to that released by rabbit neutrophils, by *de novo* synthesis.

Gery and Waksman (1972) described the effect on lymphocyte proliferation of soluble factors released in response to antigenic or mitogenic stimuli, and a few years later Dinarello and Bernheim (1981) purified the human leukocytic pyrogen from peripheral blood mononuclear cells (PBMCs) *in vitro* stimulated with heat-killed *Staphylococcus epidermidis*. Leukocytic pyrogen was also shown to enhance T cells responses to antigens and to promote synthesis of acute phase proteins (Kampschmidt et al., 1973).

Initially, the vast number of biological activities attached to a single molecule generated some confusion in the scientific community, however, with the cloning of IL-1 by Lomedico et al. (1984), the use of recombinant IL-1 established that IL-1 was indeed a pleiotropic cytokine mediating a great variety of inflammatory, as well as immunological, responses. Thanks to the seminal work of Dinarello, we now know that IL-1 is the founding member of a family of cytokines.

The IL-1 cytokine family consists of 11 members with different roles in inflammation. Seven of them, i.e., IL-1 $\alpha$ , IL-1 $\beta$ , IL-18, IL-33, IL-36 $\alpha$ , IL-36 $\beta$  and IL-36 $\gamma$ , own well-demonstrated pro-inflammatory properties, whereas four members, IL-1Ra, IL-36Ra, IL-37 and IL-38, are anti-inflammatory (Garlanda et al., 2013; Borthwick, 2016). Cytokines of the IL-1 family ligate and activate specific plasma membrane receptors, the IL-1 receptor family, comprised of 10 members, named IL-1R1, IL-1R2, IL-1R3 (IL-1RACP), IL-R4 (ST2), IL-1R5 (IL-18R $\alpha$ ), IL-1R6 (IL-R36), IL-1R7 (IL-18R $\beta$ ), IL-1R8 (SIGIRR: single Ig IL-1R-related molecule or TIR8: three Ig domain-containing IL-1R related), IL-1R9 (IL-33R), IL-1R10 (TIGIRR-1) (Garlanda et al., 2013).

Interleukin-1 $\beta$  (IL-1 $\beta$ ), a crucial factor of host defense in response to infections and injuries, is the best characterized and most extensively studied member of the IL-1 family (Dinarello, 1996). In the last decade, IL-1 $\beta$  has also emerged as a causative agent and a therapeutic target for an expanding number of systemic and local inflammatory conditions named “auto-inflammatory diseases.” The auto-inflammatory diseases include rare hereditary conditions as well as common pathologies. Recently, increasing evidence shows that the same pathogenetic mechanisms responsible for the activation of innate immunity in inherited auto-inflammatory diseases may also play a key

role in sustaining inflammation in several frequent multifactorial pathologies, such as type II diabetes, gout, pseudogout, and atherosclerosis (Ginaldi et al., 2005).

IL-1 $\beta$ , usually not expressed by healthy resting cells, is mainly produced by activated inflammatory cells of the myeloid lineage. Production of IL-1 $\beta$  is a multistep process involving synthesis of immature pro-IL-1 $\beta$ , proteolytic cleavage to mature IL-1 $\beta$  and, finally, release into the extracellular environment. Synthesis of the immature full-length pro-IL-1 $\beta$  is started with the recognition via Toll-like receptors (TLRs) of molecules derived from invading micro-organisms [pathogen-associated molecular patterns (PAMPs)] (Janeway, 2001). Once synthesized, the 31 kD pro-IL-1 $\beta$  undergoes a proteolytic cleavage catalyzed by caspase-1 (casp-1) which removes 116 N-terminal aminoacids to generate the 17 kD bioactive form, now ready to be secreted. If conversion to the 17 kD form does not occur, pro-IL-1 $\beta$  is polyubiquitinated and targeted for proteasomal degradation (Ainscough et al., 2014). Activation of casp-1, in turn, depends on assembly and activation of inflammasomes, multisubunit organelles that convert pro-casp-1 to active casp-1 (Thornberry et al., 1992; Martinon et al., 2002; Ogura et al., 2006).

The NLRP3 inflammasome has been investigated in depth and recognized as a very, likely the most, efficient machinery for pro-IL-1 $\beta$  maturation, and the biology of this cytokine has been intimately intertwined with that of the inflammasomes and of inflammasome-activating agents (Martinton et al., 2002; Di Virgilio, 2013). Inflammasomes are high molecular weight protein complexes assembled in the cytosolic compartment in response to a variety of stimuli, either of exogenous (PAMPs) or endogenous [danger/damage associated molecular patterns (DAMPs)] origin. PAMPs include bacteria- as well as virus derived components, whereas DAMPs encompass different classes of molecules normally segregated inside the cells (Venereau et al., 2015). DAMPs are released in response to invasion by micro-organisms (septic inflammation) as wells as to physical, chemical, metabolic non-infectious agents (sterile inflammation) (Gallucci et al., 1999). DAMPs released in the extracellular milieu fulfill the task of alerting surrounding cells, especially of immune lineages, of an incumbent danger or a damage (Venereau et al., 2015; Nie et al., 2016). Among DAMPs, extracellular ATP and other nucleotides play an undisputed role.

Nucleotide signaling is central in IL-1 $\beta$  maturation and release, as well as in other immune responses, such as neutrophil and macrophage chemotaxis, intracellular microbe killing, NADPH-oxidase activation, T lymphocyte proliferation and differentiation (Di Virgilio, 1995; Bours et al., 2006; Ferrari et al., 2006; Junger, 2011; Eltzschig et al., 2012; Idzko et al., 2014; Cekic and Linden, 2016). Extracellular ATP acts at plasma membrane purinergic P2 receptors, chiefly the P2X7 receptor (P2X7R) subtype, to drive NLRP3 inflammasome activation and IL-1 $\beta$  processing and release (Ferrari et al., 1997). ATP is released into extracellular environment during inflammation, ischemia, hypoxia, or other harmful events, via lytic (e.g., cell necrosis) or non-lytic (e.g., exocytosis, plasma membrane channels or pores) pathways. Pathways for non-lytic ATP release include pannexins (Dahl, 2015), connexins (Evans et al., 2006), ABC transporters (Cantiello, 2001), secretory vesicles (Sneddon and

Westfall, 1984; Wang et al., 2013), and the P2X7R (Pellegatti et al., 2005; Suadicani et al., 2006).

## THE P2X7R

Several reports underscore the pivotal role of ATP-mediated P2X7R activation in IL-1 $\beta$  release from activated immune cells (monocytes, macrophages, and microglia) (Di Virgilio et al., 1998; Pelegrin et al., 2008; Sanz et al., 2009). Macrophages from genetically modified mice lacking the P2X7R, ASC or NLRP3, do not release IL-1 $\beta$  in response to ATP (Solle et al., 2001; Mariathasan et al., 2004, 2006). Moreover, oxidized ATP, an irreversible blocker of the P2X7R (Murgia et al., 1993) abrogates ATP-induced IL-1 $\beta$  release from immune cells (Ferrari et al., 1997). P2X7R stimulation also induces fast release into the cytosol of oxidized mitochondrial DNA (mitoDNA) that promotes NLRP3 inflammasome assembly by direct interaction (Nakahira et al., 2011; Shimada et al., 2012).

The P2X7R is a bi-functional ATP-gated plasma membrane ion channel that upon sustained stimulation undergoes a transition that generates a non-selective pore permeable to aqueous solutes of MW up to 900 Da (Di Virgilio, 2000). The P2X7R is widely distributed in human tissues, the highest expression being in cells of the immune and inflammatory systems, especially of the myeloid lineage (Di Virgilio, 1995, 2015; Karmakar et al., 2016). The P2X7R is the seventh, and latest to be cloned, member of the P2X receptor (P2XR) subfamily activated by an agonist concentration about 100 fold higher than the other members of the family. P2XRs are ATP-gated channels permeable to monovalent ( $\text{Na}^+$ ,  $\text{K}^+$ ) and divalent ( $\text{Ca}^{2+}$ ) cations formed by the assembly of the same (homo) or different (hetero) P2X subunits. Six homomeric (P2X1R-P2X5R and P2X7R) and six heteromeric (P2X1/2R, P2X1/4R, P2X1/5R, P2X2/3R, P2X2/6R, and P2X4/6R) functional P2XRs have been described so far (Dubyak, 2007; North, 2016). Among P2X subunits, the P2X7 is generally thought not to assemble with the others, and thus forming P2X7 only homomeric channels. High sequence homology of P2X7R with the P2X4R (41% identity, 71% similarity), suggests a common origin by gene duplication. Therefore, the solved crystal structure for zebrafish P2X4R (Kawate et al., 2009; Hattori and Gouaux, 2012) has been used to model the 3D conformation of the P2X7R (Jiang et al., 2013). Useful insights as to ATP binding pocket, ion permeation pathway, site of antagonist binding and interaction with allosteric modulators are also derived from the crystal structure of the panda P2X7R (Karashawa and Kawate, 2016). Further information are ensued by recent 3D resolution of the human P2X3R (Mansoor et al., 2016).

The P2X subunits are characterized by a large extracellular loop, which includes agonist- and antagonist-binding sites, two short transmembrane domains, and intracellular N- and C-termini. The P2X7R with an extended C-terminal tail of 239 aa and an overall length of 595 aa, is the largest in the P2XR family. Transmembrane domains are responsible for the interactions among subunits and the formation of the ion-permeation pathway (Hattori and Gouaux, 2012; Grimes and Young, 2015).

The intracellular C-tail interacts with different intracellular molecules such as heat shock proteins (HSP), cytoskeletal components, kinases and possibly also with membrane proteins. Among these latter, pannexin-1 and connexin-43 hemichannels have been variably implicated in the formation of the P2X7R-associated large-conductance pore and therefore in P2X7R-dependent IL-1 $\beta$  secretion, and in the release of extracellular ATP (Pelegrin and Surprenant, 2007; Baroja-Mazo et al., 2013). P2X7R has also been found to interact directly with components of inflammasomes, such as NLRP2, ASC (apoptosis-associated speck-like protein containing a CARD) and NLRP3 (Minkiewicz et al., 2013; Franceschini et al., 2015; Salaro et al., 2016). P2X7R activation by ATP is one of the most potent stimuli for NLRP3 inflammasome activation (Mariathasan et al., 2006; Munoz-Planillo et al., 2013).

## THE NLRP3 INFLAMMASOME

Inflammasomes are cellular organelles with a fundamental role in inflammation and cell death (Martinon et al., 2002; Guo et al., 2015; Rathinam and Fitzgerald, 2016). The basic scaffold subunit is a nucleotide-binding and oligomerization domain (NOD)-like receptor (NLR) that contains a C-terminal leucine-rich repeat (LRR) domain, a central NACHT nucleotide-binding domain (NOD) and an N-terminal pyrin domain (a CARD domain in the NLRC4 inflammasome). The pyrin domain of the NLR scaffold subunit interacts with the pyrin domain of an adaptor molecule named ASC. NLR-driven ASC recruitment drives pro-casp-1 activation via CARD domains present on both ASC and pro-casp-1, thus resulting in pro-casp-1 cleavage and casp-1 activation. Casp-1 then cleaves pro-IL-1 $\beta$  and pro-IL-18 to produce the mature forms of both cytokines (Benko et al., 2008; Schroder and Tschoop, 2010; Broz and Dixit, 2016; Prochnicki et al., 2016). Inflammasomes play a cardinal role in innate immunity thanks to their ability to sense PAMPs and DAMPs (He et al., 2016a; Kim et al., 2016). Within the subfamily of NLRP inflammasomes (i.e., inflammasomes based on NLR scaffold molecules with an N-terminal pyrin domain) NLRP3 is currently enjoying the widest popularity as crucial sensor for a large number of danger signals and as the main platform for IL-1 $\beta$  processing. Activating stimuli for the NLRP3 inflammasome include bacterial toxins, flagellin, muramyl dipeptide, viral nucleic acids and fungal products, as well as endogenous components such as ATP, cholesterol crystals, monosodium urate, glucose and amyloid  $\beta$ , environmental pollutants, such as silica, asbestos or physical agents such as UV radiations (Kim et al., 2016).

The identity of the activating stimulus of the NLRP3 inflammasome has been a hot issue ever since its discovery. Nowadays there is basically general consensus on the key role played by  $\text{K}^+$  efflux, which seems to be the final common pathway for many different agents (Munoz-Planillo et al., 2013). Most efficient NLRP3 activators include extracellular ATP,  $\text{K}^+$  ionophores, and several extracellular crystals, all known to decrease the cytosolic  $\text{K}^+$  level. The mechanism whereby these different agents lower  $\text{K}^+$  is not entirely clear, but many converge

on P2X7R activation (Alves et al., 2014; Prochnicki et al., 2016). In fact, while P2X7R opening or nigericin, a carboxylic K<sup>+</sup> ionophore, directly allow K<sup>+</sup> efflux along its concentration gradient, the mechanism by which crystals, such as monosodium urate, deplete intracellular K<sup>+</sup> is obscure. To support the contribution of K<sup>+</sup> depletion, drugs inhibiting the Na<sup>+</sup>/K<sup>+</sup>-ATPase also trigger NLRP3 inflammasome activation (Walev et al., 1995; Munoz-Planillo et al., 2013). Albeit inhibition of Na<sup>+</sup>/K<sup>+</sup>-ATPase also causes plasma membrane depolarization, there is no evidence that depolarization itself may trigger P2X7R pore opening and/or IL-1 $\beta$  release (Di Virgilio, 2013). The central role of intracellular K<sup>+</sup> is further supported by the finding that a K<sup>+</sup> drop is also necessary to allow recruitment of the Nima-related kinase (NEK)7 protein to the NLRP3 inflammasome (He et al., 2016b). On the other hand, the mechanism by which the drop in the K<sup>+</sup> concentration drives NEK7 recruitment, NLRP3 inflammasome assembly and activation is utterly unknown.

The NLRP3 inflammasome can be also activated by a non-canonical pathway involving casp-11. Casp-11, and its human orthologs casp-4 and -5, function as cytosolic LPS sensors (Shi et al., 2014). Once activated by LPS, casp-11 induces cleavage of the plasma membrane channel pannexin-1 (Yang et al., 2015) producing two events consisting of K<sup>+</sup> efflux, that activates NLRP3, and release of ATP that acts as a P2X7R agonist to promote further NLRP3 activation and cell death (Yang et al., 2015). The casp-11/pannexin-1/NLRP3 inflammasome axis is proposed to promote IL-1 $\beta$ /IL-18 production (Yang et al., 2015). In addition, active casp-11 triggers pyroptosis via cleavage of Gasdermin D (GSDMD) leading to accumulation of free active N-terminal domains of this protein which disrupt cellular functions by forming plasma membrane pores (He et al., 2015; Vince and Silke, 2016). Casp-11-mediated cell death, like casp-1-induced pyroptosis, requires cleavage of the GSDMD pyroptotic factor (He et al., 2015; Kayagaki et al., 2015; Shi et al., 2015). Casp-11 mediated cell death is indeed abrogated in GSDMD deficient cells and, although it is not clear if GSDMD is the terminal pyroptotic factor, its N-terminal domain released following casp-11-dependent cleavage is sufficient to cause pyroptosis (Kayagaki et al., 2015; Shi et al., 2015). It has been proposed that, since casp-1 is required for both pyroptosis and IL-1 $\beta$  cleavage, IL-1 $\beta$  is passively released alongside DAMPs following plasma membrane rupture (Vince and Silke, 2016). The finding that in macrophages lack of GSDMD has no effect on NLRP3-stimulated IL-1 $\beta$  processing by casp-1 but prevents IL-1 $\beta$  secretion (He et al., 2015; Shi et al., 2015), suggests that casp-1 is necessary for IL-1 $\beta$  cleavage whereas GSDMD is indispensable for its release. Recent findings have revealed that in human monocytes stimulated with LPS casp-4 and -5 act as key determinants in one-step non-canonical NLRP3 inflammasome activation culminating with IL-1 $\beta$  release (Vigano et al., 2015). This one-step pathway has been suggested to require Syk activity and Ca<sup>2+</sup> influx due to CD14/TLR4-mediated LPS internalization (Vigano et al., 2015). NLRP3 activation and IL-1 $\beta$  release can also be driven by K<sup>+</sup> independent mechanisms involving ROS generation or RIPK1/FADD/casp-8 recruitment (Zhou et al., 2011; Heid et al., 2013; He et al., 2016a; Sanman et al., 2016). Converging experimental findings seem to rule out a role for cytosolic Ca<sup>2+</sup>

increases (Brough et al., 2003; Rada et al., 2014; Katsnelson et al., 2015). In some non-immune cells, e.g., astrocytes, IL-1 $\beta$  maturation has been reported to be due to P2X7-dependent NLRP2 stimulation via a process involving direct NLRP2, P2X7R, pannexin-1 interaction (Minkiewicz et al., 2013). Finally, IL-1 $\beta$  can also be processed independently of inflammasome/casp-1 activation, as shown in casp-1 deficient mice, where pro-IL-1 $\beta$  to IL-1 $\beta$  extracellular conversion is catalyzed by various neutrophil proteases such as elastase, proteinase-3, granzyme A and cathepsine G (Fantuzzi et al., 1997; Joosten et al., 2009).

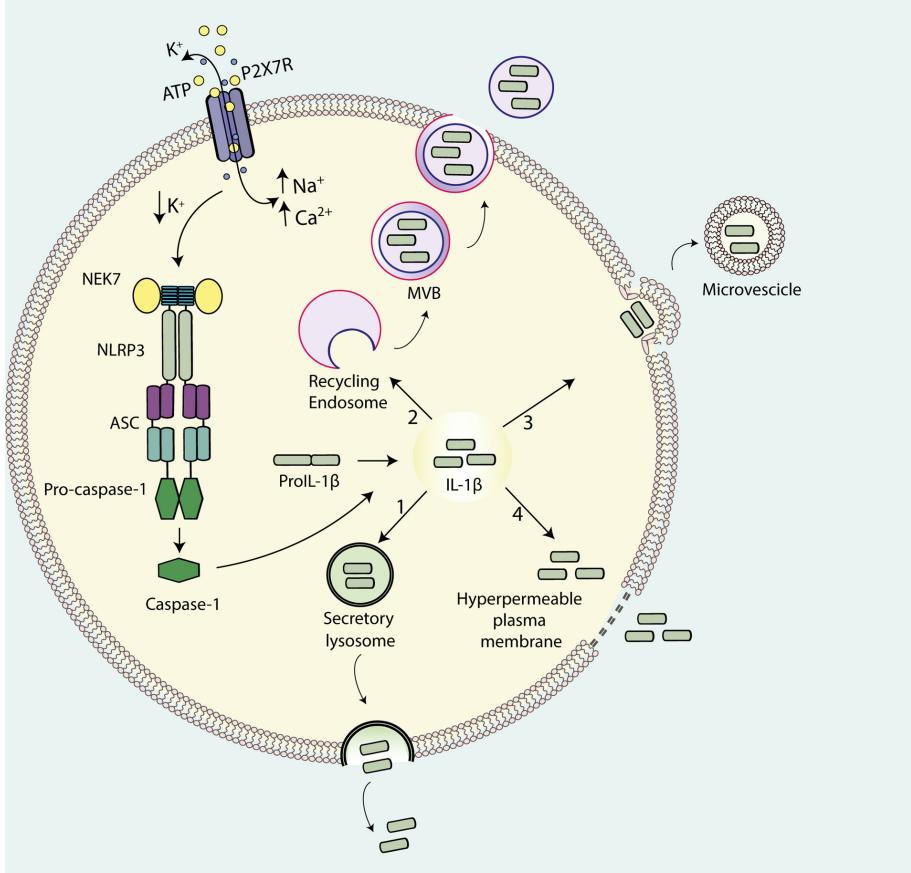
P2X7R stimulation by itself has no or little effect on pro-IL-1 $\beta$  cytoplasmic accumulation, therefore cells need priming by agents that promote IL-1 $\beta$  gene transcription, which mainly occur via NF $\kappa$ B activation. Typical priming agents are bacterial lipopolysaccharide, zymosan and poly(I:C) (Ferrari et al., 1996; Facci et al., 2014).

## IL-1 $\beta$ RELEASE

The canonical pathway for the export of cellular proteins into the extracellular space involves the ER and the Golgi apparatus that together form the endo-membrane system through which the vast majority of proteins are either targeted to the extracellular space or to specialized sub-cellular compartments. At variance with other cytokines, IL-1 $\beta$  lacks the conventional leader/signal peptide and therefore is not targeted to the conventional ER-Golgi secretory pathway (Rubartelli et al., 1990). This leads to IL-1 $\beta$  accumulation into the cytosol after translation on free ribosomes. Moreover, conversion of pro-IL-1 $\beta$  to the mature form by inflammasomes also takes place in the cytosol. Therefore, release of mature IL-1 $\beta$  requires non-classical mechanisms of export from the cytosolic compartment (Rubartelli et al., 1990; Wewers, 2004; Eder, 2009). A number of different possible mechanisms have been proposed (Dubyak, 2012) and summarized in **Figure 1**. They include exocytosis via secretory lysosomes (Andrei et al., 1999; Andrei et al., 2004), microvesicle shedding from plasma membrane (MacKenzie et al., 2001; Bianco et al., 2005; Pizzirani et al., 2007), release of exosomes (Qu et al., 2007), and, lastly, passive efflux across a leaky plasma membrane during pyroptotic cell death (Bergsbaken et al., 2009; Martin-Sanchez et al., 2016). The P2X7R has been implicated in all these processes.

## EXOCYTOSIS OF IL-1 $\beta$ -CONTAINING SECRETORY LYOSOMES

Rubartelli et al. (1990) presented the first evidence for a non-classical secretory pathway for IL-1 $\beta$  release. Blockade of protein transport and secretion through the ER-Golgi complex did not affect IL-1 $\beta$  release, thus pointing to the involvement of secretory lysosomes. Secretory lysosomes are unusual organelles found principally in hematopoietic cells with a dual-function, degradative and secretory (Blott and Griffiths, 2002). The exocytic process can be triggered by different stimuli among which ATP, possibly via the increase in the intracellular Ca<sup>2+</sup>



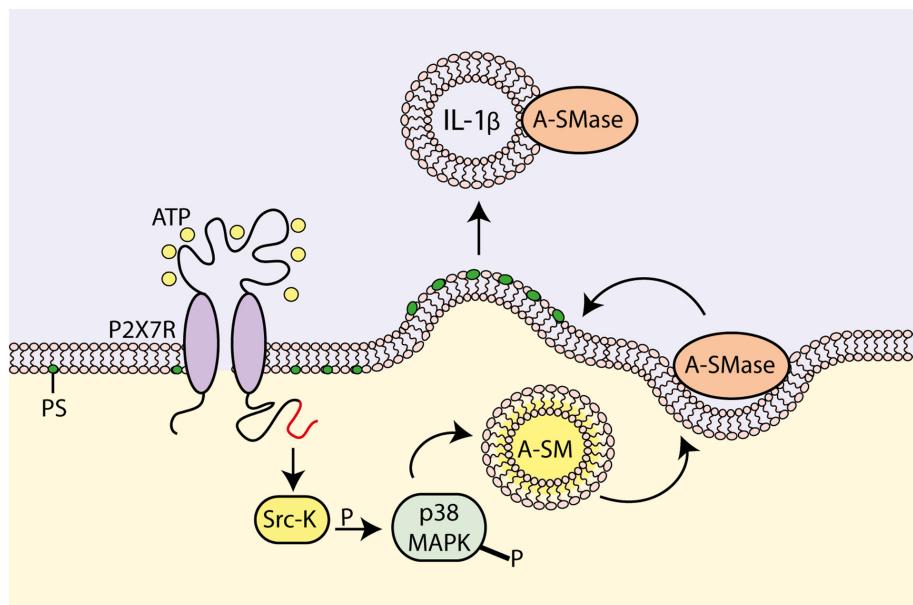
**FIGURE 1 | Pathways for IL-1 $\beta$  release from activated immune cells.** IL-1 $\beta$  maturation is catalyzed by ATP-mediated stimulation of the P2X7R that drives NLRP3 inflammasome assembly and casp-1 recruitment. Four models have been proposed for IL-1 $\beta$  release: (1) exocytosis of secretory lysosomes; (2) shedding of plasma membrane-derived microvesicles; (3) exocytosis of multivesicular body (MVB)-derived exosomes; (4) passive efflux across hyperpermeable plasma membrane during pyroptotic cell death.

concentration. Migration of exocytic lysosomes to the plasma membrane is a microtubule-dependent process that brings the lysosomes close to the plasma membrane allowing fusion and release of their content into the extracellular space. This model for IL-1 $\beta$  secretion is mainly based on morphological evidence from ATP-stimulated monocytes where IL-1 $\beta$  was found to be trapped within organelles akin to late endosomes and early lysosomes (Andrei et al., 1999). In human monocytes and mouse macrophages, ATP-stimulated, P2X7R-dependent release of mature IL-1 $\beta$  and casp-1 strongly correlated with secretion of the lysosomal markers cathepsin B, cathepsin D and lysosomal-associated membrane protein 1 (LAMP1) (Andrei et al., 1999; Carta et al., 2006). Both IL-1 $\beta$  and casp-1 are found in the extracellular medium 20 min after ATP stimulation, suggesting a similar time course. According to Rubartelli and coworkers a fraction of intracellular pro-IL-1 $\beta$  is co-stored together with pro-casp-1 within the secretory lysosomes, ready to be secreted in response to P2X7R stimulation (Rubartelli et al., 1990). The triggering stimulus is thought to be the P2X7R-induced loss of intracellular K $^{+}$ , which activates a phosphatidylcholine-specific phospholipase C, which in turn causes an increase in

cytosolic Ca $^{2+}$ , Ca $^{2+}$ -dependent phospholipase A<sub>2</sub> activation and finally exocytosis of the IL-1 $\beta$ -containing lysosomes. These events are blocked by inhibitors of phospholipase A<sub>2</sub> or phosphatidylcholine-specific phospholipase C. This model suggests that, whereas the massive K $^{+}$  efflux due to P2X7R activation has a key role in the maturation of pro-IL-1 $\beta$ , the intracellular Ca $^{2+}$  increase is more directly responsible for IL-1 $\beta$  secretion.

## SHEDDING OF IL-1 $\beta$ -CONTAINING PLASMA MEMBRANE MICROVESICLES

Surprenant and coworkers proposed a different vesicular mechanism for IL-1 $\beta$  release from THP-1 monocytes (MacKenzie et al., 2001). According to this mechanism, P2X7R stimulation induces mature IL-1 $\beta$  accumulation at discrete sub-plasmalemmal sites, where from it is then trapped into small plasma membrane blebs that are finally rapidly shed as microvesicles into the extracellular space (Figure 2). Microvesicle shedding is preceded by flip of phosphatidylserine



**FIGURE 2 | Molecular mechanism for P2X7R-dependent microvesicle shedding.** P2X7R activation promotes interaction of the C-terminal domain with a src-protein tyrosine kinase (Src-K), which in turn phosphorylates p38 MAP kinase (p38 MAPK). p38 MAPK induces flip of acidic sphingomyelinase (A-SMase) from the inner to the outer plasma membrane leaflet. On the outer plasma membrane leaflet, A-SMase hydrolyzes sphingomyelin to generate ceramide that in turn alters membrane fluidity, drives formation of plasma membrane blebs and promotes shedding of IL-1 $\beta$ -containing microvesicles (modified from Bianco et al., 2009).

(PS) to the outer leaflet of the plasma membrane. Microvesicles size ranges from 200 nm to 1  $\mu$ m, which makes them distinct from the much larger apoptotic bodies derived from apoptotic cells (1–4  $\mu$ m size), and the smaller exosomes derived from intraluminal vesicles of endosomal multivesicular bodies (MVBs). A similar mechanism for IL-1 $\beta$  release has also been observed in human monocyte-derived dendritic cells (DCs) and mouse microglia (Bianco et al., 2005; Pizzirani et al., 2007). Shed microvesicles contain (a) plasma membrane phospholipids, e.g., PS (MacKenzie et al., 2001), (b) membrane intrinsic proteins, such as P2X7R, CD63, CD39, MHC-II, LAMP1 (Andrei et al., 1999; Pizzirani et al., 2007) and (c) cytoplasmic proteins, such as pro-IL-1 $\beta$ , pro-casp-1, IL-1 $\beta$ , casp-1, casp-3 and cathepsin D (Gudipaty et al., 2003; Andrei et al., 2004; Bianco et al., 2005; Carta et al., 2006; Pizzirani et al., 2007; Qu et al., 2007). It is not clear how and if IL-1 $\beta$  finally effluxes out of the microvesicles, thus fulfilling its role as an extracellular signaling molecule, or alternatively is delivered intracellularly following microvesicle fusion with the plasma membrane of target cells. Verderio and coworkers provided ample evidence showing that microvesicles released from P2X7R-stimulated microglia fuse with the plasma membrane of target cells (e.g., neurons), deliver their content and affect target cell responses (e.g., synaptic activity) (Antonucci et al., 2012; Turola et al., 2012; Verderio et al., 2012). We reported some time ago that microvesicles shed from P2X7R-stimulated DCs express the P2X7R and are lysed by exposure to extracellular ATP, thus releasing their cargo of IL-1 $\beta$  (Pizzirani et al., 2007). This observation led us to propose that IL-1 $\beta$  is released in the vicinity of the target cell plasma membrane by ATP-stimulated and P2X7R-dependent microvesicle rupture (Pizzirani et al.,

2007). In fact, it is known that due to continuous ATP release into the extracellular space, cells are surrounded by an “ATP halo” that generates an ATP concentration higher in the vicinity of the plasma membrane than in the bulk solution. Thanks to this ATP gradient, microvesicle journey across the interstitial space should be relatively safe until they reach the target cell surface where they are supposed to find an ATP concentration sufficient to activate the P2X7R and trigger lysis.

## EXOCYTOSIS OF IL-1 $\beta$ -CONTAINING EXOSOMES

In mouse bone marrow-derived macrophages (BMDMs) the main mechanism for non-classical IL-1 $\beta$  release has been reported to be neither secretory lysosomes nor microvesicle shedding, but rather P2X7R-stimulated MVBs formation and exosome release (Qu et al., 2007). Exosomes are small vesicles (30–100 nm) released upon fusion of MVBs with the cell plasma membrane. Exosomes originate as intraluminal vesicles during the process of MVBs formation. MVBs or late endosomes are components of the endocytic pathway that range from 250 to 1000 nm in diameter. MVBs can either be degraded or fuse with the plasma membrane, releasing the intraluminal vesicles into the extracellular space. Intraluminal vesicles are then referred to as exosomes following their extracellular release. During the process of formation, transmembrane and peripheral membrane proteins are incorporated into the exosome membrane, while cytosolic components are enclosed within the vesicles. Exosomes released from macrophages, DCs or B-lymphocytes contain

soluble proteins present in the cytosol, such as pro-IL-1 $\beta$ , procasp-1 and the respective mature form IL-1 $\beta$  and casp-1, and plasma membrane proteins such as MHC I and MHC II, a feature of exosomes derived from antigen presenting cells. From P2X7R-stimulated BMDMs two distinct types of membrane-bound vesicles are shed: (a) plasma membrane-derived microvesicles carrying P2X7R and LAMP1, and (b) MVB-derived exosomes lacking both P2X7R and LAMP1. However, both types of vesicles are able to present peptide-MHCII complexes to T cells (Ramachandra et al., 2010). Secretion of IL-1 $\beta$  and MHCII are strongly inhibited in mice deleted of ASC and NLRP3, suggesting the possibility that inflammasome complex regulate the formation of MVBs and the accumulation of IL-1 $\beta$  and casp-1, although the mechanism remains unclear (Qu et al., 2009).

## IL-1 $\beta$ RELEASE AS A CONSEQUENCE OF PLASMA MEMBRANE DAMAGE AND CELL DEATH

A model for IL-1 $\beta$  release involving plasma membrane damage and cell death (whether by necrosis or apoptosis) has been proposed several years ago (Hauser et al., 1986; Hogquist et al., 1991). A major obstacle for the acceptance of this model is the need for proteolytical activation of pro-IL-1 $\beta$ , which is assumed to occur coordinately with its secretion, and the consistent observation that cytoplasmic mature IL-1 $\beta$  levels are very low (Perregaux et al., 1992). Of course, it is possible that extracellular proteases, e.g., trypsin or cathepsins might do the job, but *in vivo* relevance of extracellular pro-IL-1 $\beta$  maturation is dubious. However, in a recent paper, Pelegrin and co-workers have re-visited the cell permeabilization/cell death model for IL-1 $\beta$  release from BMDMs taking advantage of novel, highly sensitive, fluorescence-based technique to measure IL-1 $\beta$  secretion and of a novel inhibitor, punicalagin (Martin-Sanchez et al., 2016). Rigorous analysis of release of the cytoplasmic marker lactic dehydrogenase and of IL-1 $\beta$  revealed that the kinetics of two processes were closely over-imposed. Furthermore, punicalagin, a polyphenolic compound that efficiently prevents plasma membrane permeabilization in response to a number of membrane-perturbing agents, fully abolished ATP-dependent IL-1 $\beta$  secretion but not its processing, thus showing that pro-IL-1 $\beta$  cleavage and mature IL-1 $\beta$  secretion can be dissociated, and that a “leaky membrane” is needed for IL-1 $\beta$  release. Since casp-1 activation is also a major driver of pyroptotic cell death, Pelegrin and co-workers suggested that in macrophages IL-1 $\beta$  secretion occurs via a non-specific increase in plasma membrane permeability associated to cell death (Martin-Sanchez et al., 2016).

## IS THE P2X7R-TARGETING A THERAPEUTICALLY LIVE OPTION?

Several studies show that P2X7R blockade efficiently antagonize IL-1 $\beta$  release in different disease experimental models (Bartlett

et al., 2014). However, similar evidence from human studies is lacking. Measurement of serum IL-1 in autoimmune and autoinflammatory diseases is seldom significantly elevated, and is not thought to be a reliable indicator of inflammation (Dinarello, 2005). Therefore, it is not possible to verify in humans whether P2X7R blockade has any effect on IL-1 $\beta$  release. Assessing the *in vivo* effect of P2X7R blockade on IL-1, and in general, all cytokines, release, is made even more complex by the disappointing results of most clinical trials so far carried out (De Marchi et al., 2016; Jacobson and Muller, 2016).

## CONCLUSION

Extracellular ATP is now acknowledged to be one of the earliest most ubiquitous DAMPs (Di Virgilio, 2013; Kepp et al., 2014; Hammad and Lambrecht, 2015; Venereau et al., 2015). Its remarkable efficiency and plasticity as an alarm signal strongly depends on the diverse of ATP-selective plasma membrane receptors expressed by immune cells. Very interestingly, even before all ATP receptors (P2 receptors) expressed by immune cells were cloned and fully characterized, it was clear that stimulation with extracellular ATP was able to cause a dramatic acceleration of pro-IL-1 $\beta$  processing and release from monocytes/macrophages, as well as from microglial cells, and this was very likely a receptor-mediated event (Perregaux and Gabel, 1994; Di Virgilio et al., 1996; Ferrari et al., 1996). About at the same time the P2X7R was cloned (Surprenant et al., 1996), and soon after identified as the molecule responsible for ATP-dependent mature IL-1 $\beta$  release (Ferrari et al., 1997). Thus, the association between IL-1 $\beta$  and the P2X7R is rock solid and long standing. However, this has not led to the introduction of any P2X7R-targeted anti-inflammatory therapy, despite large effort by virtually all major Pharma Industries. Are we missing some crucial information of P2X7R and IL-1 $\beta$  biology, or is there a recurrent fault in P2X7R-targeting drug design and development, or both?

## AUTHOR CONTRIBUTIONS

FDV coordinated writing and reviewed the MS. AG wrote sections of the MS. AS wrote sections of the MS. SF wrote sections of the MS.

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# IL-1 Inhibition May Have an Important Role in Treating Refractory Kawasaki Disease

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Kawasaki disease (KD) is an acute inflammatory vasculitis occurring in young children before 5 years and representing at this age, the main cause of acquired heart disease. A single infusion of 2 g/kg of intravenous immunoglobulins along with aspirin has reduced the frequency of coronary artery aneurysms from 25 to 5%. However, 10–20% of patients do not respond to standard treatment and have an increased risk of cardiac complications and death. The development of more potent therapeutic approaches of KD is an urgent need. Phenotypical and immunological similarities between KD and systemic juvenile idiopathic arthritis led to the hypothesis that KD could be considered as an autoinflammatory disease. New insights regarding KD's pathogenesis have merged from the combination of genetic and transcriptomic data revealing the key role of interleukin-1 (IL-1) signaling in the pathogenesis of the vasculitis. Once activated, IL-1 $\alpha$  and IL-1 $\beta$  trigger a local proinflammatory environment-inducing vasodilatation and attracting monocytes and neutrophils to sites causing tissue damage and stress. Both IL-1 $\alpha$  and IL-1 $\beta$  have been shown to induce myocarditis and aneurysm formation in *Lactobacillus casei* cell-wall extract mouse model of KD; both being successfully improved with IL-1 blockade treatment such as anakinra. Treatment failure in patients with the high-risk inositol-triphosphate 3-kinase C genotype was associated with highest basal and stimulated intracellular calcium levels, increased cellular production of IL-1 $\beta$ , and IL-18, and higher circulating levels of both cytokines. Three clinical trials of IL-1 blockade enrolling KD patients are currently being conducted in Western Europe and in USA, they could change KD outcome.

**Keywords:** Kawasaki disease, vasculitis, pediatric, interleukin-1, coronary artery aneurysms, pediatrics, autoinflammatory disease

## INTRODUCTION

Kawasaki disease (KD) is an acute inflammatory vasculitis of the medium- and small-sized arteries generally occurring in children under 5 years old. It was first described by Kawasaki, 1967 associated with the development of coronary artery aneurysms (CAA) or ecstasies in 15–25% of untreated children. Coronary lesions may lead to ischemic heart disease and sudden death. The etiopathology of KD remains unknown though it is widely accepted that it results in an important inflammation cascade triggered by unknown infectious or other stress trigger in a genetically predisposed individual. A single infusion of 2 g/kg of intravenous immunoglobulins (IVIGs) along with aspirin is the standard treatment for KD but not all children may respond, especially the youngest ones and those predisposed

to develop CAA. Interleukin 1 (IL-1) cytokine has been shown to play a key role in the development of CAA leading to a potential use of IL-1 blockade in patients with KD.

## KAWASAKI DISEASE

Classically, KD is diagnosed in the presence of high fever lasting for at least 5 days associated to at least four principal features (**Table 1**). No blood tests are available for diagnosis of KD, therefore, a clinical algorithm has been established and validated by the American Academy of pediatrics (**Table 1**) (Newburger et al., 2004). In some cases, KD diagnosis can be made at day 4 of illness in the presence of  $\geq 4$  principal criteria. Some patients have incomplete KD, especially infants  $\leq 6$  months. In this situation, KD diagnosis is challenging and should be looked for in infants with  $\geq 7$  days of fever without explanations, even though no KD clinical criteria are found. In this case, children should, therefore, undergo laboratory testing and, if any systemic inflammation is found, an echocardiogram should be performed (Newburger et al., 2004). Echocardiographic evaluation should be performed at the time of diagnosis, at 2 weeks and at 6–8 weeks after onset of the disease. More frequent echocardiographic evaluation is needed in children at higher risk (Newburger et al., 2004). Kawasaki disease vasculitis may occur outside the heart in other medium-sized vessel such as axillary, renal and femoral arteries somewhat difficult to distinguish from infantile periarteritis nodosa (Burns and Glodé, 2004).

## Treatment in KD

A single infusion of 2 g/kg of IVIG along with aspirin has reduced CAA frequency from 25 to 5%. However, 10–20% of patients do not respond to standard treatment and have an increased risk of cardiac complications and death.

Corticosteroids (CS) as well as anti-tumor necrosis factor (TNF) agents are the two main treatments used in IVIG-resistant patients (Eleftheriou et al., 2014). Although there are no formal recommendations regarding optimal CS doses and duration (Chen et al., 2013), CS has not shown significant differences compared to an additional IVIG treatment in terms of preventing the development of CAA (Miura et al., 2008; Ogata et al., 2009). The elevated level of TNF- $\alpha$  in the sera of KD patients correlated with CAA development has led to the use of anti-TNF agent (Eleftheriou et al., 2014). The most frequently used is infliximab (IFX), a chimeric murine/human IgG1 monoclonal antibody that binds to TNF- $\alpha$ . This treatment has been administered in IVIG-resistant patients with success regarding fever and inflammatory parameters, however, with no differences regarding cardiac disease (Burns et al., 2005, 2008; Son et al., 2011). Other immunosuppressive agents have occasionally been used such as: cyclosporine, cyclophosphamide, methotrexate, and plasma exchange in resistant KD patients to IVIG, steroids, and anti-TNF $\alpha$  (Galeotti et al., 2016).

Although the use of different treatments has changed KD outcome, this disease is still lethal in certain cases. The individual prognostic factors are still poorly defined, and resistance to standard therapy represents a major risk of cardiac complications. Developing more efficient treatments and with a better action on cardiac involvement seems a priority. The phenotypical similarities between KD and systemic autoinflammatory disease (SAID) led researches to look at the role of inflammatory cytokines namely IL-1 in KD.

## Why to Use IL-1 Blockade in KD?

### KD and Systemic Juvenile Idiopathic Arthritis (SJIA): Is There a Missing Link?

Kawasaki disease and SJIA represent a major cause of fever of unknown origin in young children and share intriguing

**TABLE 1 |** Kawasaki disease (KD) clinical algorithm (Newburger et al., 2004).

Typical KD	Positive echocardiogram (1/3 conditions)
Fever persisting at least 5 days associated to at least four of the five principal features	LAD or RCA $\geq 2.5$ Z-score
Changes in the peripheral extremities:	Any coronary segment with an internal lumen diameter Z-score $\geq 2.5$
– Palm and soles erythema	
– Feet and hand edema	
– Peeling of the hands and feet at week 2 or 3	
Bilateral non-exudative conjunctivitis	3/6 features
Changes in the oral cavity:	<ul style="list-style-type: none"> <li>• Perivascular brightness</li> <li>• Lack of taperin</li> <li>• Decreased LV function</li> <li>• Mitral regurgitation</li> <li>• Pericardial effusion</li> <li>• LAD or RCA: 2–2.5 Z-score</li> </ul>
– Lips dryness	Supplementary laboratory criteria
– Erythema	<ul style="list-style-type: none"> <li>• Albumin <math>\leq 3</math> g/dl</li> <li>• Anemia for age</li> <li>• Elevation of alanine aminotransferase</li> <li>• Platelets 450,000/mm<math>^3</math> after 7 days of fever</li> <li>• White blood cell count <math>\geq 15000/\text{mm}^3</math></li> <li>• Urine white blood cells &gt;10 cells per high-power field</li> </ul>
– Strawberry tongue	
– Diffuse injection of mouth and throat mucosa	
Cervical lymphadenopathy $> 1.5$ cm diameter and usually unilateral	
Polymorphous exanthema	

Proximal right coronary artery (RCA) or left anterior descending coronary artery (LAD).

similarities. Clinically, both diseases present with high fever, macular rashes, myalgia, arthralgia, and adenopathy although arthritis seems to be specific to SJIA (Lefèvre-Utile et al., 2014); for this reason, it is difficult to differentiate KD from early SJIA especially when KD is incomplete. Early age of presentation seems to favor KD. Moreover, cardiac abnormalities have been described, especially serositis as in many SAID. Unlike KD, SJIA coronary lesions are mild (essentially hyper echogenic coronaries) with favorable evolution; no CAA are described. Because of CAA risk in incomplete KD and need for early treatment, many patients with SJIA may be initially treated as KD, with IVIG and aspirin, but without efficacy (Lefèvre-Utile et al., 2014). Looking at laboratory findings, no differences can be seen. Both present elevated C-reactive protein, leukocytosis, thrombocytosis, hypoalbuminemia, anemia, and even macrophage activation syndrome (MAS) (Lefèvre-Utile et al., 2014). Assumptions have been made that these two systemic inflammatory disorders could share common triggering agents, susceptibility factors, or immunopathogenic pathways.

When looking at sera of KD and SJIA patients, inflammatory cytokines such as IL-1, IL-6, TNF- $\alpha$ , and interleukin-18 (IL-18) are increased. IL-18 being specifically higher in SJIA compared to KD patient (Mizuta et al., 2016). These phenotypical similarities between KD and SJIA along with the immunological features led to the hypothesis that KD could be considered as an SAID as SJIA and cryopyrin-associated periodic syndrome (CAPS) (Alphonse et al., 2016).

### IL-1 Signature in SJIA and KD:

Inflammatory cytokines, especially IL-1 $\beta$ , has first been described as markedly increased in SAID such as CAPS and SJIA (Goldbach-Mansky, 2012). CAPS has allowed to understand the key role of IL-1 in the disease pathogenesis and showed striking response to IL-1-blocking therapies (Ter Haar et al., 2013). NLRP3 is a nod-like receptor (NLR) that is part of an inflammasome, which activates the caspase-1 (CASP1) and consequently the secretion of active IL-1 $\beta$  and IL-18 (Baroja-Mazo et al., 2014). NLRP3 gene mutations result in constitutive activation of the NLRP3 protein and in an amplification loop of inflammation in which normal regulatory systems, i.e., ATP and second signal requirement are debribed, and where the pro IL-1 $\beta$  may act itself as a danger signal (Koné-Paut and Galeotti, 2015).

More recently, IL-1 has been shown to play a critical role in the pathogenesis of SJIA. Pascual et al. (2005) showed three major results. First, serum from SJIA patients induces the transcription of innate immunity genes including IL-1 in peripheral blood mononuclear cells (PBMCs) from healthy volunteers. Second, when activating PBMCs of SJIA patients, a large amount of IL-1 $\beta$  is released. Finally, they showed that, the use of recombinant IL-1 receptor antagonist (IL1-RA) (anakinra) allowed complete clinical remission in seven of the nine refractory-treated patients thus, emphasizing the central role of the innate immune system (IIS), and specifically, inflammasome-derived cytokines, in the pathogenesis of SJIA (Pascual et al., 2005).

As in systemic diseases, IL-1 seems to play a key role in the physiopathology of KD and more importantly in cardiac involvement for various reasons. Alphonse et al. (2016) showed a

significant increased level of IL-1 $\beta$ , IL-18 and of their antagonists (IL-1RA and IL-18BP) in acute KD patients compared with age-matched control patients with viral or bacterial infections. Moreover, IL-1-induced inflammation has been shown to play a role in acute myocardial infarction and contributes to acute ischemic diseases. Indeed, IL-1 is known to enhance the expansion, differentiation and migration of antigen-specific CD8+ T cells as well as the induction of matrix enzymes source of major tissue damage. In the heart and brain, this inflammation can be fatal (Martignon and Tschopp, 2004). In KD, antigen-driven CD8+ T cells are known to infiltrate the coronary artery wall and contribute to the pathogenesis of CAA (Brown et al., 2001). The assumption appears all the more justified when looking at IVIG mechanism on inflammatory cytokines. In responsive KD patients treated with IVIG therapy, the level of pro-inflammatory cytokines (IL-1 $\beta$ , IL-6, and TNF- $\alpha$ ) are decreased emphasizing immunoglobulin's (IG) effect on the modulation of inflammatory cytokines namely on IL-1. Although the way IVIG acts is not perfectly understood, it is known to reduce CAA prevalence (Galeotti et al., 2010).

Interleukin-1 polymorphisms could be associated either to response or resistance to IVIG treatment (Weng et al., 2010). Interestingly elevated transcripts have been shown in IVIG-resistant KD patients, which carry the highest risk for coronary aneurysms (Fury et al., 2010). Increased transcript abundance of the neutrophil-associated calcium-binding proteins, S100A8 and A9, confirms the role of activated neutrophils in acute KD, as these proteins regulate adhesion of neutrophils and monocytes to the endothelial cell, a critical process in KD vasculitis. S100A8/9 proteins are elevated in patients who develop coronary aneurysms. The S100A8/9 heterodimer is known to activate the IL-1 receptor-associated kinase and the NF- $\kappa$ B. S100A8/9 appears to be useful biomarkers for identifying IVIG-resistant patients. Other markers of endothelial cell activation CEACAM1 (carcino embryonic antigen-related cell adhesion) and VEGF (vascular endothelial growth factor) have been detected in acute KD and may correlate with IGIV resistance and coronary vasculitis (Weng et al., 2010).

The role of IIS in the histopathology of KD has also been shown *in vivo* in mice.

### Mouse Model of CAA- and IL-1-signaling Pathways

A mouse model of coronary arteritis has been developed using intraperitoneal injection of *Lactobacillus casei* cell-wall extract (LCCWE). This mouse develops a focal, localized coronary arteritis that histopathologically mimics the coronary artery lesions found in human KD (Lehman et al., 1985). As in human CAA, the coronary lesions of LCCWE contains macrophages, activated dendritic cells, and T cells (Schulte et al., 2009). Moreover, the CAA in LCCWE mice responds to IVIG therapy as in KD children (Lehman, 1993). Although both innate and adaptive immunity have been shown as essential for the development of CAA in the LCCWE mouse model, IIS seems to play a key role. Two cytokines have been described as important in the development of CAA: NF- $\kappa$ B and IL-1. Rosenkranz et al.

(2005) have pointed out the role of toll-like receptors (TLRs), a major sensor of IIS, in KD inflammation and therefore in CAA. In LCCWE, NF- $\kappa$ B, an inflammatory cytokine, is activated and synthesized after activation of TLR-2 using a MyD88-dependent pathway (Rosenkranz et al., 2005). NF- $\kappa$ B activation coordinately controls both the innate and adaptive immune responses. To induce vasculitis in LCCWE mice, TLR2 are required as IL-1R signaling highlighting, amongst others, the importance of IL-1-signaling pathway in vasculitis (Rosenkranz et al., 2005). Both IL-1 $\alpha$  and IL-1 $\beta$  have been shown to induce aneurysm formation in LCCWE mouse model of KD; aneurysm that are successfully improved with IL-1 blocker treatment such as anakinra (Schett et al., 2016). Similar successful results were reported in recalcitrant KD children using IL-1 blockade (Alphonse et al., 2016).

Lee et al. (2012) presented a mouse model of a knock-out LCCWE mouse (CASP1-/- and IL-1R-/-) in whom KD finally developed after injection of recombinant IL1- $\beta$  protein. This mouse developed coronary arteritis, which could be prevented by injection of the IL-1 receptor antagonist (IL-1RA): anakinra, during 3–5 days. Using the LCWE mouse model, a logical progression of experiments demonstrated that (i) bone marrow-derived macrophages secrete high levels of IL-1 $\beta$  and TNF $\alpha$ ; (ii) IL-1 $\beta$  is processed from pro-IL-1 $\beta$  by CASP1 through the NLRP3 inflammasome; (iii) exogenous treatment with IL-1 $\beta$  recreates the inflammatory phenotype in CASP1 deficient mice; and (iv) IL-1R-deficient mice or mice treated with the recombinant IL-1RA, anakinra fail to develop the arteritis lesions. Of particular note, only blockade of IL-1 $\beta$ , but not blockade of TNF- $\alpha$ , reduced the myocarditis in the LCWE-injected mice (Burns, 2012; Lee et al., 2012). A recent case report showed a dramatic effect on rescuing a life-threatening case of relapsing KD (Cohen et al., 2012).

## Genetics: IL-1 Pathway and Calcium Signaling

Finally, analysis of the whole-genome expression profile of acute KD patients has pointed out the importance of IL-1 $\beta$  activation in KD inflammatory profile by showing the link between calcium concentration and inflammasome.

Inositol-triphosphate 3-kinase C (ITPKC) is a candidate gene located on chromosome 19q13.2 whose CC genotype is implicated as a determinant of both disease susceptibility and outcome in KD. ITPKC phosphorylates inositol 1, 4, 5-triphosphate (IP3) to inositol 1, 3, 4, 5-tetraphosphate (IP4), therefore, regulating the calcium response to extracellular signals. At the same time, NLRP3 inflammasome has been shown to be dependent of both extracellular and intracellular calcium concentration ( $[Ca^{2+}]_i$ ). Amazingly, ITPKC CC genotype is associated with both highest basal and stimulated  $[Ca^{2+}]_i$  levels and increased amounts of NLRP3 protein compared with other genotypes at baseline. These findings, allowed making the hypothesis of a link between the calcium level and the activation of NLRP3 in ITPKC CC genotype leading to an excess of IL-1 secretion as in SAID. Moreover, ITPKC CC genotype is associated with failure of IVIG therapy (Alphonse et al., 2016). This emphasizes the fact that phenotypic similarities between

**TABLE 2 | Clinical trials of IL-1 blockade enrolling KD patients conducted in Western Europe and in USA (Burns et al., 2016).**

Name	Type of trial	IL-1 blockade/Doses	Population KD patients	Objective	Time
Kawakinra trial (Europe) (EudraCT Number: 2014-002715-41)	Phase IIa, multi-centered trial	Anakinra: 2 mg/kg/day Dose can be increased by 2 mg/kg/24 h if persistent or recurrent fever (max 6mg/kg/d)	Children: 8 months $\geq 10$ kg)–18 years Screened: 4th–13th day of fever	Primary end points Efficacy and safety of anakinra Secondary objectives Effects of anakinra on coronary artery Disease activity and inflammation biomarkers	14 days of treatment
Anakinra trial (USA) (clinicaltrials.gov#NCT02179853)	Phase I/IIa study: Two-centered and dose escalation trial	Anakinra: 2, 4 or 8 mg/kg Persistent or recrudescence fever after $\geq 36$ h and $<7$ days following the end of intravenous immunoglobulin (IVIG) infusion	Children ( $\geq 8$ -M-old) with acute KD and with coronary artery $L^2$ -score $\geq 3.0$ in the RCA or LAD abnormalities	Safety, tolerability, and pharmacokinetics of anakinra	2–6 weeks
Canakinumab trial (Europe)	Phase II trial: Two-arm, multi-centered and carried out in seven European countries	Canakinumab: 6 mg/kg IV Group 1: Complete fever resolution Canakinumab (1 or 2 SC injections) at 4 and 8 weeks Depending on the clinical and CRP course Group 2 Fever remains after 48–72 h of canakinumab IVG	Naïve KD patients or IVIG-resistant KD patient	The presence or absence of fever will be looked-for:	

IVG, intravenous immunoglobulin; RCA: right coronary artery; LAD, left anterior descending coronary artery; SC, subcutaneous.

KD and AID are anchored by the common immunobiological processes associated with inflammasome activation.

## Experience of IL-1 Blockade in KD Patients

Nowadays, three IL-1 blockades have been approved: anakinra, rilonacept, and canakinumab. Anakinra (Kineret<sup>®</sup>) was the first IL-1 blockade agent administered initially in rheumatoid arthritis (1993) and is now used in numerous diseases such as hereditary SAIDs (Schett et al., 2016). It is an IL1-RA blocking both IL-1 $\alpha$  and IL-1 $\beta$  (Carter et al., 1990). In 2008, rilonacept (Hoffman et al., 2008), a soluble IL-1 decoy receptor, that neutralizes either IL-1 $\alpha$  or IL-1 $\beta$ , received US Food and Drug Administration (FDA) approval in CAPS patients, and Canakinumab (Ilaris<sup>®</sup>) in 2009 (Chakraborty et al., 2012). The latter is a humanized monoclonal antibody that specifically blocks IL-1 $\beta$  (Dinarello et al., 2012). In pediatrics, only anakinra ( $\geq 8$  months and 10 kg) and canakinumab ( $\geq 24$  months and  $\geq 7.5$  kg) have FDA and European Medicines Agency (EMA) approval for CAPS disease. These IL-1 blockades are safe and well tolerated with a low-adverse event rate (Rossi-Semerano et al., 2015). Anakinra is preferred for it has a remarkable record of safety with over 150,000 patients treated daily for over 10 years (Bresnihan et al., 2004; Fleischmann et al., 2006). In addition, drug level significantly drops 1 h after discontinuation of treatment (Dinarello et al., 2012).

In SJIA, the three IL-1 blockers have been tested so far and were proven as effective and safe, although only canakinumab is currently approved for use (Giancane et al., 2016). In addition, anakinra has been demonstrated as efficient in severe SJIA with MAS (Miettunen et al., 2011) a severe complication that can occur in up to half of SJIA patients. In KD, MAS is probably a frequently under-recognized complication situation which could benefit from IL-1 blockers (Wang et al., 2015).

For now, two case reports showing promising results with anakinra in severe KD patient have been published. The first one is an 11-week-old Caucasian female that presented with severe KD complicated by MAS. Diffuse enlargement of the entire coronary artery system was revealed by echocardiogram. IVIG, aspirin and CS were inefficient. High doses of anakinra (3 mg/kg/dose, twice daily for 3 days) were introduced at day 6 because of bad clinical outcome and biological signs of MAS. IFX and methylprednisolone were added at day 9 because of cardiac failure despite favorable clinical and biological course. The evolution was favorable allowing CS to be tapered off over 10 days following discharge and under anakinra over the next 5 months. At 8 months, the echocardiogram was normal (Shafferman et al., 2014).

The second one is a 2-year-old boy diagnosed with KD who developed secondarily cardiac failure (shortening fraction

of 20%) without CAA under IVIG (2 g/kg) treatment. A second IVIG perfusion was administered as well as multiple methylprednisolone pulses with little effect and worsening of cardiac involvement. Extracorporeal membrane oxygenation was performed from day 14 until day 17 and subcutaneous anakinra (1 mg/kg/day) was introduced at day 18 until day 24 with success. Relapse was seen three days after anakinra's last injection with progression to giant CAA. Anakinra was therefore reinitiated for 6 weeks with normalization of the coronary lesions at 6 months (Cohen et al., 2012).

## What Could Be the Place of Anti-IL1 in the Current Standard of KD Treatments?

Considering current knowledge, it seems reasonable to use IL-1 blockade in resistant KD with CAA before IFX which has not proven its efficiency in coronary disease. A new approach could be the early use of IL-1 blockade associated with CS in patients at high risks of severe KD depending on validated risk scores, in Japanese patients. Apart from IVIG's, anti-IL1 are the only therapies that have proven their effect on CAA. It should be considered whether their use should not be generalized to all patients. Indeed, IL-1 blockers seem to better prevent CAA development than IVIG, especially if used at diagnosis. In this idea three clinical trials of IL-1 blockade enrolling KD patients are currently being conducted in Western Europe and in the US (Table 2) (Burns et al., 2016). Their conclusion may help to better define, in the future, the place of IL-1 blockade in KD treatment in association or in replacement of IGIV and CS.

## CONCLUSION

Kawasaki disease clinical and immunological features mimic SAID. These similarities have allowed looking at new inflammatory cytokines such as IL-1. Better understanding of IL-1 involvement in KD and specifically in CAA with the use of IL-1 blockers, has brought hope for resistant and severe patients. Doses and time to introduce IL-1 therapy has still to be defined. Another challenge is the need to better define patients with a higher risk of CAA, allowing better medical care and the use of new treatment strategies. We hope that results of clinical trials using IL-1 blockade will allow to better understand the respective roles of IL-1 $\alpha$  and  $\beta$ , and to pursue with phase III trials.

## AUTHOR CONTRIBUTIONS

Both the authors had a substantial contribution to the work. PD wrote the first draft. PD and IK-P were involved in drafting the article or critically revising it for important intellectual content and approved the final version to be published.

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# Atherosclerosis Is an Inflammatory Disease which Lacks a Common Anti-inflammatory Therapy: How Human Genetics Can Help to This Issue. A Narrative Review

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Atherosclerosis is a multifactorial disease triggered and sustained by different risk factors such as dyslipidemia, arterial hypertension, diabetes mellitus, smoke, etc. Since a couple of decades, a pivotal role for inflammation in its pathogenesis has been recognized and proved at molecular levels, and already described in many animal models. Despite all this knowledge, due to the complexity of the specific inflammatory process subtending atherosclerosis and to the fact that inflammation is also a protective response against microorganisms, no anti-inflammatory therapy has been rendered available in the therapeutic armamentarium against atherosclerosis and vascular events till 2017 when canakinumab in the first *ad-hoc* randomized clinical trial (RCT) proved for the first time that targeting specifically inflammation lowers cardiovascular (CV) events. From the genetic side, in the 90's and early 2000, several genetic markers in inflammatory pathway have been explored searching for an association with athero-thrombosis which gave seldom consistent results. Then, in the genomic era, plenty of genetic markers covering most of the genome have been analyzed at once without *a priori* information. The results coming from genome wide association studies (GWAS) have pinpointed some loci closed to inflammatory molecules consistently associated with atherosclerosis and CV consequences revamping the strict link between inflammation and atherosclerosis and suggesting some tailored target therapy. Whole-exome and whole-genome sequencing will come soon showing new and old loci associated with atherosclerosis suggesting new molecular targets or underlying which inflammatory pathway could be most attractive to target for blocking atherosclerosis even in its early stages.

**Keywords:** atherosclerosis, inflammation, genetics, anti-inflammatory drugs, genome wide association study, DNA sequencing

## INTRODUCTION

Atherosclerosis is an inflammatory disease (Ross, 1999; Libby, 2012). Accumulation of leukocytes in the subendothelial space is an early step in the formation of atherosclerotic lesions. Then, other inflammatory cells follow and participate in all the processes starting from the "fatty streak" and leading to advanced atherosclerotic lesions triggering clinical events. In particular a pivotal role has

been recognized for monocyte-derived macrophages, which become “foam cells,” dendritic cells, lymphocytes (both T and B), and mast cells. Lots of adhesion molecules or receptors for leukocytes expressed on the surface of the arterial endothelial cell probably participate in the recruitment of leukocytes to the nascent atheroma. Proinflammatory cytokines can act at different stages in the process: interleukin 1 (IL-1) and tumor necrosis factor (TNF) can regulate the expression of adhesion molecules involved in early and late leukocyte recruitment. Indeed, IL-1 and TNF can induce local production of growth factors, including fibroblast growth factors (FGF) and platelet-derived growth factor (PDGF) which attract smooth-muscle cells from the tunica media into the intima. Finally, other cytokines and growth factors may be important in the evolution to a more advanced fibrous plaque which may be protective against plaque rupture: i.e., the transforming growth factor  $\beta$  (TGF- $\beta$ ) stimulates whereas interferon  $\gamma$  (IFN- $\gamma$ ) counteracts interstitial collagen production by smooth-muscle cells (Ross, 1999; Libby, 2012).

C-reactive protein (CRP) is an acute phase reaction protein, produced by the liver and triggered especially by interleukin-6 (IL-6) which is often used in the clinic as an inflammatory marker in “classical” inflammatory disease such as infections, vasculitis, tumors. When all these conditions are excluded, CRP, even in the lower range of detection, could be useful to monitor subclinical inflammatory state deriving from atherosclerosis (Libby and Ridker, 2004).

The Jupiter trial have already shown that patients with 2.0 mg/L or higher level of CRP benefit from statin therapy to lower their CV risk. But of course in that trial not only CRP (by 37%) but especially LDL cholesterol decreased (nearly by 50%) along with all the end-points: myocardial infarction (MI), stroke, arterial revascularization, hospitalization for unstable angina, or death from CV causes (Ridker et al., 2008). Thus, after this trial it was still plausible that the reduced LDL-cholesterol levels and not the reduced grade of inflammation mostly contributed to this beneficial result.

Secretory phospholipase A2 (sPLA2) and lipoprotein-associated phospholipase A2 (Lp-PLA2) were identified in animal and observational studies in humans, as potential risk factors for coronary heart disease due to their putative effects on lipids and inflammation (Rosenson et al., 2010; Wang et al., 2011). Nevertheless, two randomized clinical trials (RCTs), exploring the possible efficacy of darapladib, a Lp-PLA2 inhibitor, in patients after an acute coronary syndrome (ACS) or stable coronary atherosclerosis, did not find any difference in CV and cerebrovascular events as compared to placebo (O'Donoghue et al., 2014; STABILITY Investigators et al., 2014). A RCT, testing varespladib, a sPLA2 inhibitor, failed to demonstrate a beneficial CV effect and showed instead a possible increase in coronary events (Nicholls et al., 2014). A genetic variant, rs11573156 of the sPLA2 gene was associated with lower level of sPLA2 but not with major vascular events. Thus, also Mendelian randomization analysis fails to indicate sPLA2 as a possible target for preventing CV diseases (Holmes et al., 2013).

## TARGETING INFLAMMATION WITH OLD DRUGS

Beside the current large availability of anti-inflammatory drugs in the medical field, targeting specifically inflammation in humans remains challenging. In fact, most of available anti-inflammatory medications have adverse effects which render their use, as a chronic therapy to prevent CV events, not feasible and some of them have proved to be deleterious.

Nevertheless, at least a clue can be drawn by exploring clinical trials (which in *post-hoc* analyses evaluated these drugs for their potential role in CV risk) or observational surveys related to patients which need these drugs for other indications, such as chronic inflammatory or degenerative diseases.

Among the anti-inflammatory therapies, the non-steroidal anti-inflammatory drugs (NSAIDs) are the most common used drugs worldwide in acute inflammatory disease, chronic therapy for osteoarthritis or other painful debilitating diseases. NSAIDs effects have been explored in many trials and their potential CV effect evaluated many times. Quite recently, in a network meta-analysis including 31 trials which analyzed either NSAIDs and coxibs, both rofecoxib and lumiracoxib were associated with an increased risk of MI whereas ibuprofen and diclofenac with the risk of stroke.(Trelle et al., 2011) Two years later a comprehensive meta-analysis collected 280 trials where different anti-inflammatory agents were tested vs. placebo and 474 trials where the comparison was between anti-inflammatory agents and another NSAIDs (including coxibs) (Coxib and traditional NSAID Trialists' (CNT) Collaboration et al., 2013). Major vascular events, and especially coronary events were increased by coxibs, diclofenac, and ibuprofen. Only high-dose naproxen was associated with less vascular risk than other NSAIDs (Coxib and traditional NSAID Trialists' (CNT) Collaboration et al., 2013).

Methotrexate (MTX) is an anti-inflammatory drug widely used for the treatment of chronic inflammatory disorders such as rheumatoid arthritis and psoriasis. A systematic review and meta-analysis exploring the effect of MTX on major CV outcomes searched for cohorts, case-control studies, and randomized trials (Micha et al., 2011). In many observational studies MTX was associated with lower risk for CVD (21% reduction) and MI (18% reduction; Micha et al., 2011). The authors suggested that MTX could be a useful drug to decrease CV risk and these findings were in line with other meta-analyses (Rouille et al., 2015).

About possible effects of corticosteroids, Rouille and co-authors explored studies in patients with rheumatoid and psoriatic arthritis. Corticosteroids were associated with an increased risk of cardiovascular events regardless of the inflammatory disease (Rouille et al., 2015). This was probably due to the well-known adverse cardiometabolic effects of this class of drugs. The same meta-analysis indicated that in rheumatoid arthritis, TNF inhibitors can reduce the risk of CV events. These data confirmed findings of previous meta-analyses and large registries (Barnabe et al., 2011; Westlake et al., 2011; Low et al., 2017) but other studies did not find any significant difference (Ryan et al., 2011; Ljung et al., 2012).

Thus, TNF may represent an anti-inflammatory drug for atherosclerosis, even though its potent adverse effects and high cost makes its use for this treatment unlikely.

Another biological therapy for chronic plaque psoriasis targets interleukin 12 (IL-12) and interleukin 23 (IL-23). Two meta-analyses explored their possible effect on CV risks. The conclusion of the first meta-analysis is that anti IL-12/23 therapy is not statistically different from placebo regarding CV events, but it is underlined that 10 major adverse CV events were registered in the intervention arm as compared to 0 in the placebo group. (Ryan et al., 2011) A successive meta-analysis, including some more trials, concluded that IL-12/23 therapy increases the risk of a CV outcome (Tzellos et al., 2013).

In a phase II RCT 182 patients with NSTE-ACS, recruited in the first 48 h from onset of chest pain, were allocated to either placebo or subcutaneous IL-1 receptor antagonist (IL-1ra) for 14 days. The IL-1ra group, significantly reduced hs-CRP and IL-6 levels ( $P = 0.02$ ). The study was underpowered to detect a difference in CV endpoints. Nevertheless, MACE despite being similar till the 3rd month were higher in the IL-1ra group at 1 year (Morton et al., 2015).

## THE CANTOS STUDY AND THE PROOF OF CONCEPT

In September 2017, the results of the Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) trial interrupted in the scientific field unequivocally proving for the first time that targeting specifically inflammation is useful for patients with atherosclerosis (Ridker et al., 2017).

More than 10,000 patients with a previous history of MI and levels of hs-CRP  $>2$  mg/L were randomly assigned to three different dosages of canakinumab, an antibody which binds and blocks selectively the IL-1beta receptor (no effect observed for IL-1alpha). A control placebo group was included in the study. Due to financial considerations, the final sample size was reduced but the follow-up was extended. Interestingly the primary endpoint was reached in significantly less patients assigned to canakinumab than placebo. On the other hand, more deaths from infections were detected in the canakinumab group (Ridker et al., 2017).

Thus, a definitive point for the inflammatory hypothesis has been finally reached: atherosclerosis is an inflammatory disease which can be targeted by a specific anti-inflammatory therapy. But the drug to use remains still undefined. In fact, at least for canakinumab, there are simple considerations which will limit its widespread use: first the weight of the increase of deaths from infections may counterbalance the decrease in CV events; second the price of this therapy is still not easily affordable (Harrington, 2017).

Clinical trials to test a cheaper anti-inflammatory drug for atherosclerosis, the MTX, are still ongoing (Everett et al., 2013; Moreira et al., 2013).

Anti-inflammatory targets are probably multiplex and many drugs could target different steps in the inflammatory process. In this regard, many genetic studies and especially genome wide association studies (GWAS) have pinpointed

new molecular candidates that might represent specific targets for the inflammatory process in atherosclerosis rather than other inflammatory disease. Thus, genetics could be one of the key to detect molecular targets which specifically address CV inflammation. By searching in the Pubmed database, on the 15<sup>th</sup> of November 2017 and using as key words: “genome wide association study” OR “coronary” AND “atherosclerosis,” 2,858 citations were retrieved. In the remaining of the review, we focus our attention on some interesting examples between the screened studies where inflammatory loci were identified by the genome wide strategy to support the inflammatory hypothesis. Even if we acknowledge that our search is not exhaustive, since it is not a systematic review, we have found and reported beyond some significant examples of genes and SNPs in inflammatory loci potentially implicated in coronary disease.

## GENETIC VARIANTS IN INFLAMMATORY GENES AND CORONARY ARTERY DISEASE (CAD)

Regarding genes involved in inflammatory pathway, C-X-C motif ligand 12 (CXCL12), SH2B adaptor protein 3 (SH2B3), AB0, Human Leukocyte Antigen (HLA), interleukin 6 receptor (IL-6R), IL-5, Platelet endothelial cell adhesion molecule 1 (PECAM1), Protein C Receptor (PROCR) and antisense non-coding RNA in the INK4 locus (ANRIL) have been identified in different GWAS and sometimes confirmed in case-controls studies as significantly associated to coronary artery disease (CAD) (Table 1).

### CXCL12 Polymorphisms

*CXCL12* is a gene located on 10q11.1 encoding for a member of the alpha chemokine protein family, also called stromal cell-derived factor-1 (SDF-1). It is involved in vascular repair and remodeling through endothelial progenitor cell recruitment, and it is expressed in atherosclerotic lesion by contributing to macrophages migration that promote formation of complicated and unstable plaques by maintaining a pro-inflammatory microenvironment (Farouk et al., 2010).

The association between two SNPs (rs501120 and rs1746048) on 10q11.21 and CAD has been originally shown in the GWAS performed in the Wellcome Trust Case Control Consortium Study (WTCCC) involving 1,926 CAD patients and 2,938 controls (Burton et al., 2007) but not emphasized. In fact, both rs501120 [per allele odds ratio (OR) 1.24] and rs1746048 (OR 1.21) resulted associated with CAD but not at a genome wide significant level (Burton et al., 2007). In the same year, data of the Wellcome Trust Case Control Consortium and of the German Myocardial Infarction Family Study (enrolling 875 MI patients lower than 60 years and 1,644 healthy controls) were combined and confirmed previously published results (Samani et al., 2007) about these SNPs.

Kathiresan et al. published the results of a GWAS conducted in 2,967 early-onset MI cases (men  $\leq 50$  years and women  $\leq 60$  years) and in 3,075 age- and sex-matched controls enrolled in the Myocardial Infarction Genetics Consortium (MIGen), other than in an additional 6 studies comprising in total 5,469 MI cases and

**TABLE 1 |** Single nucleotide polymorphisms in inflammatory gene related to CAD.

Locus	Gene(s)	Polymorphism(s) and association	Population(s)	References
1	<i>IL6R</i>	rs4845625 (intron) $P = 3.64 \times 10^{-10}$	CARDIoGRAMplusC4D Consortium ( $n = 63,746$ CAD patients; $n = 130,681$ HC)	CARDIoGRAMplusC4D Consortium et al., 2013
5q31.1	<i>IL-5</i>	rs2706399 (near gene) $P = 2.1 \times 10^{-6}$ (combined)	IBC 50K CAD Consortium Discovery: ( $n = 15,596$ European and South Asian CAD patients; $n = 34,992$ European and South Asian HC) Replication: ( $n = 17,121$ CAD patients; $n = 40,473$ HC)	IBC 50K CAD Consortium, 2011
6p21.3	<i>HLA-C</i> <i>HLA-B</i> <i>HCG27</i>	rs3869109 (near gene) $P = 1.12 \times 10^{-9}$ (combined)	Discovery: 5 European case control meta-analysis (OHGS_A, OHGS_CCGB_B, DUKE, WTCCC, ITH) ( $n = 7,123$ CAD patients; $n = 6,826$ HC) Replication: 5 CAD European case control studies (GerMIFS1, GerMIFS2, PennCath, MedStar, OHGS_CCGB_S) ( $n = 5,211$ CAD patients; $n = 5,821$ HC)	Davies et al., 2012
6p21.3	<i>HLA-C</i> <i>HLA-B</i> <i>HCG27</i>	rs3869109 (near gene) $P < 0.05$	Chinese Han population ( $n = 422$ patients including 210 cases with coronary stenosis $\geq 50\%$ or previous MI; $n = 212$ HC)	Xie et al., 2013
9p21.3	<i>ANRIL</i>	rs1333049 (near gene) $1.8 \times 10^{-14}$	Wellcome Trust Case Control Consortium (WTCCC) ( $n = 1,926$ CAD patients; $n = 2,938$ HC)	Burton et al., 2007
9p21.3	<i>ANRIL</i>	rs1333049 (near gene) $P = 1.80 \times 10^{-14}$ for WTCCC $P = 3.40 \times 10^{-6}$ for German study	WTCCC study ( $n = 1,988$ MI patients or coronary revascularization $< 66$ years; $n = 3,004$ HC) German study ( $n = 875$ MI patients $< 60$ years; $n = 1,644$ HC)	Samani et al., 2007
9p21	<i>ANRIL</i>	rs2383207 (intron variant) $P = 2.0 \times 10^{-16}$ (combined) rs10757278 (near gene) $P = 1.2 \times 10^{-20}$ (combined)	Iceland A ( $n = 1,607$ MI patients; $n = 6,728$ HC) Iceland B ( $n = 665$ MI patients; $n = 3,533$ HC) Atlanta ( $n = 596$ MI patients; $n = 1,284$ HC) Philadelphia ( $n = 582$ MI patients; $n = 504$ HC) Durham ( $n = 1137$ MI patients; $n = 718$ HC) All groups ( $n = 4,587$ MI patients / $n = 12,767$ )	Helgadottir et al., 2007
9p21	<i>ANRIL</i>	rs10757274 (near gene) $P = 3.9 \times 10^{-6}$ (combined) rs2383206 (intron variant) $P = 3.9 \times 10^{-6}$ (combined)	Copenhagen City Heart Study (CCHS) ( $n = 1,525$ CHD patients; $n = 9,053$ HC) Dallas Heart Study (DHS) ( $n = 154$ CHD patients; $n = 527$ HC) Ottawa Heart Study population (OHS-3) ( $n = 647$ CHD patients; $n = 847$ HC)	McPherson et al., 2007
9p21.3	<i>ANRIL</i>	rs10965215 (intron variant) $P = 0.020$ rs10738605 (nc transcript variant) $P = 0.019$	Chinese Han population ( $n = 286$ MI patients; $n = 646$ HC)	Cheng et al., 2017
9q34.2	<i>ABO</i>	rs579459 (near gene) $P = 4.08 \times 10^{-14}$ (combined)	14 GWAS Discovery: ( $n = 22,233$ CAD patients; $n = 64,762$ European HC) Replication: ( $n = 30,949$ CAD patients; $n = 27,674$ HC)	Schunkert et al., 2011
9q34.2	<i>ABO</i>	rs514659 (near gene) $P = 7.62 \times 10^{-9}$ and other 8 SNPs at genome wide significant level	$n = 5,783$ patients who had angiographic CAD and MI; $n = 3,644$ patients who had angiographic CAD but no MI	Reilly et al., 2011
9q34.2	<i>ABO</i>	rs579459 (near gene) $P = 2.66 \times 10^{-8}$	CARDIoGRAMplusC4D Consortium ( $n = 63,746$ CAD patients; $n = 130,681$ HC)	CARDIoGRAMplusC4D Consortium et al., 2013
10q11.21	<i>CXCL12</i>	rs501120 (near gene) $P = 1.31 \times 10^{-3}$ (combined) rs1746048 (near gene) $P = 2.96 \times 10^{-3}$ (combined)	WTCCC study ( $n = 1,926$ MI patients or coronary revascularization $< 66$ years; $n = 2,938$ HC)	Burton et al., 2007

(Continued)

**TABLE 1 |** Continued

Locus	Gene(s)	Polymorphism(s) and association	Population(s)	References
10q11.21	CXCL12	rs1746048 (near gene) $P = 5.73 \times 10^{-7}$ (combined) rs501120 (near gene) $P = 9.46 \times 10^{-8}$ (combined)	WTCCC study ( $n = 1,988$ MI patients or coronary revascularization <66 years; $n = 3,004$ HC) German study ( $n = 875$ MI patients <60 years; $n = 1,644$ HC)	Samani et al., 2007
10q11.21	CXCL12	rs1746048 (near gene) $P = 8.14 \times 10^{-11}$ (combined)	Discovery: MiGen ( $n = 2,967$ early-onset MI cases (men $\leq 50$ years and women $\leq 60$ years); $n = 3,075$ HC) Replication: 6 other studies ( $n = 5,469$ MI patients; $n = 5,469$ HC)	Kathiresan et al., 2009
10q11.21	CXCL12	rs501120 (near gene) $P = 4.34 \times 10^{-4}$ (combined)	Coronary Artery Disease Consortium ( $n = 11,550$ CAD patients; $n = 11,205$ HC)	Coronary Artery Disease Consortium et al., 2009
10q11.21	CXCL12	rs501120 (near gene) $P = \text{ns}$ (combined) Only women: $P = 8.36 \times 10^{-3}$	Chinese Han population ( $n = 2,335$ CAD patients; $n = 1,078$ HC)	Xie et al., 2011
10q11.21	CXCL12	rs1065297 (3' UTR) all $P = 0.01$ ;	Chinese Han population ( $n = 597$ CAD patients; $n = 685$ HC)	Zhang et al., 2017
12q24	SH2B3	rs3184504 (Trp60Arg) $P = 8.6 \times 10^{-8}$	Six populations ( $n = 6,650$ MI patients; $n = 40,621$ HC)	Gudbjartsson et al., 2009
12q24	SH2B3	rs3184504 (Trp60Arg) $P = \text{ns}$	FGENTCARD population ( $n = 1520$ CAD patients; $n = 424$ HC)	Saade et al., 2011
12q24	SH2B3	rs3184504 (Trp60Arg) $P = 5.44 \times 10^{-11}$	CARDIoGRAMplusC4D Consortium ( $n = 63,746$ CAD patients; $n = 130,681$ HC)	CARDIoGRAMplusC4D Consortium et al., 2013
12q24	SH2B3	rs653178 (intron variant) $P = 2.2 \times 10^{-6}$	CARDIoGRAM ( $n = 52,120$ MI patients; $n = 34,875$ HC)	Olden et al., 2013
12q24	SH2B3	rs79105258 (near gene) $P = 2.5 \times 10^{-5}$	TAICHI Consortium ( $n = 3,133$ Taiwan CAD patients; $n = 5,423$ HC)	Assimes et al., 2016
17	PECAM1	rs1867624 (near gene) $P = 3.98 \times 10^{-8}$	CARDIoGRAMplusC4D, EPIC-CVD, + 8 other studies and six studies from the Myocardial Infarction Genetics Consortium (MiGen) ( $n = 88,192$ CAD patients; $n = 162,544$ HC)	Howson et al., 2017
20	PROCR	rs867186 (Ser219Gly) $P = 2.70 \times 10^{-9}$	CARDIoGRAMplusC4D, EPIC-CVD, + 8 other studies and six studies from the Myocardial Infarction Genetics Consortium (MiGen) ( $n = 88,192$ CAD patients; $n = 162,544$ HC)	Howson et al., 2017

ANRIL, antisense non-coding RNA in the INK4 locus; CAD, coronary artery disease; CXCL12, C-X-C motif ligand 12; HC, healthy controls; HLA, Human Leukocyte Antigen; IL-5, interleukin 5; IL-6R, interleukin 6 receptor; MI, myocardial infarction; PECAM1, Platelet endothelial cell adhesion molecule 1; PROCR, Protein C Receptor; SH2B3, SH2B adaptor protein 3.

5,469 controls (Kathiresan et al., 2009). The association between rs1746048 of the CXCL12 gene and MI resulted highly significant (combined  $p$ -value  $8.14 \times 10^{-11}$ ).

By analyzing 11,550 cases and 11,205 controls from 9 European studies, the Coronary Artery Disease Consortium reported for the SNP rs501120 a nominally significant association ( $P = 4.34 \times 10^{-4}$ ) and observed a possible sex interaction with a significant effect in women (OR = 1.29, 95% CI: 1.15–1.45,  $P = 1.86 \times 10^{-5}$ ) but not in men (OR = 1.03, 95% CI: 0.96–1.11,  $P = 0.387$ ; Coronary Artery Disease Consortium et al., 2009).

Successively, this observation has been replicated in a Chinese Han population of 2,335 coronary atherosclerosis patients and 1,078 controls undergoing coronary angiography. Accordingly, rs501120 at 10q11.21 was associated with coronary atherosclerosis in females ( $P = 8.36 \times 10^{-3}$ ) but not in males (Xie et al., 2011).

In the same year, it was shown that SNPs rs1746048 and rs501120 of the CXCL12 gene, are associated with high CXCL12

mRNAs and high CXCL12 plasma levels (Mehta et al., 2011) suggesting their functionality.

Very recently, in a case-control study involving 597 Chinese Han CAD patients and 685 healthy control, Zhang et al., by genotyping six SNPs (rs1065297, rs1801157, rs266089, rs197452, rs2839693, and rs10793538), identified three different SNPs (rs1065297, rs266089, and rs10793538) of CXCL12 associated with the risk of CAD (Zhang et al., 2017). Stratified according to gender, in the allele model, rs266089 and rs2839693 in CXCL12 gene were associated with the risk of CAD in men, while rs1065297 and rs10793538 in CXCL12 gene were associated with the risk of CAD in women. However, after adjustment by sex and age this association resulted not yet significant.

Anyhow, although this gene appears to be quite interesting, most of the tested SNPs are not functional, and the conflicting results obtained prompt to be cautious about a solid implication of this gene in coronary atherosclerosis.

## SH2B3 Polymorphisms

*SH2B3*, also known as *LNK*, encodes for an intracellular adaptor protein expressed in vascular endothelial cells and functions as a negative regulator in many pathways, as cytokine signaling pathways (Fitau et al., 2006; Gueller et al., 2011). In a mouse model of atherosclerosis, it has been demonstrated that this gene is up-regulated over five-fold as endothelial cells change from normal to the atherosclerotic disease state (Erbilgin et al., 2013).

Gudbjartsson et al., by investigating sequence variants affecting eosinophil counts in blood of 9,392 Icelanders and in six different populations (6,650 cases and 40,621 controls) found that the SNP rs3184504 (R262W) in exon 3 of *SH2B3* located at 12q24, resulted significantly associated with MI (Gudbjartsson et al., 2009). Since eosinophils are leukocytes involved in initiation and propagation of inflammatory responses, the accumulation of eosinophils in the thrombus could contribute to the genesis and progression of thrombus (Jiang et al., 2015).

Saade et al. performed a replication study of nine previously identified CAD/MI susceptibility loci in a total of 2,002 subjects of the FGENTCARD population (Saade et al., 2011). Previously reported results about rs3184504 and rs653178 were not confirmed in this population.

The SNP rs653178 near *SH2B3* showed, in a very large population including participants from the CARDIoGRAM ( $n = 86,995$ ) and the ICBP ( $n = 69,395$ ), a significant association with CAD ( $P = 2.2 \times 10^{-6}$ ; Olden et al., 2013).

With the aim to investigate the genetic susceptibility to CAD, in a large study (63,746 CAD cases and 130,681 controls) performed by combining genome-wide data from the CARDIoGRAM and C4D consortia, Deloukas and colleagues found evidence that only the SNP rs3184504 at the *SH2B3* locus displayed statistically significant association with CAD (CARDIoGRAMplusC4D Consortium et al., 2013).

Different results were more recently reported by Assimes et al. by genotyping 5,423 HC and 3,133 CAD patients from Taiwan (Assimes et al., 2016). Despite the association of rs79105258 ( $P = 2.5 \times 10^{-5}$ ), it was observed that the rs79105258 of the *SH2B3* contributes to CAD only in the presence of the rs2289800 of *COL4A1*.

Ji et al. conducted a study on a Chinese Han population of 456 CAD patients (291 men, 165 women) and 685 age-matched controls (385 men, 300 women) to determine the influence of sex on the association between *SH2B3* polymorphisms and CAD. Despite *SH2B3* polymorphisms resulted associated with CAD susceptibility in both women and men (Ji et al., 2017), the authors observed that the *SH2B3* haplotypes were associated with decreased CAD risk in women ( $P = 0.007$ ) but increased CAD risk in men ( $P = 0.047$ ). Interestingly enough, the rs3184504 at the *SH2B3* locus is one of the 29 SNPs identified in the first consortium able to discover genetic loci consistently associated with BP/hypertension at a genome significant level depicting a possible link between hypertension and atherosclerosis (Ehret et al., 2011).

Also for this gene the first findings were not constantly replicated but, differently from *CXCL12*, at least the rs3184504 determines an amino acid change and is located inside the gene.

Even if more research is needed, it sounds also attracting the common positivity in GWAS for hypertension.

## HLA Polymorphisms

HLA is a set of cell surface proteins essential for the acquired immune system by playing an important role in inflammation and T cell responses and mediating the interactions between leucocytes.

An association between a *HLA* locus, 6p21.3, and CHD was demonstrated for the first time by a meta-analysis of 5 GWAS including 13,949 subjects of European ethnicity (7,123 cases, 6,826 control subjects). The SNP significantly associated with CAD, the rs3869109, was located in an intragenic region between *HLA-C* and *HCG27* (HLA complex group 27; Davies et al., 2012).

To investigate whether this SNP confers the risk of premature CAD in the Chinese Han population, this association was explored in a total of 422 patients including 210 cases with coronary stenosis  $\geq 50\%$  or previous MI (male  $< 55$  years and female  $< 65$  years) and 212 controls without documented CAD (Xie et al., 2013). The authors showed that rs3869109 G-carriers have a higher risk of premature CAD than AA homozygotes (OR: 1.997, 95% CI: 1.166–3.419,  $P = 0.012$ ; OR 1.695, 95% CI: 1.044–2.752,  $P = 0.033$ ; respectively) and that there was a significant association between rs3869109 genotypes and the severity of disease.

Sinisalo et al. by conducting a large-scale genetic analysis on a case-control cohort comprising 5,376 ACS cases and 4,852 unrelated controls from 4 populations of 2 European countries, found a risk haplotype for the disease containing single nucleotide polymorphisms from *BTNL2* and *HLA-DRA* genes and the *HLA-DRB1\*01* allele (Sinisalo et al., 2016).

Taking together, the results of these studies are not convincing about a pivotal role of HLA in atherosclerosis.

## ABO

Among the 13 loci newly associated with CAD ( $P < 5 \times 10^{-8}$ ) in the very large meta-analysis performed by Schunkert et al. in 14 GWAS comprising 22,233 CAD patients and 64,762 controls of European descent followed by genotyping of 56,682 additional individuals (Schunkert et al., 2011), the risk allele on chromosome 9q34.2 (rs579459) in the *ABO* gene resulted associated with CAD ( $P = 4.08 \times 10^{-14}$ ) and weakly with increased LDL ( $P = 0.0049$ ) and total cholesterol ( $P = 0.0038$ ).

To identify loci that predispose to MI, Reilly et al. compared in a GWAS 5,783 patients who had angiographic CAD and MI and 3,644 patients who had angiographic CAD but no MI. They identified a novel association at the *ABO* locus: 11 *ABO* SNPs resulted significantly related to MI risk (Reilly et al., 2011). The authors hypothesized that the *ABO* association could be attributable to the glycosidase-deficient enzyme that encodes the AB0 blood group 0 phenotype previously proposed to protect against MI (Reilly et al., 2011). It has been hypothesized that blood group antigens could be directly involved in atherosclerotic inflammatory process (Wu et al., 2007). The same considerations as for the HLA locus apply also to the *ABO* locus.

## Other “Inflammatory” Loci Associated with CAD

In 2007 the locus 9p21 was identified as the strongest genetic susceptibility locus for CAD and MI independently by three different research groups (Burton et al., 2007; Helgadottir et al., 2007; McPherson et al., 2007; Samani et al., 2007). One year later, Schunkert confirmed the association between chromosome 9p21.3 and CAD in 7 case-control studies involving 4,645 patients and 5,177 healthy controls (Schunkert et al., 2008).

The variants identified in these studies are located in a locus that contains several genes, like *CDKN2A* [coding for p16(ink4a) and p14(ARF)], *CDKN2B* [coding for p15(ink4b)], *MTAP* (Holdt et al., 2011). These proteins are cyclin-dependent kinase inhibitors involved in the regulation of cell proliferation, aging, senescence and apoptosis in many cell types and result abundantly expressed in atherosclerotic lesions (Holdt et al., 2011). In this locus also the gene antisense non-coding RNA in the INK4 locus (*ANRIL*) maps also called the *CDKN2B* antisense RNA 1.

Several SNPs in the 9p21.3 locus have been showed associated with CAD risk (Burton et al., 2007; Samani et al., 2007; Schunkert et al., 2011).

More recently, a consistent association between two variants in the 9p21.3 locus (rs10965215 and rs10738605) and CAD has been reported also in the Chinese Han population (Cheng et al., 2017). For rs10965215, unconditional logistic regression analysis revealed that the G allele increased MI risk with OR of 1.37 (95% CI = 1.05–1.78,  $P = 0.020$ ). For rs10738605, C allele conferred increased MI risk with OR of 1.38 (95% CI = 1.06–1.80,  $P = 0.019$ ) as compared to the G allele after adjustment for conventional risk factors (Cheng et al., 2017).

*ANRIL* is expressed in endothelial cells, smooth muscle cells, and inflammatory cells and is involved in the regulation of expression of adjacent protein coding genes, including *MTAP*, *CDKN2A* (p15INK4b), and *CDKN2B* (p16INK4a) through multiple mechanisms, including RNA interference, gene silencing, chromatin remodeling, or DNA methylation (Holdt et al., 2010).

This locus remains still one of the most promising for coronary atherosclerosis, being detected in most of the GWAS performed and subtending genes of putative effect. Nevertheless, the exact mechanism and the gene responsible is to be definitely proved.

The IBC 50K CAD Consortium examined 49,094 genetic variants in 2,100 genes in 15,596 CAD cases and 34,992 controls and replicate putative novel associations in an additional 17,121 CAD cases and 40,473 controls (IBC 50K CAD Consortium, 2011). Among the new variants identified, SNP (rs2706399) in interleukin 5 (*IL5*) gene (5q31.1) resulted statistically associated with CAD in the discovery and replication study (combined  $p$ -value  $2.1 \times 10^{-6}$ ). *IL5* is an interleukin produced by T helper-2 cells with a role in the inflammatory process characterizing the development and progression of atherosclerosis and CAD (Hansson, 2005).

The association between rs4845625 of the *IL6R* and CAD ( $P = 3.64 \times 10^{-10}$ ) was demonstrated in the CARDIoGRAMplusC4D Consortium, in which the

CARDIoGRAM population (22,233 cases and 64,762 controls) was expanded with 34 additional European or south Asian population comprising 41,513 cases and 65,919 controls (CARDIoGRAMplusC4D Consortium et al., 2013). The IL6-mediated activation of IL6R, a receptor located on the leukocytes and hepatocytes membrane, stimulates the proinflammatory cascade including hepatic production of acute phase reactants C-reactive protein and fibrinogen (Schuett et al., 2009).

Moreover, the Asp358Ala (rs2228145) in IL6R was not associated with several traditional risk factors such as blood pressure, adiposity, hyperglycaemia, cholesterol, and smoking but with the concentration IL-6, C-reactive protein, fibrinogen, and coronary heart disease implying a causal role for this inflammatory pathway (IL6R Genetics Consortium Emerging Risk Factors Collaboration et al., 2012).

Howson et al. genotyped 56,309 participants and performed meta-analysis of results with 194,427 participants previously genotyped, totalising 88,192 CAD cases and 162,544 controls. The authors identified 25 new SNP-CAD associations ( $P < 5 \times 10^{-8}$ ) from 15 genomic regions, including SNPs in *PECAM1* (rs1867624,  $P = 3.98 \times 10^{-8}$ ) and in *PROCR* (rs867186,  $P = 2.70 \times 10^{-9}$ ) genes, that are involved in cellular adhesion, leukocyte migration and inflammation (Howson et al., 2017).

In particular PECAM-1 is important to maintain the integrity of the vascular barrier. Accordingly, the consequence of a not conserved barrier can be the development of chronic inflammatory diseases such as atherosclerosis (Privratsky et al., 2011).

These are a few examples of how genetics can and will be able to in the near future identify molecules or pathways as tailored anti-inflammatory therapeutic targets in atherosclerosis.

## CONCLUSION

Despite the inflammatory core of atherosclerosis has been well described, a straightforward anti-inflammatory therapy for targeting inflammation is still missing in the physician’s armamentarium. Therapy with biological drugs is attractive but probably too expensive to be largely available. Newer targets are needed and genetics can help with this issue. So far, the results coming from GWAS have pinpointed a few loci closed to inflammatory molecules and/or pathways consistently associated with atherosclerosis and CV events, revamping the strict link between inflammation and atherosclerosis suggesting some tailored target therapy.

In some cases Mendelian randomization studies can help in detecting which of them is a real primary therapeutic goal and which should be considered secondary (Ridker and Lüscher, 2014).

In the upcoming genomic era, both whole-exome and whole-genome sequencing will pinpoint new and old loci associated with atherosclerosis identifying new molecular targets or characterizing which inflammatory pathway could be a suitable target to block atherosclerosis even in its early stages.

## AUTHOR CONTRIBUTIONS

Both authors contributed to the drafting and completion of the final version of the manuscript.

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# Molecular Mechanisms of T Cells Activation by Dendritic Cells in Autoimmune Diseases

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The interaction between T cell and dendritic cells (DCs) that leads to T cell activation affects the progression of the immune response including autoimmune diseases. Antigen presentation on immune cell surface, formation of an immunological synapse (IS), and specific identification of complex by T cells including two activating signals are necessary steps that lead to T cell activation. The formation of stimulatory IS involves the inclusion of costimulatory molecules, such as ICAM-1/LFA-1 and CD28/B7-1, and so on. Some fusion proteins and monoclonal antibodies targeting costimulatory molecules have been developed and approved to treat autoimmune diseases, including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), multiple sclerosis (MS), type I diabetes (T1D), inflammatory bowel disease (IBD), and psoriasis. These biological agents, including CTLA-4- and LFA-3-Ig, anti-CD3 monoclonal antibody, could prevent the successful engagement of DCs by T cell with significant efficacy and safety profile. In this article, we reviewed the molecular mechanisms of T cell activation during the interaction between T cells and DCs, and summarized some biological agents that target costimulatory molecules involved in the regulation of T cell activation.

**Keywords:** T cell, dendritic cells, activation, autoimmune diseases, immunological synapse, biological agents

## INTRODUCTION

Various organ specific autoimmune diseases are mediated by an imbalance of T cell subsets, e.g., an absence of regulatory T cells, or tissue injury driven by pathological T helper cell responses. Examples are rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), multiple sclerosis (MS), type I diabetes (T1D), and inflammatory bowel disease (IBD) (Fletcher et al., 2010; Burmester et al., 2014; Geem et al., 2015; Suarez-Fueyo et al., 2016; Pugliese, 2017). In an inflammatory environment, autoreactive T cells are activated initially by dendritic cells (DCs). Like macrophages and B cells, DCs are professional antigen-presenting cells (APCs). However, DCs have the unique property of inducing the differentiation of naïve CD4<sup>+</sup> T cells into helper and effector T cells with a unique combination of abilities, which allows DCs to effectively process antigen, strongly express costimulatory molecules, secrete cytokines, and migrate to tissues or lymphoid organs to prime T cells (Steinman, 2007). Therefore, DCs emerged as critical players in the initiation and development of immune response.

In identifying pathogen-associated cues, DCs undergo a series of functional changes known as maturation. Mature DCs present antigenic peptides in the context of major histocompatibility complex (MHC) class II to the T cell receptor and express co-stimulatory molecules CD40 and B7. Mature DCs are characterized by the production of cytokines, such as IL-12, and by the expression of homing receptors, such as CCR7, which directs the migration of DCs into the T-cell regions of secondary lymphoid organs. Together these changes enable DCs to effectively activate naïve T cells. At the same time, DCs induce the expression of the corresponding costimulatory molecules of CD40L, CD28, on T cells. Mature DCs promote naïve T cells differentiate into Th1, Th2, Th17, or Treg cells in a stimulus-dependent manner by secreting cytokines. In a mouse model of EAE *in vivo* and *in vitro*, DCs which express aberrant P38 can promote the differentiation of Th17 cells by inducing the secretion of IL-17 and the expression of IL-23 receptors (IL-23R) (Huang et al., 2012). Detection of mature DCs producing large quantities of IL-12 and IL-23 strongly supports the notion that DCs play a key role in autoimmune diseases by promoting a deleterious imbalance between Th1, Th2, and Th17 cells (Lebre et al., 2008; Tournadre et al., 2012; Segura et al., 2013). The majority of DCs exist in the inflammatory synovia fluid of RA patients, expressing CD1a and secreting IL-23 (Segura et al., 2013). Furthermore, DCs with a gene deletion of interleukin 1 receptor related kinase M (IRAK-M) show strong antigen presenting ability, resulting in the abnormal activity of diabetogenic T cells and autoimmune reaction *in vitro* and the rapid onset of T1D *in vivo* in immunodeficient NOD mice when cotransferred with diabetogenic T cells (Tan Q. et al., 2014).

DCs include immunogenicity DCs and tolerogenic DCs according to function. Interactions between tolerogenic DCs and CD4<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (Tregs) play a critical role in maintaining peripheral tolerance and preventing activation of T cells (Audiger et al., 2017). Peripheral tolerance is associated with a high activity of Tregs and a reduced inflammatory profile of Th cells (Min et al., 2006; Li et al., 2008). CD4<sup>+</sup> Treg in both the spleen and lymph node help to maintain tolerogenic status of DCs through the expression of CTLA-4 in mice (Wing et al., 2008).

DCs from different location exert different functions. Plasmacytoid DCs secrete large amounts of type I interferons (such as IFN-alpha and IFN-beta) after identification of the viruses through TLR7 and TLR9, which are located in intracellular compartments (Gilliet et al., 2008). The central role of plasmacytoid DC in autoimmune diseases is emphasized by its association with type I-interferon signal. Type I interferons produced by plasmacytoid DC from human PBMCs also supports IL-17 secretion and Th17 responses (Lombardi et al., 2009). Furthermore, human plasmacytoid DCs enhance thymic Treg expansion and generation of peripheral Treg through the production of indoleamine 2, 3-dioxygenase (IDO) and the expression of programmed death-ligand 1 (PD-L1) *in vivo* and *in vitro* (Chen et al., 2008; Amarnath et al., 2011; Creusot et al., 2014). Lymphoid-resident DCs rapidly extracts antigens from lymph and blood for presentation to T cells (Sixt et al., 2005). In particular, CD205<sup>+</sup> DCs in the spleen of mice are able to

induce the tolerance of CD4<sup>+</sup> T cell under suboptimal activation conditions (Yamazaki et al., 2008).

The interaction between T cells and DC leads to the formation of immunological synapse (IS) and is maintained by highly expressing adhesion molecules (LFA-1, LFA-3, ICAM-1, ICAM-2), cytokines and chemokines (Lee et al., 2002; Tseng et al., 2008). In this article, we reviewed the molecular mechanism of T cells activation by DCs and immunotherapy targeting T cell activation in autoimmune diseases.

## MOLECULAR MECHANISMS OF T CELL ACTIVATION BY DCs

There are three stages during T cells activation by DCs, namely antigen presenting, antigen recognition of T cells and two signals formation. In addition, IS formation between T cells and DCs plays an important role in T cell activation.

### Antigen Presenting

Germline encoded pattern recognition receptors (PPR) specific for pathogen-associated patterns (PAM) are present on immature DCs. An engagement of these membrane-bound receptors trigger a maturation of DCs and lead to an up-regulation of costimulatory molecules (Kabelitz and Medzhitov, 2007). Mature DCs in mice express chemokine receptor 7 (CCR7) and begin to migrate into regional lymph nodes after an encounter with antigen (Ritter et al., 2004).

For a presentation with MHC class II, antigen is degraded by DCs to a suitable length (approximately 12 amino acids) utilizing proteasomes in the endogenous pathway. These antigenic peptides bind to specific grooves in the MHC class II molecules (Jones et al., 2006). Peptide-MHC II complexes are formed in the rough endoplasmatic reticulum and transported to the cell surface for presentation (Vyas et al., 2008; Neefjes et al., 2011). At the local draining lymph node, DC present complexes of processed peptides together with MHC class II to naïve CD4<sup>+</sup> T cells which bind to this combination with their TCR and initiate signaling. The peptide binding to MHC class I and the subsequent presentation to CD8<sup>+</sup> T cells is similar in many aspects and will not be discussed in detail. Overall, antigen presentation with MHC class II and MHC class I are mainly two modes for DCs.

A third mode of antigen presentation utilizing the conserved non-classical MHC class I molecule CD1 plays an important role in microbial infection and the immune response to lipid antigens (Shao et al., 2005; Barral and Brenner, 2007). CD1 has 30% homology with MHC class I molecules, and there are main five types of CD1 in humans, termed CD1a-e (Barral and Brenner, 2007). Probably the best studied member of the CD1 family is CD1d which presents predominantly lipids to CD1-restricted T cells that have a limited repertoire of TCR and have been termed Natural Killer (Kang et al., 2014). Although interferon (IFN)-gamma secretion by CD1-restricted T cells during infection had been shown before, the function of CD1 restricted T cells was not entirely understood for a long time (Gilleron et al., 2004). Only recently, it was demonstrated that the expression of human class I CD1 molecules in transgenic mice caused a rapid response of

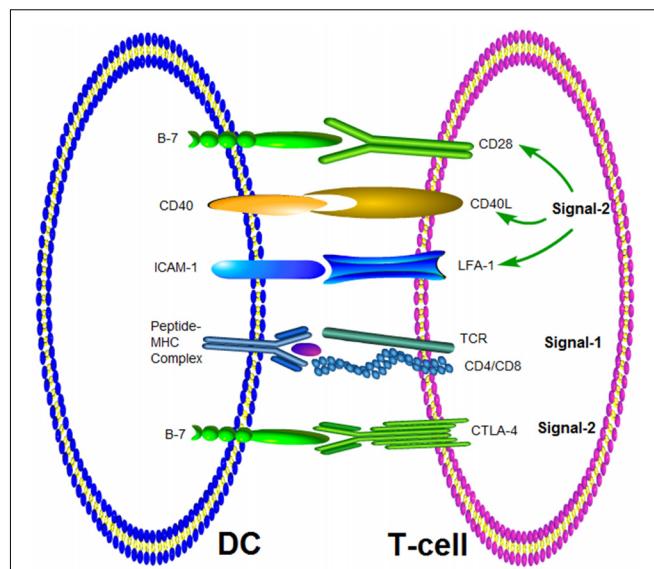
CD1-restricted T cells after re-exposure to antigen, suggesting a protective effect of CD1-restricted T cells (Felio et al., 2009). Natural sebum can be used as a headless antigen and presented to autoreactive T cells through CD1a (de Jong et al., 2010). In addition, researchers have found a group of highly conserved T cells in tuberculosis (TB) patients. These conserved T cells could recognize specifically mycobacterial antigens presented by CD1b (Van Rhijn et al., 2013). These findings suggest that the antigen-presenting molecule CD1 plays an important role during special antigen presentation.

## Antigen Recognition of T Cells

T cell receptor (TCR) on T cells not only identify peptide-MHC complexes derived from host cells infected by pathogens, but also recognize similar structures derived from healthy host cells, so called autoantigens. The specificity of the TCR for antigen is located in the V segment, which is composed of N-terminal of two TCR polypeptides (Govers et al., 2010). Both V alpha and V beta have 3 hypervariable regions and are also known as the complementarity determining region, namely CDR1, CDR2, and CDR3. CDR3 is a largest hypervariable region and directly determines the antigen binding specificity of TCR (Feng et al., 2015). The TCR identifies simultaneously the entire complex of antigenic peptide and MHC molecule as a first step in T cell activation (Reiser et al., 2000). The comparison between the TCR conformation and the conformation of TCR bound to the peptide-MHC complex indicates that CDR3 region undergoes a large conformational transition in order to obtain a diagonal position that allows the binding to peptide-MHC complex (Garcia, 2012). When the V segment of the TCR identifies an antigen/MHC complex, the TCR heterodimer deliver the activation signal to the cell nucleus through the constant transmembrane components of the CD3 complex. (Schamel et al., 2005). Therefore, TCR signaling has a key role in determining T cell fate. TCR-peptide-MHC complexes appear to support a model of physical specificity between TCR germline V regions and MHC.

## Two Signals Are Necessary for Activation of Naïve T Cells

Naïve T cell activation by DCs requires two signals, termed signal-1 and signal-2 (Anderson and Siahaan, 2003). Signal-1 is equivalent to the binding of TCR to peptide-MHC complex (Garg et al., 2010; Vesely et al., 2011; Manikwar et al., 2012). Signal-2 requires the interaction of costimulatory molecules at the interface between DCs and T cells (B7/CD28, LFA-1/ICAM-1 and ICAM2, CD2/LFA-3) (Figure 1). The combination of TCR and peptide-MHC complex (signal 1) will lead to phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) on CD3, which is closely adjacent to TCR by Lck kinase (Rossy et al., 2013). T cells receive signal-1 when the activation cascade leads to signaling through multiple TCR for several hours (Frauwirth and Thompson, 2004). This sustained signaling is necessary for the effective activation of signal transduction pathways that lead to the activation of nuclear transcription factors. The clustering of TCR in IS at the interface between T cells and DC is necessary



**FIGURE 1 |** Molecular interactions at the interface of T-cell and APC. Signal-1 is provided by the interaction between TCR and the MHC-peptide complex. The co-stimulatory signal (Signal-2) can be delivered by different pairs of molecules.

for continuous signal transduction and will be discussed in more detail later. Signal-2 was initialized by the interaction of costimulatory molecules expressed DCs and T cells. Positive signals, such as CD28/B7-1 (CD80) and CD28/B7-2 (CD86), and negative signals, such as CTLA-4/CD80 and CTLA-4/CD86 have been identified (Huang et al., 2012; Manikwar et al., 2012). As mentioned above pairs of costimulatory molecules (CD80/CD28, LFA-1/ICAM-1, or ICAM2, CD2/LFA-3) provide signal 2. The activation signal of these costimulatory molecules is delivered to T cells via the ITAM of the cytoplasmic domain, which enhances the TCR response to antigen (Acuto et al., 2008). It was two signals model for T-cell activation. T cells could be activated in simultaneously receiving signal-1 from T-cell recognition of antigen and signal-2 from costimulatory molecular. However, it was an off signal to T cells in only receiving signal-1, and T cells would translate into tolerance, clone incompetent or deletion.

CD28/CD80 was a pair of co-stimulatory molecules that mediated and enhanced immune responses, but these molecules were not directly involved in memory immune responses (Kopf et al., 2000). Furthermore, the co-stimulatory signal of CD28 was related mainly to initiating interaction between DCs and T cells. The CD28/CD80 signal activate T cells to express multiple other costimulatory molecules, these costimulatory molecules control the balance of immune response and the stability of internal environment. The interaction between cytotoxic T lymphocytes (CTL) and Th or Th and B cells rests on other costimulatory molecules, such as OX40 (CD134), inducible T-cell costimulator (ICOS) (Bansal-Pakala et al., 2004). The costimulatory molecule 4-1BB and its ligand 4-1BBL can control adaptive immunity (Lee et al., 2008). Treg cells up-regulate the expression of 4-1BB in response to IL-2 and suppressed T cell proliferation. At the same time, the synergy of 4-1BB and CD28 signal can affect cell

polarization, and promote Th0 cells differentiation into Th1 cells which are characterized by the production of IFN-gamma (Elpek et al., 2007).

CTLA-4 (CD152) is homologous to CD28 and also expressed on activated T cells, but the cytoplasmic domain of CTLA-4 has an immunoreceptor tyrosine-based inhibitory motif (ITIM) (Topalian et al., 2015). Therefore, CTLA-4 binds to CD80 in competition with CD28 with an affinity that is 20 times higher than CD28/CD80 and can send an inhibitory signal to activated T cells through its ITIM motif to restore the balance of immune response (Pentcheva-Hoang et al., 2004; Vogel et al., 2015). CTLA-4 activates protein tyrosine phosphatase (PTP) through the ITIM structure and inhibits T cell activation signal transduction, leading to a negative regulation of T cell activation (Chemnitz et al., 2004). Additionally, CTLA-4 inhibits the expression of IL-2 receptor alpha chain, IL-2 secretion and IL-2 mRNA accumulation, also resulting in an inhibition of the activation of T cells in preclinical mouse models (Hannani et al., 2015). Hence, costimulatory signals mediated by costimulatory molecules, including positive signals and negative signals, play important role in regulating interaction between T cell and DCs and maintaining the balance of immune response.

## IS Formation Plays an Important Role in T Cell Activation

IS play an important role in T cells activation, and IS formation involves a variety of costimulatory molecules, such as ICAM-1/LFA-1, CD28/B7-1, and so on (Schwartz et al., 2002).

## Formation Mechanisms of IS

In the process of T cell and DC interaction, a variety of transmembrane molecules accumulate in a “raft” structure that is rich in sphingomyelin and cholesterol, and are clustered at the interface of T cell and DC contact. This special “raft” structure has been termed IS. Before the formation of IS, T cells form pseudopods in search of peptide MHC complexes on APCs. After the initial contact the formation of IS is a dynamic process that has been described to depend on a planar lipid bilayer. IS formation includes three phases: (i) The first stage is the connection of TCR and peptide MHC complex. The adhesion molecules such as LFA-1/ICAM-1 and CD2/LFA-3 are recruited to the nascent rafts (Barreiro et al., 2007); (ii) The second stage has been termed the peptide MHC complex transfer stage. In the early stages of IS formation, TCR-peptide MHC complex is transported to the central region of IS, while LFA-1/ICAM-1 is transferred to the peripheral region to form mature IS; (iii) The third stage is the formation of a stable contact region at the interface of T cell and antigen presenting cell. In this stage, the super molecular structure of a mature IS can be maintained for 1 h, while PKC theta, Bcl10, IKK beta are recruited to IS by cytoskeleton changes (Dustin, 2005).

## Molecular Structure of IS

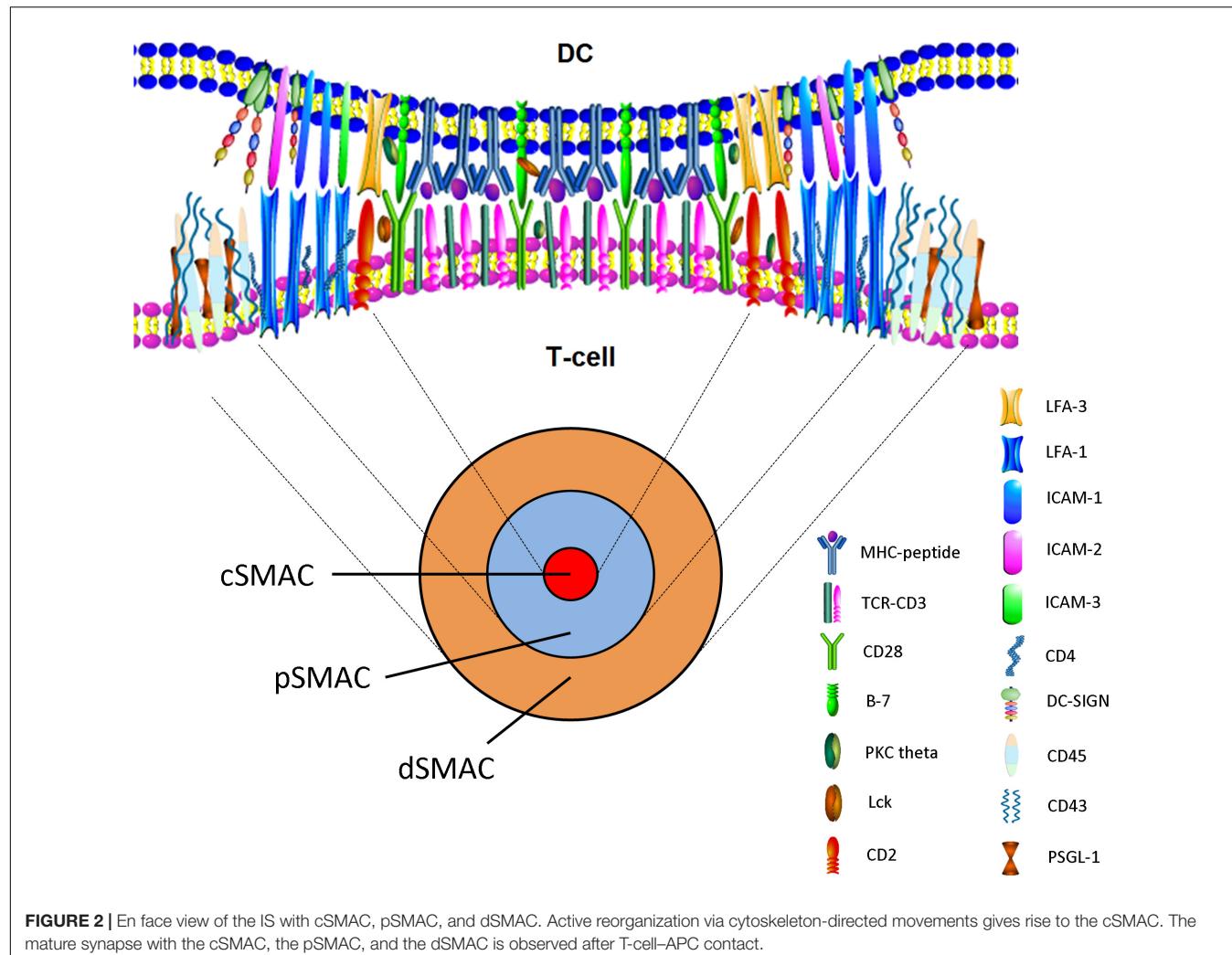
The molecular structure of IS include three areas, namely central supermolecular activation cluster (cSMAC), peripheral SMAC (pSMAC), and distal SMAC (dSMAC). The molecules in cSMAC

area mainly includes TCR-peptide MHC complex, CD3, CD28, and signal transduction molecules such as PKC theta and Lck (Valitutti, 2008). Adhesion molecules such as LFA-1/ICAM-1 or DC-SIGN surround pSMAC area (Dustin, 2005). CD2/LFA-3 is located between cSMAC and pSMAC, and CD43, CD45, and PSGL-1 are located at dSMAC. The number of TCRs in cSMAC is only double that of in pSMAC, but the number of LFA in pSMAC is almost 6 times that of in cSMAC. In fact, cSMAC and pSMAC do not show obvious boundaries. Although cSMAC and pSMAC can be maintained for several hours, the numbers of receptors and molecules in IS are changed dynamically (Dustin, 2005). CD45 is a unique molecule that is transferred to dSMAC from cSMAC, which may be related to the activation of Lck at the stage of early IS formation (Grigorian et al., 2009). cSMAC take part in the reuse and degradation of TCRs, which down-regulate the TCR and attenuate signals (Figure 2). Thus it can be seen that the molecular structure of IS was very complex involving in many molecules and signals, which take part in IS formation through interaction and dynamically balance.

## Factors That Influence IS Formation

TCR signaling is necessary for the maintenance of IS. TCR-microclusters (TCR-MCs) are formed immediately after the TCR on T cells adheres to peptide MHC complex including many signal molecules such as CD3, SLP-76, TCR, and ZAP-70 (Campi et al., 2005; Saito and Yokosuka, 2006). TCR-MCs are the activation site of initial signal. TCR stimulation, calcium influx, tyrosine activation occur before the formation of cSMAC. TCR-MCs are continuously produced, even after IS formation (Bardaa-Saad et al., 2005). TCR is rapidly internalized about 5 min after the exposure to DC, but TCR stimulation will continue for several hours, which results in the activation signal in the peripheral MCs rather than cSMAC. Blocking TCR-peptide-MHC complex within 10 hours results in IS dissolution, decreasing the level of calcium influx and causes cell separation. These findings indicate that the maintenance of TCR signaling is necessary for the maintenance of IS and the full activation of T cells.

CD4 molecule could promote IS formation. CD4 can improve the sensitivity of T cells to antigens and can accumulate Lck to the center region of IS after the initial exposure of DCs to T cells. CD4-deficient T cells have a reduced proliferative response to antigen stimulation and the effect of IS formation is also significantly reduced. The cells expressing CD4 or displaying a peptide MHC complex have a strong binding, suggesting that CD4 is not only an auxiliary receptor but also contributes to cell adhesion. The actual lipid raft is the key components of a functional IS. The accumulation of lipid rafts was observed at cSMAC, indicating that the formation of IS was accompanied by the movement of lipid rafts to cSMAC and gathered on the contact interface of cells (Henel et al., 2006). Lck and LAT are linked to lipid rafts by deacylation. Other signaling molecules such as PLC gamma, SLP76, and Vav are transferred to IS by binding to phosphorylated tyrosine on LAT (Phee et al., 2005; Braiman et al., 2006; Soares et al., 2013). CTLA-4 limits the accumulation of lipid rafts, thereby inhibiting T cell proliferation and cytokine secretion (Teft et al., 2006). Actin movement is associated with the transportation of cytoskeleton and can be



blocked by the myosin inhibitor butanedione monoxime (van der Honing et al., 2010).

Overall, TCR signaling, CD4 molecule, lipid raft, PLC gamma and CTLA-4, and so on not only involve in IS information but also modulate IS information. Any abnormal signals or the imbalance among molecules would lead to abnormal T cell activation in autoimmune diseases. These molecules might be new drug targets, and it would offer new therapy strategies through developing new drugs targeting above molecules.

## IMMUNOTHERAPY TARGETING T CELLS ACTIVATING IN AUTOIMMUNE DISEASES

Detailed insights into the molecular mechanisms of the interaction between T cells and DCs are helpful to design immunotherapy strategies that target T cell activation in autoimmune diseases. At present, some biological agents, such as CTLA-4Ig (Abatacept), Anti CD3 monoclonal antibody, LFA-3 Ig fusion protein (Alefacept) that target co-stimulation molecules

on T cell have been developed and approved to treat autoimmune diseases.

### CTLA-4Ig Modulates Co-stimulatory Signals and Inhibits T Cell Activation

The recombinant fusion protein, CTLA-4Ig (Abatacept) that comprises the extracellular domain of human CTLA4 and a fragment of Fc domain of human IgG1 belongs to a new type of selective co-stimulatory modulators. Abatacept prevents complement fixation and modulates the necessary co-stimulatory signal for T cell activation. Furthermore, it binds to CD80 and CD86, thus competing with CD28 and reducing T cell activation (Cutolo and Nadler, 2013; Keating, 2013). The fusion protein affects multiple downstream pathways through modulation of upstream events of T cells activation. Additionally, Abatacept inhibits T-cell proliferation and the secretion of IFN-gamma, IL-1, IL-6, and TNF-alpha (Koenders et al., 2012; Whitfield et al., 2017).

As therapy, Abatacept is mainly used in RA treatment (Genovese et al., 2005; Dorner et al., 2010). It has been proven to

be efficient, safe, and tolerable in combination with methotrexate (MTX) in clinical trials with RA patients when the response to MTX was inadequate (Kremer et al., 2005). In Europe, Abatacept is approved for the treatment of patients with highly active and progressive RA, who have never received MTX treatment. It is also approved for the treatment of patients with moderate to severe active RA, who have shown inadequate responses in previous therapies with at least one conventional disease-modifying antirheumatic drug (cDMARDs) such as MTX or a TNF inhibitor. In phase III clinical trials, intravenous or subcutaneous injection regimens of Abatacept were beneficial for RA symptoms, disease activity, structural damage progression and physical function of the joint. In a long-term follow-up, the efficacy could be shown to be maintained. The drug-free remission rates following discontinuation of all RA treatment were significantly higher after treatment of Abatacept plus methotrexate than of methotrexate alone. Intravenous and subcutaneous injections of Abatacept were generally well tolerated and showed low immunogenicity (Blair and Deeks, 2017). Previous studies using synovial tissue from RA patients treated with Abatacept found the inhibition of B-cell proliferation and down regulation of the expression of B-cell markers (Buch et al., 2009).

Abatacept was also used to treat lupus nephritis by inhibiting CD28 engagement on T cells and plasma cells (Bahlis et al., 2007). This mechanistic rationale is strongly supported by the studies in SLE murine models, in which treatment with Abatacept or other forms of CTLA4-Ig have been shown to arrest and even reverse established lupus nephritis. Treatment with Abatacept induced remission by binding to CD80 on renal podocytes in patients with focal segmental glomerulosclerosis (Yu et al., 2013; Group, 2014).

### Anti-CD3 mAbs by Induce Anergy and Apoptosis of Activated T Cells

Anti CD3 monoclonal antibodies are an immunosuppressant. Muromonab-CD3 is a murine IgG2, which specifically binds to CD3 on T cells and blocks proliferation and function of T cells. Tolerance induction by anti-CD3 mAbs is related to the induction of Tregs that control pathogenic autoimmune responses preferentially by inducing anergy or apoptosis in activated T cells while ignoring Tregs (You et al., 2008; Penaranda et al., 2011). Consequently, anti-CD3 mAb therapy is associated with an increase in number and function of Treg and regulatory cytokines such as TGF-beta and IL-10. The heterogeneity of TCR expression by different T-cell subsets might explain the differential effect of anti-CD3 mAb on effector, regulatory or naïve T cells (Valle et al., 2015). At the same time anti-CD3 mAb-induced signaling through the CD3/TCR complex can render the T cell anergic or trigger apoptosis. Various studies have shown that anti-CD3 mAbs effectively treat chronic inflammatory and autoimmune diseases, such as IBD, T1D and MS. Intravenous administration of anti-CD3 mAb has been successfully tested in numerous animal models of autoimmunity, including the experimental autoimmune encephalomyelitis (EAE) model of MS, diabetic NOD mice, TNP-KLH induced colitis (a model of IBD) and collagen-induced arthritis (Kohm et al., 2005;

Chatenoud and Bluestone, 2007; Notley et al., 2010; Wu et al., 2010).

Biological agents targeting CD3 include teplizumab, otezixizumab, and visilizumab. It has been observed that administration of otezixizumab, teplizumab, or visilizumab result in positive clinical responses (Keymeulen et al., 2005, 2010; Plevy et al., 2007). Otezixizumab and teplizumab were foremost tested in T1D patients, while visilizumab and foralumab were mainly studied in IBD (Yu et al., 2008; Daifotis et al., 2013). In clinical trials, the tolerogenic activity of humanized anti-CD3 mAb (visilizumab) in T1D was found to be excellent. In a second Phase I/II trial, teplizumab improved insulin production and metabolic control in patients with recent onset T1D. A phase II trial that assessed the safety and efficacy of visilizumab in patients with severe corticosteroid-refractory ulcerative colitis had promising results (Plevy et al., 2007). In general, non-FcR binding anti-CD3 mAb are promising models for treatment of autoimmune and inflammatory diseases (Herold et al., 2003).

### LFA-3 Ig (Alefacept) and Anti-LFA-1 Antibody (Efalizumab) Inhibit CD2 Signaling in T Cells

It had been demonstrated that an LFA-3 Ig fusion protein (Alefacept) could reduce psoriasis lesions (Nickoloff and Nestle, 2004). Alefacept competes with LFA-3 for binding to CD2 on T cells and efficiently interferes with LFA-3/CD2 binding, consequently stopping T cell activation. Furthermore, the Ig part of Alefacept binds to immunoglobulin receptor Fc-gamma-RIII on the surface of natural killer cells and some T cell subpopulations inducing apoptosis of memory T cell subgroups (da Silva et al., 2002; Rigby et al., 2015). Finally, administered intramuscularly or intravenously Alefacept inhibits T cell activation and proliferation, and induces the apoptosis of memory-effector ( $CD45RO^+$ ) T cells (Konig et al., 2016).

In psoriasis the presence of LFA-1 in IS is very important. A separate anti-LFA-1 antibody (Efalizumab) has been shown to block adhesive interaction in the treatment of psoriasis. The antibody reduced skin lesions by blocking the adhesion molecule on T cells and was well tolerated and resulted in significant improvement in patients with moderate to severe plaque psoriasis (Papp et al., 2006).

## CONCLUSION

In summary, the interaction between T cells and DCs involves in the pathogenesis of autoimmune disease. Autoreactive T cells are activated by autoantigens presented by DCs during the interaction between T and DC (Tan T. et al., 2014). The underlying molecular mechanisms of T cell activation by DCs have been well understood. Three stages during T cells activation by DCs, including antigen presenting, antigen recognition of T cells, and two signals formation have been investigated in great detail. T cells could be activated in two signals model by simultaneously receiving signal-1 from T-cell recognition of antigen and signal-2 from costimulatory molecular. In addition, IS formation between

T cells and DCs plays an important role in T cell activation. cSMAC, pSMAC, and dSMAC form the molecular structure of IS. IS molecular structure is very complex involving in a variety of molecules and signals, which take part in IS formation through interaction and dynamically balance.

Understanding the molecular mechanisms of the interaction between T cells and DCs is helpful to discover new drug targets and design immunotherapy strategies that target T cell activation in autoimmune diseases. At present, some recombinant fusion protein and monoclonal antibodies targeting costimulatory molecules, such as CTLA-4- and LFA-3-Ig, anti-CD3 monoclonal antibody, and so on have been developed and approved to treat autoimmune diseases, such as RA, SLE, IBD, MS, and psoriasis. These biological drugs show a significant efficacy and have a high safety profile. More biological agents that modulate T cell activation will be developed based on a better understanding of the molecular mechanisms of T cell activation in the near future.

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## AUTHOR CONTRIBUTIONS

YT and QW collected data and wrote this paper. HK and LZ revised the article. WW designed the work.

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# The Role of Nrf2 in Liver Disease: Novel Molecular Mechanisms and Therapeutic Approaches

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Oxidative stress and inflammation are the most important pathogenic events in the development and progression of liver diseases. Nuclear erythroid 2-related factor 2 (Nrf2) is the master regulator of the cellular protection *via* induction of anti-inflammatory, antioxidant, and cyto-protective genes expression. Multiple studies have shown that activation or suppression of this transcriptional factor significantly affect progression of liver diseases. Comprehensive understanding the roles of Nrf2 activation/expression and the outcomes of its activators/inhibitors are indispensable for defining the mechanisms and therapeutic strategies against liver diseases. In this current review, we discussed recent advances in the function and principal mechanisms by regulating Nrf2 in liver diseases, including acute liver failure, hepatic ischemia–reperfusion injury (IRI), alcoholic liver disease (ALD), viral hepatitis, non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), and hepatocellular carcinoma (HCC).

**Keywords:** Nrf2, oxidative stress, cytoprotective genes, acute liver injury, viral hepatitis, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, hepatocellular carcinoma

## INTRODUCTION

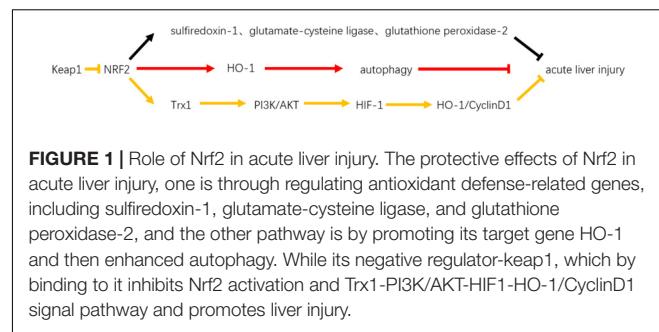
Oxidative stress and inflammation are the most important pathogenic events in liver diseases. During liver injuries, the unregulated production of free radicals and/or ROS leads to damage of important biomolecules and cells and generation of proinflammatory genes. Antioxidant and anti-inflammatory therapy has been considered to be beneficial in liver diseases. Nrf2 is the master regulator of the primary means of cellular defense through mediation of antioxidant response, anti-inflammatory and cytoprotective properties, and dysregulation of Nrf2 activity has been revealed to correlated with the development of chronic inflammatory diseases (Alam et al., 1999; Vomund et al., 2017; Bellezza et al., 2018; Hennig et al., 2018). The protective effects of Nrf2 signaling pathway has been identified in a number of disease models, including acute kidney, lung or neurons

**Abbreviations:** ALD, alcoholic liver disease; ALP, alkaline phosphatase; ALT, aspartate aminotransferase; CDDO-Im, CDDO-imidazolidine; CYP2E1, cytochrome P450 2E1; D3T, 3H-1,2-dithiole-3-thione; D-GalN, D-galactosamine; DAMP, damage-associated molecular pattern; DILI, drug-induced liver injury; FGF19, fibroblast growth factor 19; GST, glutathione S-transferase; HBV, hepatitis B virus; HBx, HBV stimulates by its X protein; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IRI, ischemia–reperfusion injury; JNK, c-Jun N-terminal kinase; Keap1, Kelch-like ECH-associated protein; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NQO1, NAD(P)H quinone dehydrogenase one; Nrf2, nuclear erythroid 2-related factor 2; NS, non-structural; PBC, primary biliary cholangitis; PPAR $\gamma$ , peroxisome proliferator-activated receptor- $\gamma$ ; ROS, reactive oxygen species; VLDLR, very-low density lipoprotein receptor.

injury, emphysema, and sepsis (Thimmulappa et al., 2007; Reddy et al., 2009; Sussan et al., 2009; Zhang et al., 2012; Liu et al., 2014). Accumulating evidence also has implicated this transcription factor in various liver diseases, including acute hepatotoxicity, NAFLD, NASH, ALD, DILI, viral hepatitis, liver fibrosis, hepatic IRI, and primary hepatic malignancies (Klaassen and Reisman, 2010; Tang et al., 2014). Under acute and chronic oxidative stress and inflammatory conditions, Nrf2 is activated and prevents oxidative and inflammatory diseases by modulating genes expression of cytoprotective proteins and enzymes, which decreases ROS levels, inflammation, and cell death (Bataille and Manautou, 2012). However, the function of Nrf2 is not always protective in diseases, recent studies have identified that the gene expression of Nrf2 was associated with the pathogenesis, progression, and metastasis of cancer, resistance to cancer therapy, and the regulation of cancer cells metabolism, thereby suggesting that Nrf2 is a pleiotropic transcriptional factor (Karin and Dhar, 2016; Rojo de la Vega et al., 2018). In this review, we summarized up-to-date studies in the understanding of the roles and mechanisms of Nrf2 and the therapeutic approaches by targeting Nrf2 in liver diseases.

## ACTIVATION OF Nrf2 ATTENUATES ACUTE LIVER INJURY

Study has shown that activation of Nrf2 attenuates acute liver injury. Wu et al. (2012) compared serum ALT, LDH, hepatic hemorrhage, and necrosis levels between Nrf2-null and Nrf2-enhanced mice in cadmium-induced acute liver injury mice model; they found that Nrf2-enhanced mice were associated with lower ALT and LDH levels and with fewer morphological alterations. The mRNA levels of cytoprotective genes, including sulfiredoxin-1, glutamate-cysteine ligase, and glutathione peroxidase-2 were expressed only in Nrf2-enhanced mice, suggesting that Nrf2 activation prevents oxidative stress and acute liver injury through modulation of antioxidant defense-associated genes (Figure 1). Subsequently, the protective effects of Nrf2 was tested in LPS and D-GalN-induced liver injury mouse models by treatment with mangiferin, which could upregulate the gene expression of Nrf2 in a dose-dependent manner (Pan et al., 2016). Mangiferin treatment suppressed serum levels of ALT, AST, IL-1 $\beta$ , TNF- $\alpha$ , and ROS levels, adding evidences that activation of Nrf2 pathway protects against acute liver injury. Biochanin A, morin, curcumin, andrographolide, oxymatrine, and madecassoside were also found to play a protective role via activation of Nrf2 in LPS and D-GalN-induced acute liver injury in mice (Liu et al., 2016; Pan et al., 2017; Tian et al., 2017; Xie et al., 2017; Wang et al., 2018). In addition, the antioxidant pathway of Nrf2 was further tested and found to be effective in carbon tetrachloride-induced and acetaminophen-induced mouse acute liver injury models (Huang et al., 2016; Cao et al., 2017; Peng et al., 2018; Shen Z. et al., 2018). The role of Nrf2 in hepatic IRI was also identified by several studies (Ke et al., 2013; Kudoh et al., 2014; Rao et al., 2015; Ge et al., 2017; Xu et al., 2017). Ke et al. (2013) showed that the Keap1-Nrf2 complex could alleviate oxidative injury in



**FIGURE 1 |** Role of Nrf2 in acute liver injury. The protective effects of Nrf2 in acute liver injury, one is through regulating antioxidant defense-related genes, including sulfiredoxin-1, glutamate-cysteine ligase, and glutathione peroxidase-2, and the other pathway is by promoting its target gene HO-1 and then enhanced autophagy. While its negative regulator-keap1, which by binding to it inhibits Nrf2 activation and Trx1-PI3K/AKT-HIF1-HO-1/CyclinD1 signal pathway and promotes liver injury.

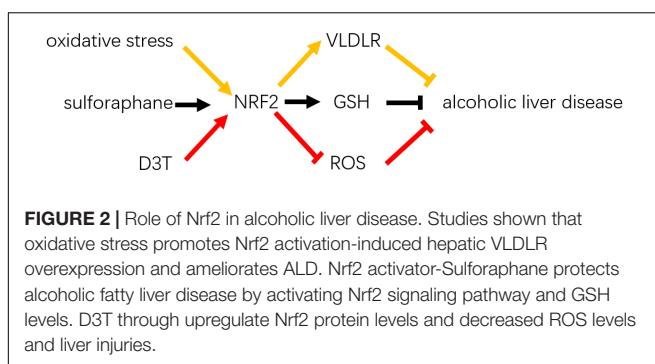
mouse orthotopic liver transplantation through Keap1 signaling (Figure 1). The protective effects were identified by limiting hepatic inflammatory responses and hepatocellular necrosis. Recently, our research identified cytoprotective effects of CDDO-Im, a potent activator of the Nrf2 pathway, in hepatic IRI, through inducing Nrf2 target gene HO-1 expression leads to enhanced autophagy in hepatocytes, which results in increased clearance of damaged mitochondria, reduced mtDNA release and ROS production leading to reductions in DAMP release-induced inflammatory responses and subsequent secondary hepatocyte injury (Xu et al., 2017). Despite accumulating evidences, Nrf2-based treatment is yet to enter clinical trials in the USA<sup>1</sup> for patients with acute liver failure.

## ACTIVATION OF Nrf2 AMELIORATES ALCOHOLIC LIVER DISEASE

Alcohol consumption has been revealed to be significantly associated with the development and progression of liver diseases over decades (Shepard et al., 2010). Alcohol metabolism in the liver includes ethanol oxidation by alcohol dehydrogenase in hepatocytes and microsomal oxidation promoted by CYP2E1 (Bae et al., 2011; Wang et al., 2014a). Alcohol dehydrogenase-associated ethanol metabolism results in acetaldehyde, which gives rise to some downstream effects, such as depletion of glutathione, lipid peroxidation, and generation of ROS (Dey and Cederbaum, 2006). In addition, the dysregulation of antioxidant glutathione by Nrf2-dependent regulation was found to contribute to the development of ALD by providing pathological conditions, whereas the Nrf2-mediated antioxidant response provided protection against alcohol-induced oxidative stress by regulating glutathione metabolism (Harvey et al., 2009; Lu, 2013; Rejitha et al., 2015). Furthermore, the oxidative stress-induced upregulation of Nrf2 is considered to positively modulate expression of VLDLR, which contributes to ALD (Wang et al., 2014b).

In ethanol-exposed mice, the role of Nrf2-induced antioxidant factors was first tested by the Nrf2 inducer D3T (Dong et al., 2008). Upregulation of Nrf2 by D3T treatment has significantly decreased generation of ethanol-induced ROS and apoptosis, which indicated that the activation of Nrf2 could diminish ethanol-induced apoptosis and ameliorate the disease status.

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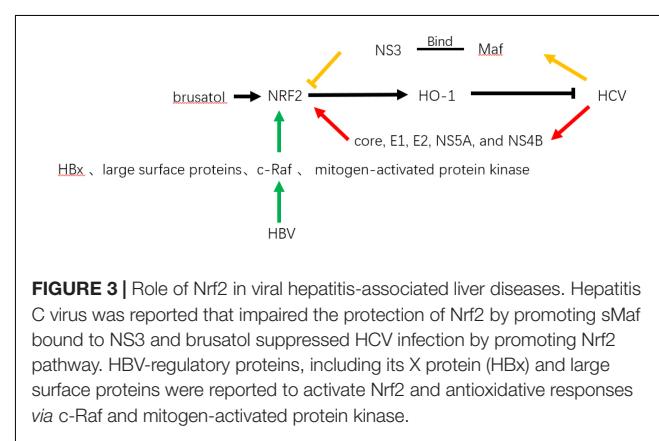
Moreover, Zhou et al. (2014) verified that Nrf2-mediated cytoprotective enzymes could ameliorate alcohol-induced liver steatosis both in *in vivo* and *in vitro* models. They further administered sulforaphane, which is an activator of Nrf2 and present in considerable quantities in brassica vegetables including broccoli, cabbage, and kale, and found it to be effective in improving alcohol-induced liver steatosis (Figure 2). Furthermore, recent advances indicated that activation of the Nrf2 pathway was protective in alcohol-induced liver fibrosis and hepatotoxicity, whereas knockdown of Nrf2 was associated with enhanced alcohol-induced hepatocyte necrosis (Song et al., 2015; Lu et al., 2016; Ni et al., 2017). By contrast, a more recent study demonstrated that ethyl pyruvate, which has multi-effects including antibacterial, anti-inflammatory, antiviral, vasodilatory, antioxidant, and antiapoptotic effects, decreases ALT, AST, hepatic morphological changes, triglycerides, free fatty acids, and the expression of proinflammatory factors and increases the expression of anti-inflammatory factors and peroxisome proliferator-activated receptor- $\alpha$  mRNA which through downregulation of the ROS-Nrf2 signaling pathway, thereby alleviating ALD in mice (Fawcett et al., 1999; Harding et al., 2000; Shen F. et al., 2018). Taken together, these evidences showed that Nrf2 activation plays essential protective role in the development of ALD and that simultaneous downregulation of Nrf2 with ROS and VLDLR may also be effective in the amelioration of ALD (Figure 2). Further studies are required to demonstrate the extent of amelioration between upregulation and downregulation of Nrf2 when ROS and VLDLR expression levels are downregulated in ALD.

## PROTECTIVE EFFECTS OF Nrf2 ON VIRAL HEPATITIS-INFECTED CELLS AGAINST OXIDATIVE DAMAGE

Oxidative stress has been shown to be implicated in viral hepatitis-associated liver diseases, including HBV and HCV infections (Bolukbas et al., 2005; Ivanov et al., 2013). A previous study indicated that HCV could mediate the phosphorylation and activation of Nrf2, which was regulated by the mitogen-activated protein kinases. The authors further suggested that the activation of Nrf2-derived survival of HCV-infected cells may provide favorable circumstances for carcinogenesis (Burkette et al.,

2010). Another study showed that the inhibition of Nrf2 and antioxidant response elements is regulated by the core proteins of HCV-replicating cell-triggered delocalization of small Maf proteins, which were bound to NS proteins NS3, thus reducing the expression of cytoprotective genes (Carvajal-Yepes et al., 2011). From the authors' point of view, inhibition of Nrf2 and antioxidant response element-regulated genes may contribute to HCV-associated pathogenesis due to impaired induction of reactive oxygen intermediates caused by cytoprotective genes, which giving rise to host cell DNA damage and promoting the genetic variability of the viral genome. Moreover, Ivanov et al. (2011) found that the antioxidant-protective Nrf2/antioxidant response element pathway is activated by HCV proteins, including core, E1, E2, NS5A, and NS4B, in an ROS-dependent and -independent manners (Figure 3). In addition, a strong upregulation of the antioxidant-protective system was modulated in the earliest stage, indicating that Nrf2 is activated to protect against HCV-induced oxidative stress in the acute stage of HCV infection. In addition, replication of HCV has been reported to be suppressed by Nrf2-mediated heme oxygenase-1 (HO-1) inducible factor, which is a phytocompound isolated from *Lindera erythrocarpa* Makino fruits (lucidone), and a quinone methide triterpene isolated from *Tripterygium wilfordii* root extract (celastrol) (Chen et al., 2013; Tseng et al., 2017). Furthermore, an *in vitro* cell line study from Japan found that knockdown of Nrf2 significantly reduced HCV infection and steatosis (Sugiyama et al., 2014). Most recently, the authors further confirmed that an Nrf2 inhibitor (brusatol) had anti-HCV effects *in vitro* (Murakami et al., 2018) (Figure 3).

Hepatitis B virus infection, which causes acute or chronic liver inflammation and contributes to the development of HCC, has been shown to induce activation of Nrf2 and antioxidative response elements *in vivo* and *in vitro* by HBV-regulatory proteins, including HBx and large surface proteins, *via* c-Raf and mitogen-activated protein kinase (Hildt et al., 2002; Schaedler et al., 2010). In addition, the HBx protein-mediated activation of Nrf2 has been introduced to trigger the upregulation of glucose-6-phosphate dehydrogenase, thereby reprogramming metabolism of glucose, and may participate in the development of HCC (Liu et al., 2015). Therefore, Nrf2 is not only a crucial factor that is activated to defend against viral hepatitis-induced

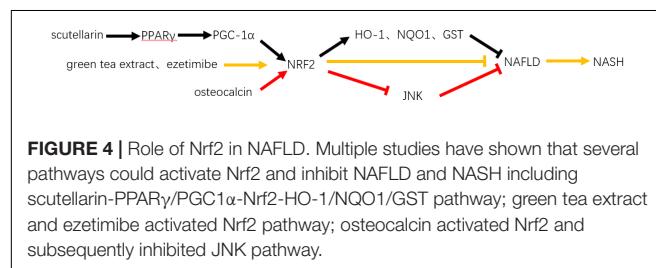


oxidative stress but also a protective factor that is involved in the survival of viral hepatitis-infected cells and may contribute to hepatocarcinogenesis.

## PROTECTIVE IMPACT OF Nrf2 IN NON-ALCOHOLIC FATTY LIVER DISEASE

Non-alcoholic fatty liver disease is a progressive disease arising from the accumulation of lipids in hepatocytes and has an increasing incidence worldwide (Satapathy and Sanyal, 2015). Approximately one-third of patients with NAFLD progress to severe NASH, which is linked with inflammation and cirrhosis (Tarantino and Finelli, 2013; Dietrich and Hellerbrand, 2014). Recent studies indicated that ROS and electrophiles are associated with the pathogenesis of NASH; thus, induction of Nrf2 seemed to be promising in the prevention and treatment of NAFLD (Chambel et al., 2015). Du et al. (2016), explored the therapeutic impact of Nrf2 activation by using osteocalcin, and found that it could improve NAFLD by ameliorating oxidative stress and inhibiting the JNK pathway, which is an important pathway involved in the pathogenesis of NAFLD. A recent study demonstrated that scutellarin, a flavonoid glycoside that has an antioxidative stress effect, significantly reduced blood lipid levels and enhanced the antioxidative capacity by activating PPAR $\gamma$  and its coactivator-1 $\alpha$ , Nrf2, HO-1, GST, and NQO1, and suppressing nuclear factor  $\kappa$ B and Keap1 at the mRNA and protein levels, thus ameliorating NAFLD (Zhang et al., 2018) (Figure 4). In addition, a modulator of PPAR $\gamma$ , apigenin, was also revealed to attenuate NAFLD by Nrf2-associated regulation of oxidative stress and hepatocyte lipid metabolism (Feng et al., 2017). Moreover, for the prevention of NAFLD, scutellarin, which is a natural drug with active components of breviscapine, was shown to be effective by enhancing the Nrf2-mediated antioxidant system in high-fat diet- and chronic stress-subjected rats (Fan et al., 2017).

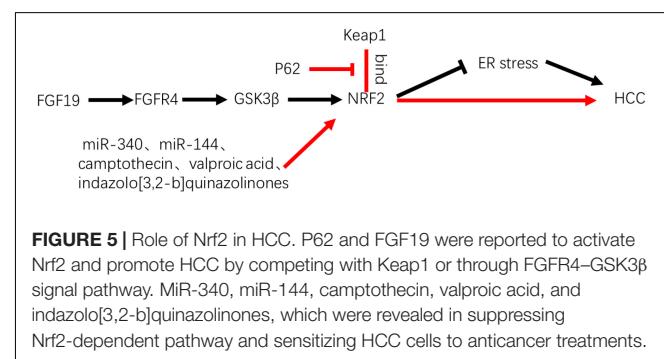
Nuclear erythroid 2-related factor 2 has been found to be a key regulator in the protection against NASH (Gupte et al., 2013). By contrast, loss of Nrf2 or deletion of Nrf2 has been found to cause benign steatosis to develop into NASH and contribute to exacerbation of disease status (Chowdhry et al., 2010; Wang et al., 2013). Ramadori et al. (2017) indicated that overactivation of Nrf2 suppressed the hepatocyte-specific c-met deletion (an accelerative factor for NASH)-induced deleterious impact on the progression of NASH and suggested that Nrf2 repaired liver damage in hepatocyte-specific c-met-deficient mice via



maintaining balance in cellular redox homeostasis. To date, green tea extract and ezetimibe (an inhibitor of Niemann-Pick-C1-Like 1) have been revealed to promote the protective impact of Nrf2 against lipid accumulation and the inflammatory response during NASH (Lee et al., 2016; Li et al., 2016) (Figure 4). However, Nrf2-associated therapeutic approaches for NASH remain to be implemented in a real-world clinical manner in the near future.

## Nrf2 IN PRIMARY LIVER CANCER

Hepatocellular carcinoma is the most common primary liver cancer, accounting for more than 80% of all hepatic malignancies (Forner et al., 2018), with molecular alterations in HCC arising in the very early stage of carcinogenesis (Pitot, 2007). Among the changes, activation of Nrf2 was found to be the prominent pathway that contributes to the progression of preneoplastic lesion to malignancy, which was confirmed by *in vivo* detection of the inhibition of the Nrf2 pathway that accompanied the regression of cytokeratin 19-positive nodules (Petrelli et al., 2014). The persistent activation of this transcription factor was found to be associated with the accumulation of p62, thus participating in the development of HCC (Inami et al., 2011). This finding was further supported by Saito et al. (2016) who confirmed the promotive impact of p62 in HCV-positive HCC through Nrf2-dependent metabolic reprogramming. In addition, Nrf2 was also found to participate in protection of HCC cells by facilitating the survival response of FGF19 to endoplasmic reticulum stress (Teng et al., 2017; Tian et al., 2018) (Figure 5). Thus, advances were made to regulate the Nrf2 pathway in HCC, including identification of miR-340, miR-144, camptothecin, and valproic acid, which were revealed to be effective in suppressing the Nrf2-dependent pathway, thereby sensitizing HCC cells to anticancer treatments (Shi et al., 2014; Zhou et al., 2016; Chen et al., 2017; Yu et al., 2017) (Figure 5). Moreover, indazolo[3,2-b]quinazolinones were revealed to attack HCC cells by suppressing Nrf2/antioxidative response elements and inducing mitochondrial-dependent apoptosis simultaneously (Zhang et al., 2016). In a clinical retrospective study, patients with high expression levels of Nrf2 ( $n = 48$ ) had significantly reduced overall (median, 13.87 months) and disease-free survival (median, 11.24 months) compared with patients with low expression levels of Nrf2 ( $n = 17$ ), who exhibited median overall survival of 30.40 months and



disease-free survival of 24.43 months ( $P < 0.01$ ) (Zhang et al., 2015). The relative risk of high Nrf2 levels in overall survival was 5.96 with 95% confidence interval of 2.46–14.69 ( $P < 0.01$ ). However, regarding the sample size and retrospective nature, a large-sized prospective clinical study is required to confirm the prognostic impact of Nrf2 in patients with HCC.

## CONCLUSION

In this review, we briefly summarized the biology characteristics of Nrf2 pathway and discussed the potential therapeutic applications of targeting Nrf2 in liver diseases. To date, there are currently few pharmacological options available to prevent or treat liver diseases. Recently, in clinical trial, NGM282, an engineered FGF19 analog, could significantly reduce liver fat content in patients with NASH and remarkably improve ALP and transaminase levels in patients with PBC (Harrison et al., 2018; Mayo et al., 2018). The small molecule PRI-724 also identified the anti-fibrotic effects in a phase 1 trial in patients with HCV cirrhosis (Kudo et al., 2018).

A link between liver diseases and oxidative stress is indispensable. The Nrf2 antioxidant pathway is activated to protect the liver by modulating defensive genes, which even

protect viral hepatitis-infected cells and HCC cells. A number of preclinical studies have detected regulatory factors for Nrf2; however, further identification of Nrf2 activators for liver injury/failure and Nrf2 inhibitors for viral hepatitis, and HCC is promising for the establishment of extensive and effective approaches to improve the prognosis of liver diseases. Regarding the great potential of this transcription factor, there is an unmet need for prospective clinical trials to explore the therapeutic impact of Nrf2 regulation in patients with liver diseases.

## AUTHOR CONTRIBUTIONS

DX and MX wrote the manuscript. SJ and YQ wrote some part of the manuscript and made language retouching for our manuscript. HW revised the manuscript. QX and XK designed and revised the manuscript.

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# The Protectin Family of Specialized Pro-resolving Mediators: Potent Immunoresolvents Enabling Innovative Approaches to Target Obesity and Diabetes

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A western type diet and lifestyle play an important role in the development of chronic diseases, yet little insight into the precise cellular and biomolecular mechanisms has emerged. It is known that an unbalanced diet may result in obesity and diabetes. Sufficient amounts and proper balance of omega-6 and omega-3 polyunsaturated fatty acids is key for maintenance of health. The resolution of inflammation is now held to be a biosynthetically actively driven process precisely regulated and controlled by a superfamily of specialized pro-resolving mediators. Specialized pro-resolving mediators are biosynthesized from both omega-6 and omega-3 polyunsaturated fatty acids and are resolution agonists acting on distinct G-coupled protein receptors. These mediators display potent anti-inflammatory and pro-resolving bioactions with EC<sub>50</sub>-values in the low nanomolar to picomolar range. The protectin (PD) family of specialized pro-resolving mediators is biosynthesized from the two omega-3 polyunsaturated fatty acids docosahexaenoic acid (DHA) and n-3 docosapentaenoic acid (n-3 DPA). All of the PDs display interesting bioactions as anti-inflammatory and pro-resolving agents. This review covers the bioactions, G-coupled protein receptors pharmacology, biosynthesis, and medicinal chemistry of the PD family of specialized pro-resolving mediators with an emphasis on obesity and anti-diabetic effects. In order to enable drug development and medicinal chemistry efforts against these diseases, stereoselective total organic synthesis of each of these mediators is required for confirmation of structure, stereochemical biosynthesis, and their functions. We provide an overview of our ongoing efforts and the current knowledge.

**Keywords:** protectins, specialized pro-resolving mediators, obesity, diabetes, G-protein coupled receptors, immunoresolvents, resolution pharmacology, western society diseases

## INTRODUCTION

### Lipid Mediators in the Acute Inflammatory Response: Uncontrolled Inflammation and Neutrophil Responders

Obesity and diabetes are two highly prevalent pathological conditions of western society due to incorrect diet, tobacco use, alcohol consumption, and an increased sedative lifestyle (Leonard, 2008). Among these factors, the general medical opinion is that diet is a significant factor increasing incidence and mortality of these diseases (Leonard, 2008). An elevated intake of western type diet rich in red meat and processed food, i.e., a poor and pro-inflammatory diet, might develop into acute or chronic inflammation (Carrear-Bastos et al., 2011). This type of diet is high in omega-6 and rather low in omega-3 PUFAs (Simopoulos, 2006). Studies of the molecular, cellular and pharmacological processes involved in inflammation have revealed that such PUFAs are biosynthetically transformed to potent oxygenated lipid mediators that participate in the inflammatory processes (Cotran et al., 1999). The acute inflammatory responses are host-protective to contain foreign invaders and in health, are self-limited (Malawista et al., 2008). If uncontrolled, chronic inflammation may result in numerous diseases, including obesity and diabetes (Serhan, 2004). In both diseases, peripheral blood markers of inflammation are present in elevated levels after intake of a pro-inflammatory western type diet (Calder, 2017).

Studies led by Samuelson and co-workers on the biomolecular understanding of inflammation resulted in the identification of PGs, LTs and thromboxanes that act as pro-inflammatory mediators when formed in excess (Samuelsson, 2012). Examples are LTs B<sub>4</sub> and C<sub>4</sub> that are stereoselectively biosynthesized from the omega-6 PUFA AA in the presence of LOXs, while COXs form PGs (Samuelsson et al., 1987). AA is also involved in the biosynthesis of the anti-inflammatory and pro-resolving lipid mediators named LX A<sub>4</sub> (LXA<sub>4</sub>) and LX B<sub>4</sub> (LXB<sub>4</sub>) (Serhan, 1997). LTs, PGs and LXs act via individual GPCRs and play key roles in the early events and in the initiation of the inflammatory response by activating neutrophils (polymorphonuclear leukocytes, PMNs) (Samuelsson et al., 1987;

**Abbreviations:** AA, arachidonic acid; COX, cyclooxygenase; DHA, docosahexaenoic acid; n-3 DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; ePD, epoxide biosynthesized from DHA and an intermediate in protectin D1 biosynthesis; ePD<sub>n-3</sub> DPA, epoxide biosynthesized from n-3 DPA and an intermediate in protectin D1 biosynthesis; GPCR, G-protein coupled receptor; LC/MS-MS, liquid chromatography tandem mass spectrometry; LM, lipid-derived mediators; LOX, lipoxygenase; LT, leukotriene; LX, lipoxin; LXA<sub>4</sub>, lipoxin A<sub>4</sub> (5S, 6R, 15S-trihydroxy-eicos-7E, 9E, 11Z, 13E-tetraenoic acid); LXB<sub>4</sub>, lipoxin B<sub>4</sub>: (5S, 14R, 15S-trihydroxy-eicos-6E, 8Z, 10E, 12E-tetraenoic acid); Maresin, macrophage-derived resolution mediator of inflammation; MaR1, maresin 1 (7R, 14S-dihydroxy-docosa-4Z, 8E, 10E, 12Z, 16Z, 19Z-hexaenoic acid); MCTR, maresin conjugates in tissue regeneration; PCTR, protectin conjugates in tissue regeneration; PD, protectin; PD1, protectin D1 (10R, 17S-dihydroxy-docosa-4Z, 7Z, 11E, 13E, 15Z, 19Z-hexaenoic acid), also named neuroprotectin D1 (NPD1); PD1<sub>n-3</sub> DPA, PD1<sub>n-3</sub> DPA biosynthesized from n-3 DPA (10R, 17S-dihydroxy-docosa-7Z, 11E, 13E, 15Z, 19Z-pentaenoic acid); PG, prostaglandin; PLP, phospholipid; PMN, polymorphonuclear leukocyte; PUFA, polyunsaturated fatty acid; RCTRs, resolin conjugates in tissue regeneration; Rv, resolin, bioactive omega-3 derived resolution phase interaction products; sEH, soluble epoxide hydrolase; SPM, specialized pro-resolving mediators; TG, triglyceride.

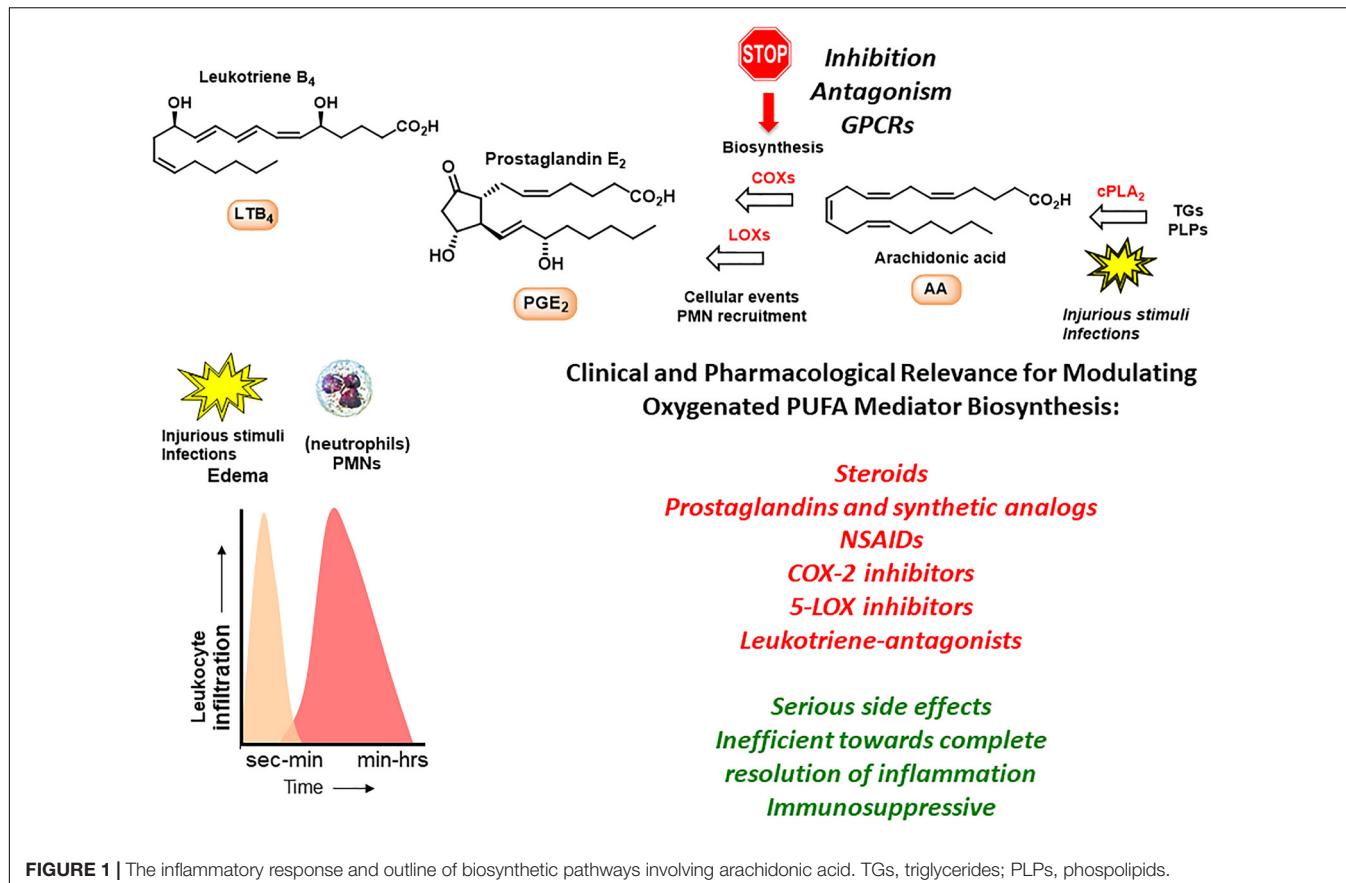
Serhan, 1997). PMNs are the first cellular responders to the site of inflammation and aim to neutralize and clear foreign invaders. During the inflammatory response the biosynthesis of PGs and LTs occurs within seconds to minutes and increases with time (Figure 1; Samuelsson et al., 1987; Serhan, 1997; Buckley et al., 2014).

Several drugs have been developed that target chronic conditions and work toward dampening the effects from inflammatory markers. Steroids, such as cortisol, provided the first leads, but later the LTs and PGs have been used as lead compounds for the development of several anti-inflammatory drugs (Samuelsson, 2012). The LXs have also been the topic of drug discovery efforts (Petasis et al., 2005; Fetterman and Zdanowicz, 2009). Examples of anti-inflammatory drugs are the two non-selective COX-inhibitors ibuprofen and acetylsalicylic acid, the selective COX-2 inhibitor celecoxib, the leukotriene-antagonist montelukast and the 5-LOX inhibitor zileuton (Samuelsson, 2012).

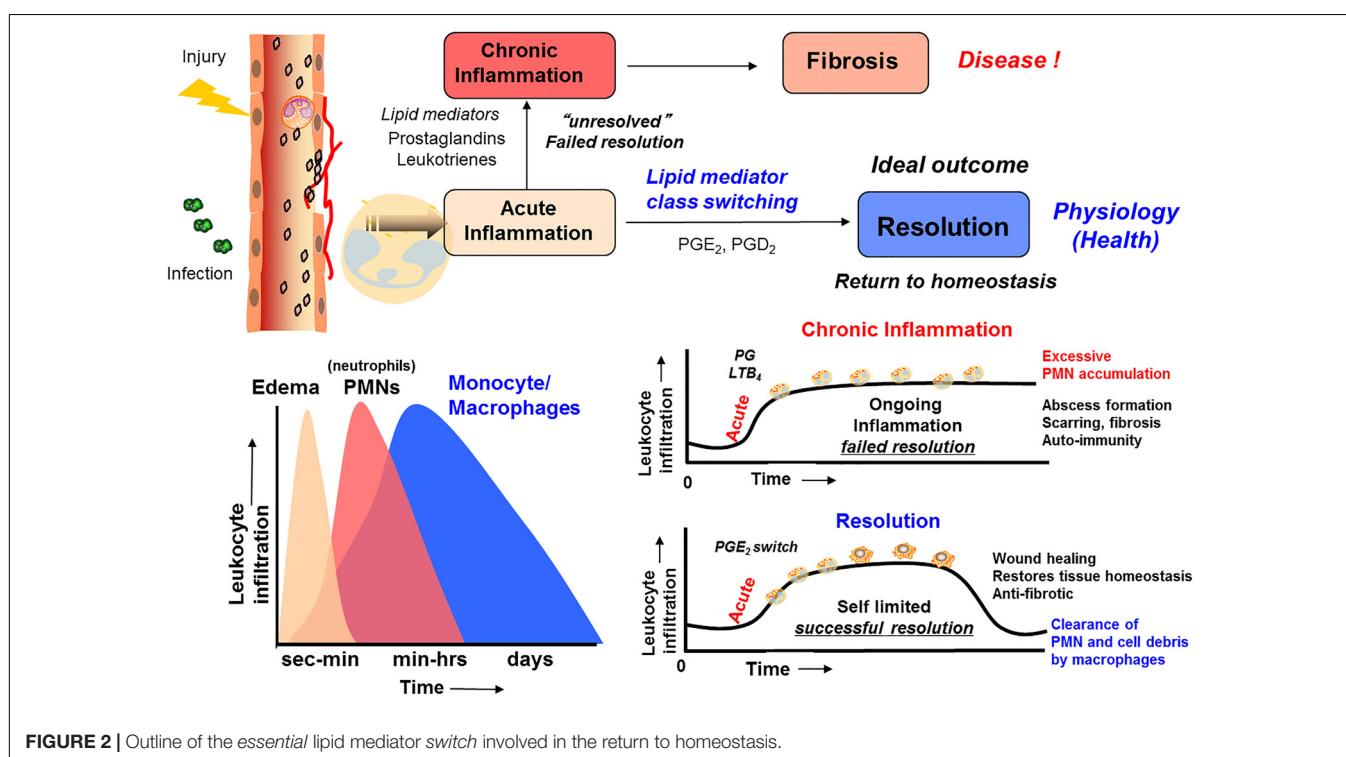
### Beneficial Roles of Dietary Omega-3 Fatty Acids in Inflammatory Processes. Biosynthesis of Specialized Pro-resolving Mediators and Resolution

The omega-3 PUFAs EPA, n-3 DPA, and DHA, abundant in fatty fish and several dietary supplement products, have been attributed with several health benefits, including prevention of obesity and diabetes (Cotran et al., 1999). These PUFAs are essential as they are produced in only very limited amounts *de novo* in humans and thus must be obtained from our diets (Simopoulos, 2006; Calder, 2017). The cellular, pharmacological and biochemical modes of actions these PUFAs display in modulating these diseases are still under investigation. Of note, it was believed earlier that the host response was passive (Bannenberg et al., 2005; Serhan and Savill, 2005; Gordon, 2016) during resolution, and that eicosanoids (LT B<sub>4</sub>, PGs) (Bannenberg et al., 2005; Serhan and Savill, 2005), complement products (Ward, 2010) chemokines, and cytokines directed PMNs to local tissue sites (Medzhitov, 2015) with all of these mediators simply diluting over time within tissues (Figure 2). This dilution would then limit additional PMN recruitment and eventually enabling tissues to restore physiology (Bannenberg et al., 2005). However, numerous studies have shown that the LXs, biosynthesized from AA, function as potent and active stop signals for PMN influx characteristics of SPMs (Takano et al., 1998; Serhan et al., 2000) indicating that the resolution response is a biosynthetically active process (Serhan and Savill, 2005).

The omega-3 PUFAs EPA and DHA, abundant in fat fish and used in dietary supplements, have been associated with many health benefits (Calder, 2017). SPMs may constitute the molecular basis for such positive claims in a wide range of clinical indications. Evidence has been provided over the last two decades on the detailed cellular and biochemical mechanisms showing that during self-limited inflammatory response a **switch** in the biosynthesis of pro-resolving SPMs occurs (Figure 2; Levy et al., 2001). This active biosynthesis increases with time. The switch in the biosynthesis of pro-resolving SPM autacoids provides



**FIGURE 1 |** The inflammatory response and outline of biosynthetic pathways involving arachidonic acid. TGs, triglycerides; PLPs, phospholipids.



**FIGURE 2 |** Outline of the essential lipid mediator switch involved in the return to homeostasis.

a cellular, biochemical and detailed enzymatic mechanistic explanation on how the resolution of inflammation occurs and completes in order to regain a new homeostasis in contained inflammatory exudates (**Figures 2, 3**; Bannenberg et al., 2005). The molecular, biochemical and cellular events involved in the return to homeostasis have been coined catabasis (Serhan and Savill, 2005). For a schematic overview, please consult **Figure 3**. When the resolving secretory phospholipases cPLA<sub>2</sub>-IID and ZPLA<sub>2</sub>-III are stimulated (Takano et al., 1998) the PUFAs EPA, DHA and n-3 DPA are released from phospholipids enabling biosynthetic production of SPMs in specific organs (**Figure 4**; Levy et al., 2001). In exudates, unesterified omega-3 PUFAs are delivered from blood via edema proteins for enzymatic conversion to SPMs (Kasuga et al., 2008), thus providing novel mechanisms for substrate availability for SPM biosynthesis to terminate further expansion of the cellular exudates (Murakami et al., 2015).

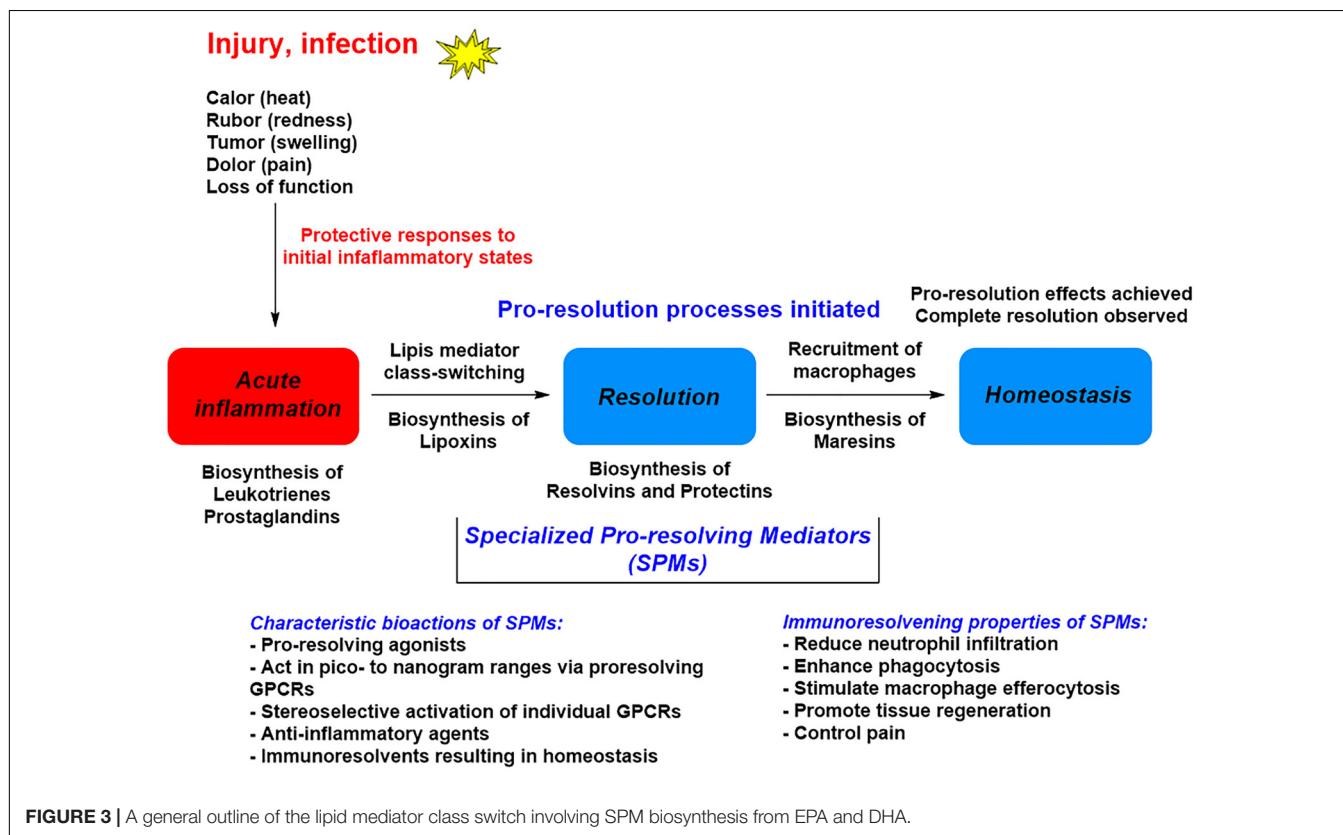
## Specialized Pro-resolving Mediators Are Resolution Agonists Acting on G-Protein Coupled Receptors

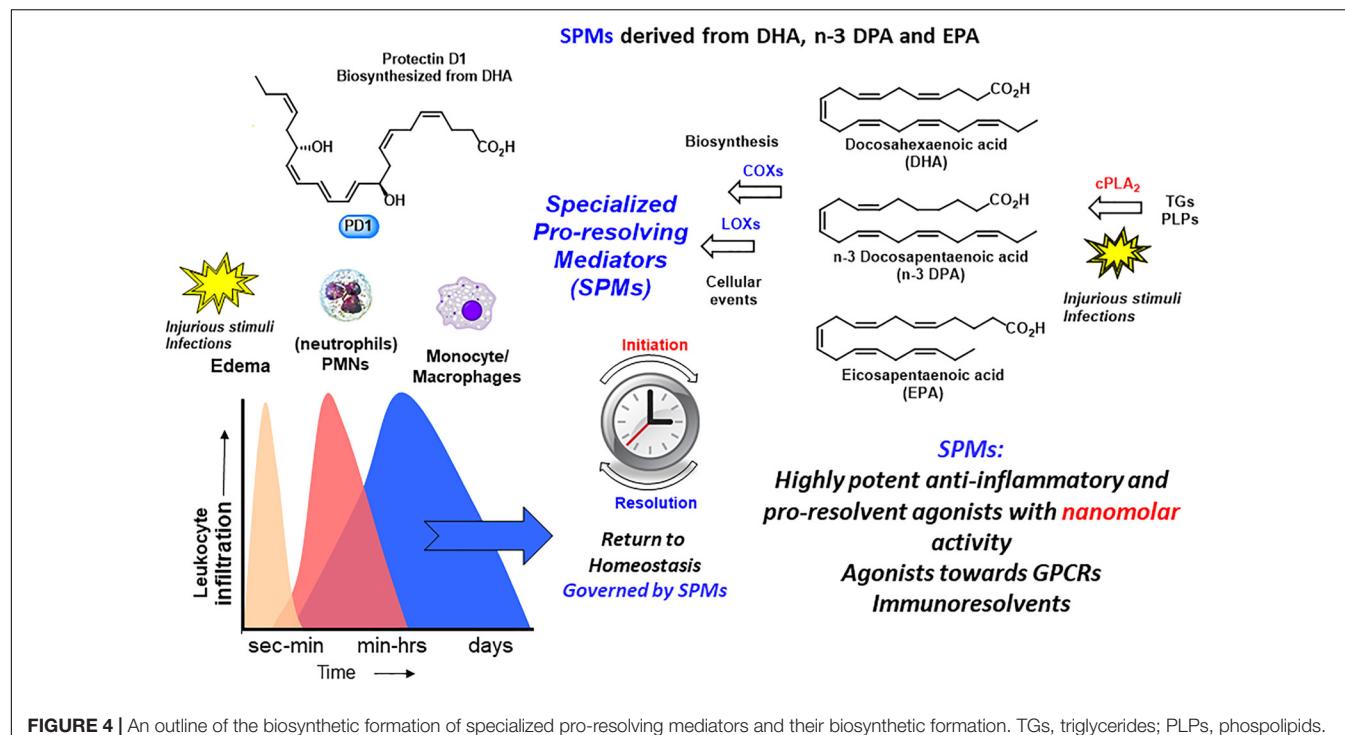
The SPMs display potent nanomolar agonist actions *in vivo*, that are stereoselective and act as ligands for individual GPCRs (Serhan and Chiang, 2013). The activation of one or several GPCRs induces cellular functions that carry out the potent bioactivities of the SPMs. Initial studies on the receptors for PD1 revealed cell-type specific activity that was also structure

dependent (Levy et al., 2007). By using radiolabelled PD1 specific binding toward leukocytes was observed (Marcheselli et al., 2010). The identification that PD1 elicit signaling responses toward GPR37 was very recently reported by Bang et al. (2018). These investigators also reported that GPR37 activation in macrophages increased phagocytosis, altered cytokine release and promoted resolution of inflammatory pain. **Table 1** lists the known GPCRs that SPMs activate to evoke resolution of inflammation *in vivo* in experimental animal models. Hence, it is possible that PD1 has additional receptors on neurons that directly regulate pain signaling.

## THE PROTECTIN FAMILY OF SPMs

As mentioned, the resolution of inflammation is now held to be a biosynthetically active process, regulated by biochemical mediators and receptor-signaling pathways governed by SPMs. The Serhan group employed lipid mediator proteomics, metabololipidomics (LC/MS-MS) and cell trafficking in self-limited inflammatory exudates to identify three new families of SPMs (Serhan et al., 2002, 2009; Hong et al., 2003; Dalli et al., 2013, 2014, 2015a,b; Ramon et al., 2016) coined the resolvins (*resolution phase interaction products*), PDs, and maresins (*macrophage mediators in resolving inflammation*). Each family is structurally distinct and biosynthesized from the n-3 essential fatty acids EPA, n-3 DPA, or DHA (**Figure 5**; Serhan et al., 2002, 2009; Hong et al., 2003; Dalli et al., 2013, 2014, 2015a,b;





**FIGURE 4 |** An outline of the biosynthetic formation of specialized pro-resolving mediators and their biosynthetic formation. TGs, triglycerides; PLPs, phospholipids.

**TABLE 1 |** Reported receptors for specialized pro-resolving mediators.

Specialized pro-resolving mediator	Receptors	Human	Mouse
Lipoxin A <sub>4</sub>	ALX/FPR2; GPR32	Yes; Yes	Yes; n/a*
Resolvin E1	BLT1; CMKLR1; ERV	Yes; Yes	Yes; Yes
Resolvin D1	ALX/FPR2; GPR32; DRV1	Yes; Yes	Yes; n/a*
Resolvin D2	GPR18, DRV2	Yes; Yes	Yes; Yes
Resolvin D3	ALX/FPR2; GPR32; DRV1	Yes; Yes	Yes; Yes
Protectin D1 (Neuroprotectin D1)	GPR37, Pael-R	Yes	Yes

\*Not available.

Ramon et al., 2016). PDs belong, together with the LXs, resolvins, maresin, as well as the sulfido-conjugates RCTRs, PCTRs, and MCTR (maresin conjugates in tissue regeneration), to the super families of mediators (Figure 5). The PDs are structurally unique from the other SPMs because they possess a conjugated triene and their biosynthesis is initiated from the enzymatic production of a 17*H*pDHA intermediate.

## Pro-resolving and Anti-Inflammatory Actions of Protectins

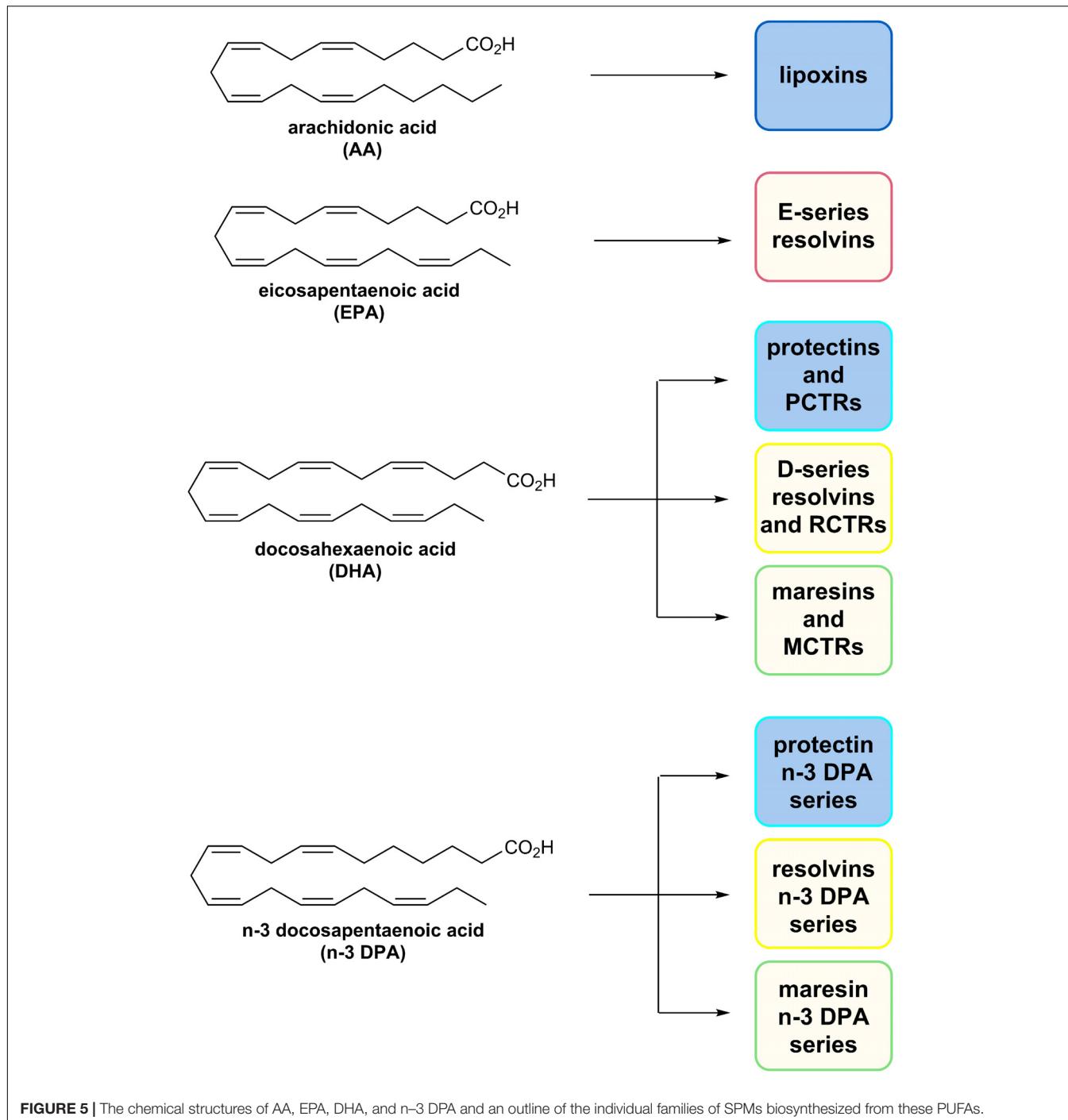
The protectin family of SPMs have attracted considerable interest from the biomedical community as resolution leads (Serhan, 2014; Fullerton and Gilroy, 2016; Dalli and Serhan, 2018). The precise cellular events, biochemical pathways and molecular mechanisms of the PDs in the resolution of inflammation is of interest in pharmacology

and medicinal chemistry enabling drug development (Serhan, 2014; Fullerton and Gilroy, 2016; Dalli and Serhan, 2018). This SPM-subfamily is chemically characterized by two chiral secondary alcohols separated by an *E,E,Z*-triene moiety. Figure 6 depicts the distinct members of the PD family of SPMs.

The protectins are biosynthesized from DHA (Serhan et al., 2006; Aursnes et al., 2015) while the n-3 PDs are biosynthesized from n-3 DPA (Primdahl et al., 2017), with the individual biochemical steps presented in detail below. In addition to PMNs, PD1<sub>n-3</sub> DPA is produced by macrophages (Serhan et al., 2006; Dalli and Serhan, 2012) and eosinophils (Yamada et al., 2011; Katakura et al., 2015) and its production is reduced in severe asthma patients (Miyata et al., 2013). When PD1 is produced in neural systems, the name NPD1 is used to denote the location of the potent protective actions in retina, brain, and induction of pain (Bazan et al., 2010; Marcheselli et al., 2010; Asatryan and Bazan, 2017). Remarkable potent pro-resolving actions in mice with peritonitis was observed as only 1 ng of PD1 caused a reduction of PMN infiltration by approximately 40% (Serhan et al., 2006). PD1 display potent pro-resolution agonist effects with EC<sub>50</sub> ~ 1 nM and a K<sub>d</sub>-value of ~31 pmol/mg of cell protein (Marcheselli et al., 2010).

## AT-PD1

The biosynthetic pathways mediated by human 15-LOX for the omega-6 AA derived LXs and their aspirin-triggered 15-epimeric forms are well established and studied (Serhan, 1997). PD1/NPD1 is biosynthesized predominantly in the 17S configuration by 15-LOX, but aspirin acetylation of COX-2



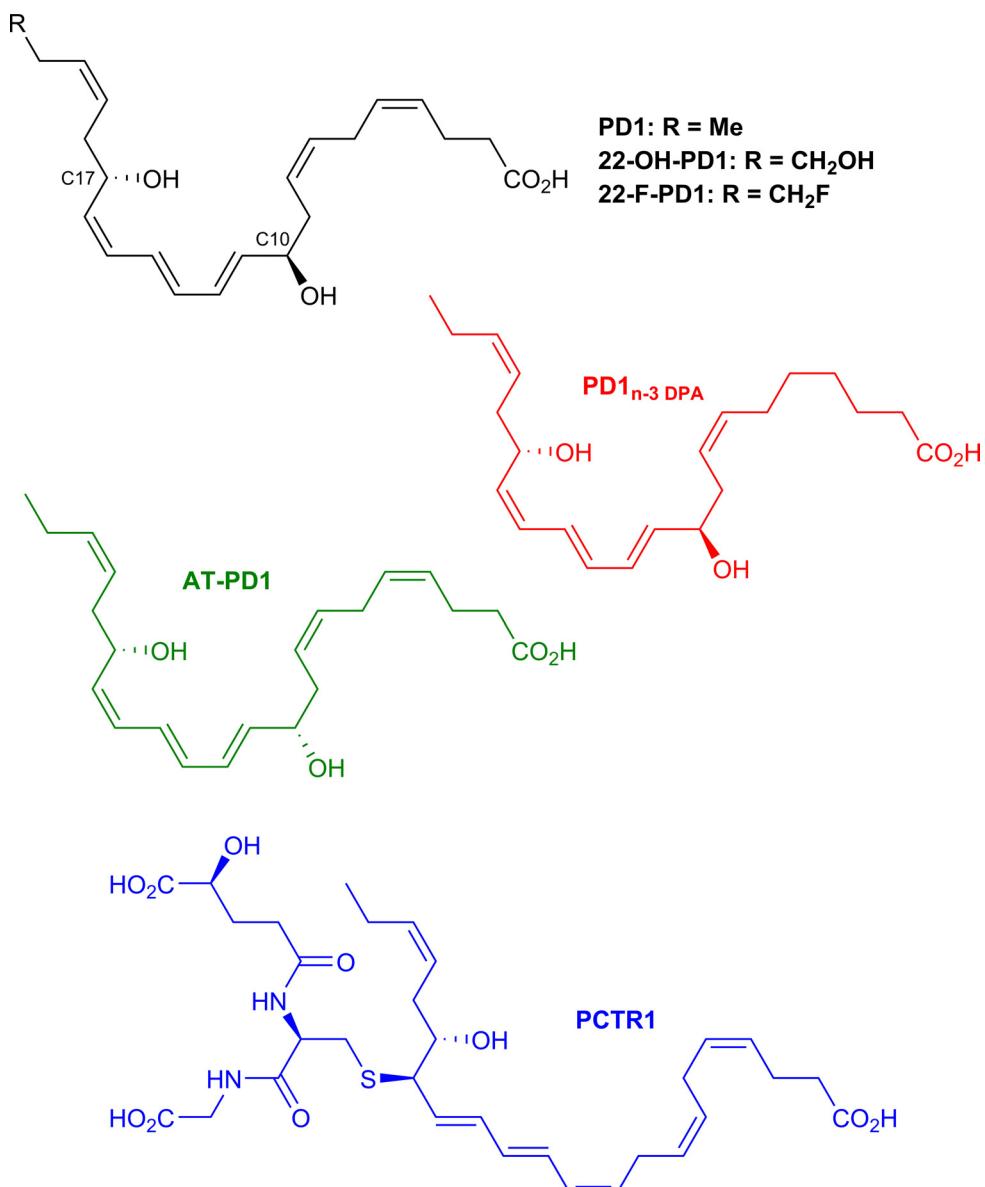
**FIGURE 5 |** The chemical structures of AA, EPA, DHA, and n-3 DPA and an outline of the individual families of SPMs biosynthesized from these PUFAs.

produces the hydroperoxide intermediate predominantly in the *R*-configuration at the 17-carbon position. This epimeric hydroperoxide is converted to the 17*R* epimer 17*R*-PD1, that is coined AT-PD1 (Serhan et al., 2002, 2011). The *R*-epimer is longer acting than the *S*-epimer PD1, most likely due to the stereochemical preference of the eicosanoid oxidoreductase enzymes for *S*-configured alcohols in the metabolism of oxygenated PUFAs (Serhan et al., 2011). AT-PD1 also display potent pro-resolving and anti-inflammatory actions

as well as neuroprotective properties (Serhan et al., 2002, 2011).

### The PD1 Further Metabolite 22-OH-PD1 and the Synthetic Analog 22-F-PD1: Medicinal Chemistry Efforts

The further metabolism of PD1 once it is produced locally has not been studied *in vivo* in humans, but one study has



**FIGURE 6 |** Chemical structures of PD1 and the epimer AT-PD1, PD1<sub>n-3</sub> DPA, the metabolite 22-OH-PD1, the synthetic analog 22-F-PD1 and PCTR1. The chiral secondary alcohols are positioned at carbon atoms 10 and 17 for all protectins except PCTR1, while the primary alcohol in the metabolite 22-OH-PD1 is at carbon atom 22.

reported a metabolite named 22-OH-PD1 (**Figure 6**) formed by  $\omega$ -oxidation at the carbon atom number 22 (C-22) in PD1 (Serhan et al., 2002). This metabolite was prepared by total synthesis (Tungen et al., 2014). *In vivo* experiments in mice revealed that 22-OH-PD1 displayed potent pro-resolving and anti-inflammatory activities (Tungen et al., 2014). It is likely that additional further metabolic pathways of PD1 are mediated via eicosa oxidoreductases in the same way as for some of the other SPMs (Serhan and Petasis, 2011), although further studies are needed. The potent *in vivo* pro-resolution actions in the nanomolar range toward efferocytosis and phagocytosis that 22-OH-PD1 displayed spurred our interest in

preparing the synthetic analog 22-F-PD1 depicted in **Figure 6** (Tungen et al., 2018). When administered via intraperitoneal injection at 100 ng/mouse following *Escherichia coli* infection, 22-F-PD1 reduced PMN recruitment, enhanced macrophage phagocytosis and reduced bacterial load at similar levels to PD1 (Tungen et al., 2018). Overall, these results verified that the synthetic analog and putative medicinal chemistry agent 22-F-PD1 exhibited both potent anti-inflammatory and pro-resolving actions similar to native PD1. Macrophage phagocytosis and efferocytosis are both key pro-resolving biological actions of interest in drug discovery and clinical development (Serhan et al., 2002).

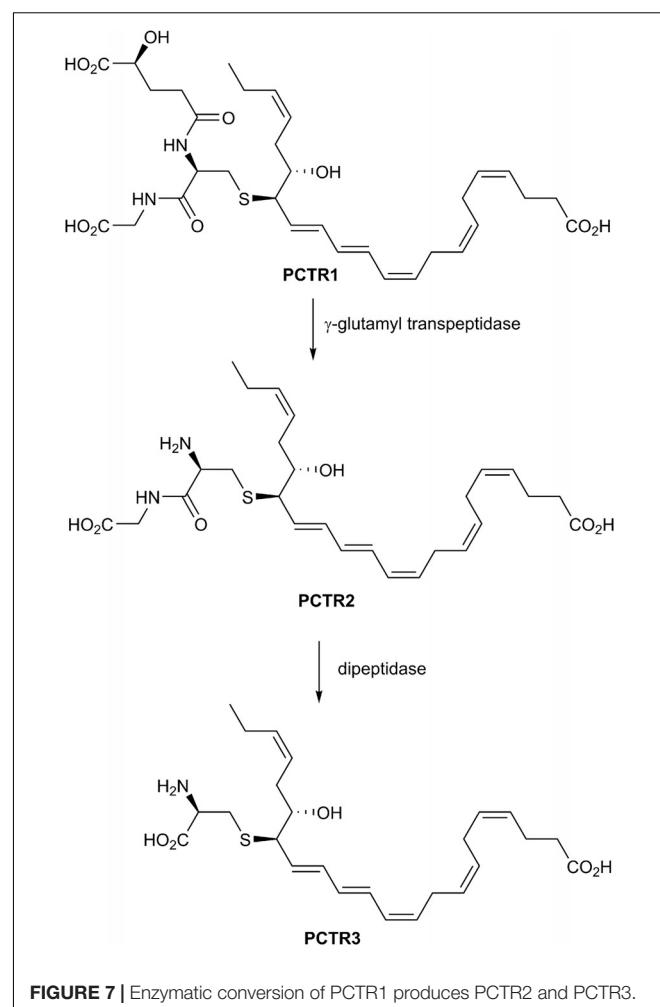
## The Novel Protectin PD1<sub>n-3</sub> DPA

Recently, Dalli, Colas and Serhan in Boston, United States demonstrated that n-3 DPA is also a substrate for the biosynthesis of potent bioactive mediators that each correspond to the novel families of SPM (Dalli et al., 2013). While appreciated as an intermediate in omega-3 PUFA biosynthesis in humans, it is interesting that this PUFA is also a precursor to SPMs with 22 carbons and five double bonds (Dalli et al., 2013; Aursnes et al., 2014a). These n-3 immunoresolvents belong (**Figure 5**) to the three sub-families resolvins<sub>n-3</sub> DPA, PD<sub>n-3</sub> DPA, and maresins<sub>n-3</sub> DPA each demonstrating potent pro-resolving actions as identified in human subjects (Aursnes et al., 2014a; Markworth et al., 2016; Gobbetti et al., 2017; Hansen et al., 2017). To obtain further evidence for the complete structure of PD1<sub>n-3</sub> DPA it was essential to assess that the synthetic material carried the potent biological actions described for PD1<sub>n-3</sub> DPA, see below for a discussion on its synthesis (Aursnes et al., 2014a). Administration of synthetic material using 10 ng per mouse significantly reduced neutrophil recruitment during peritonitis following zymosan A challenge (Aursnes et al., 2014a). These bioactions were comparable to those displayed by PD1. Moreover, synthetic material of PD1<sub>n-3</sub> DPA stimulated human macrophage phagocytosis and efferocytosis in the pico- to nanomolar range. Overall, these results verified that PD1<sub>n-3</sub> DPA exhibited both potent anti-inflammatory and pro-resolving actions, confirming the potent immunoresolvent properties of this SPM (Aursnes et al., 2014a). Potent protective bioactions for PD1<sub>n-3</sub> DPA and RvD5<sub>n-3</sub> DPA were demonstrated in mouse colitis and in reducing human PMN adhesion to endothelium (Gobbetti et al., 2017). In another recent study, PD1<sub>n-3</sub> DPA promotes resolution of neuroinflammation and arrests epileptogenesis potently due to a marked delay in the neuroinflammatory response (Frigerio et al., 2018). These studies were also the first to report that PD1<sub>n-3</sub> DPA regulates neuroinflammation (Frigerio et al., 2018). Recently Dalli and co-workers found that the biosynthetic pathway of the n-3 DPA PDs regulated the differentiation of human monocytes, altering macrophage phenotype, efferocytosis, and bacterial phagocytosis (Pistorius et al., 2018).

## Protectin Conjugates in Tissue Regeneration: PCTR1, PCTR2 and PCTR3

In 2014 and 2015 three new classes of SPMs were discovered and elucidated that carry potent tissue regenerative properties and possess anti-inflammatory and pro-resolving bioactions (Dalli et al., 2013, 2014). In **Figure 6** the chemical structure of PCTR1 is depicted, and this novel SPM belongs to the novel peptide-conjugated PDs that contain a sulfido-bond at the carbon atom 16 and were identified from self-resolving *E. coli* infections in mice and in human spleen. The biosynthesis is presented below. Enzymatic conversion of PCTR1 in the presence of  $\gamma$ -glutamyl transpeptidase produces PCTR2 and dipeptidase actions yield PCTR3 (**Figure 7**; Dalli et al., 2014).

PCTR1 enhances resolution of infectious inflammation and is produced by human M2 macrophages (Dalli et al., 2015b). In



**FIGURE 7 |** Enzymatic conversion of PCTR1 produces PCTR2 and PCTR3.

addition, PCTR1 promoted human monocyte and macrophage migration potently and dose-dependently in the 0.001–10 nM range (Dalli et al., 2015b). Furthermore, PCTR1 increased macrophage and monocyte migration, enhanced macrophage efferocytosis, and accelerated tissue regeneration in planaria (Dalli et al., 2015b). It was also reported that PCTR1 is temporally regulated during self-resolving infection. At the peak of inflammation, PCTR1 enhanced macrophage recruitment and phagocytosis of *E. coli*, decreased PMN infiltration, and counter-regulated inflammation-initiating lipid mediators, including PGs (Dalli et al., 2015b). These findings demonstrated that PCTR1 is a potent monocyte- and macrophage-agonist regulating key anti-inflammatory and pro-resolving processes during bacterial infection.

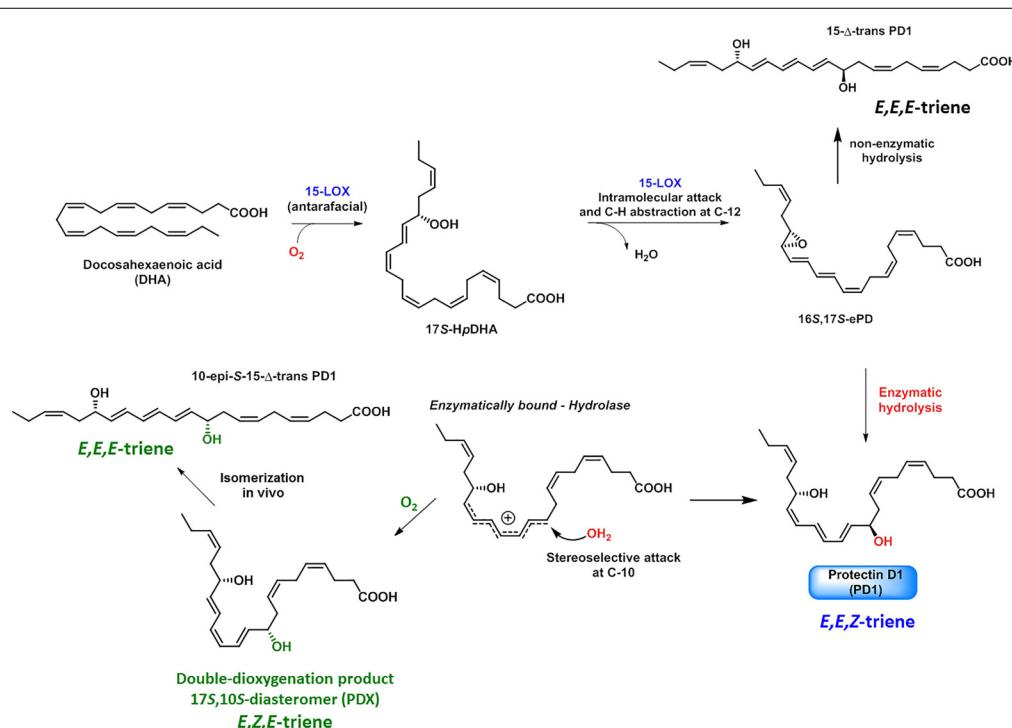
## PROTECTINS IN OBESITY AND DIABETES

As mentioned, a pro-inflammatory western type diet results in an increased level of inflammatory cellular and biomolecular markers, including those biosynthesized from omega-6 and

omega-3 PUFAs (Simopoulos, 2006). An expansion of adipose tissue mass associated with a low-grade type of inflammation has been observed with an excess intake of diet and nutrition. This chronic and unresolved inflammation of adipose tissue is harmful and may result in diabetes, insulin resistance and non-alcoholic fatty liver disease, all increasing maladies in western societies (Clària et al., 2017). White adipose tissue plays essential roles in balancing metabolic and energy homeostasis (Clària et al., 2017). This balance is affected by AA, EPA, and DHA. In this setting, Clària and co-workers reported the first investigations on SPM biosynthesis in white adipose tissues given elevated levels of EPA and DHA (Gonzales-Periz et al., 2006, 2009; Clària et al., 2017). These studies showed that dietary amplification of DHA results in increased biosynthesis of PD1 and its precursor 17S-HpDHA (Gonzales-Periz et al., 2006), enzymatically reduced *in vivo* to 17S-HDHA (Serhan and Petasis, 2011). Using a transactivation assay, 17S-HDHA was shown to be a PPAR $\gamma$ -agonist (Gonzales-Periz et al., 2006). This finding is of interest since several PPAR $\gamma$ -agonists, such as the glitazones, have been developed as anti-diabetic drugs (Gonzales-Periz et al., 2006; Clària et al., 2017). Clària and co-workers also demonstrated that administration of DHA diminished the presence of pro-inflammatory PGs and LT B<sub>4</sub> (LTB<sub>4</sub>) (Gonzales-Periz et al., 2006). Synthetic 17-HDHA stopped genotoxic and oxidative damage in hepatocytes and diminished 5-LOX expression in macrophages. In further studies, these authors reported that the biosynthetic formation of SPMs was severely deregulated in inflamed white adipose tissues as well as in obese mice (Gonzales-Periz et al., 2009). PD1 and RvD1

were reported to be the dominant DHA-derived SPMs based on LC/MS-MS metabololipidomic analyses. They also reported that reduced insulin resistance was observed in white adipose tissues, observations that were in parallel with initiation of phosphorylation of adenosine monophosphate and adiponectin, important regulators of systemic energy balance (Gonzales-Periz et al., 2009).

A skewed biosynthetic process was also observed when these investigators used white adipose tissue from patients with peripheral vascular diseases. In these patients the inflammatory status of white adipose tissue is severely altered (Clària et al., 2013). Within the setting of obesity and diabetes these observations could be due to the diminished tissue levels of omega-3 PUFAs since it has been reported that an increased intake of omega-3 PUFAs enhance SPM biosynthesis (Mas et al., 2012). The reduced level of SPMs quantified could also be explained by an enhanced catabolism or metabolism followed by conversion to further inactive metabolites of SPMs. Interestingly, in obese adipose tissue the enzyme eicosanoid oxidoreductase (15-PG-dehydrogenase) is markedly up-regulated (Gonzales-Periz et al., 2009). This enzyme is involved in the metabolic formation of 17-oxo-RvD1 and 7-oxo-RvD2 from RvD1 and RvD2 (Serhan and Petasis, 2011), respectively, that also occurs in white adipose tissue (Clària et al., 2012). The enzyme soluble epoxide hydrolase 2 (sEH) converts epoxides (Haeggström and Funk, 2011; Serhan and Petasis, 2011), some of which are intermediates in PD biosynthesis, formed from EPA and DHA, into diols with lower pro-resolving and anti-inflammatory properties (Haeggström and Funk, 2011; Serhan and Petasis,



**FIGURE 8 |** Biosynthesis of PD1, PDX, and isomers formed *in vivo*.

2011). The enzyme sEH is found invariably overexpressed in obese mice (Lopez-Vicario et al., 2015). Overall, the studies from Clària et al. (2017) showed that an unbalanced level of SPMs are directly connected to insufficient tissue resolution in both *in vitro* and *in vivo* models of diabetes and obesity.

Of interest, Kuda et al. (2016) reported the isolation and characterization of new DHA-derived fatty acid esters of hydroxy fatty acids present in both serum and white adipose tissue after supplementation with DHA. LC/MS-MS results supported the assigned structures without information on the absolute configurations of the compounds. They performed experiments using mice as well as serum from obese patient with diabetes. The novel compound named 13-DHAHLA showed anti-inflammatory properties at much higher concentrations than SPMs (Kuda et al., 2016). These authors also found that 13-DHAHLA hindered the increase in several pro-inflammatory markers, such as interleukin-6, tumor necrosis factor- $\alpha$ , and PGs. In addition, 13-DHAHLA enhanced phagocytosis in zymosan A induced in an *in vitro* bone marrow derived macrophage assay (Kuda et al., 2016). For the assignment of absolute configuration of these branched DHA esters of hydroxyl substituted fatty acids, stereoselective total synthesis will be required. With synthetic material in hand, investigations toward which GPCR(s) these novel compounds activate, but also thorough *in vivo* experiments can be performed toward elucidation of any pro-resolving and anti-inflammatory activities these compounds may display.

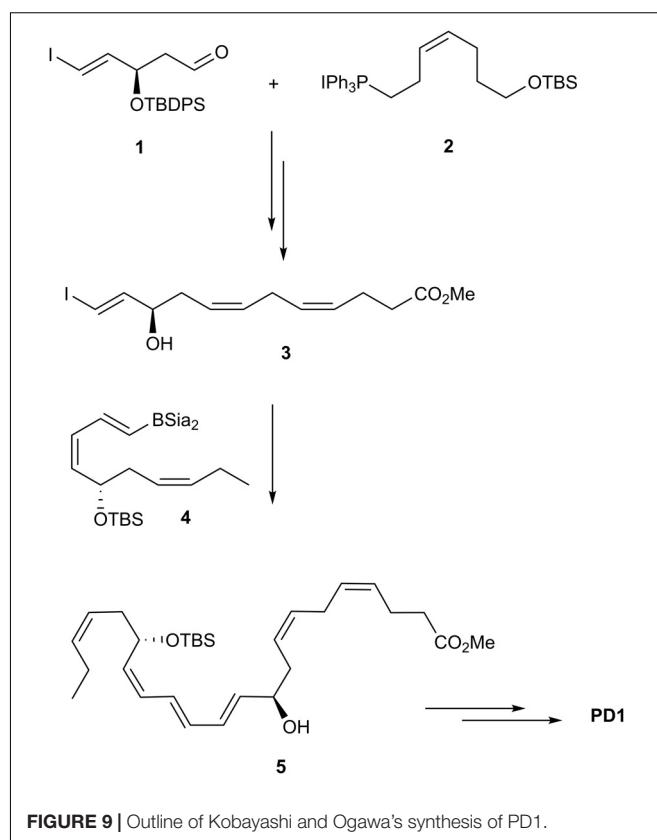
An isomer of PD1, named protectin DX (PDX), see below and **Figure 8** for structure, has been confirmed formed and isolated from white adipose tissue (White et al., 2014). This DHA-derived compound has been reported to alleviate insulin resistance in db/db mice (White et al., 2014, 2015). Of note, PDX did not resolve white adipose tissue inflammation (White et al., 2014). However, *in vivo* studies with mice showed that both PD1 and PDX were able to modulate PPAR $\gamma$  transcriptional activity (White et al., 2015).

## Structural Elucidation and Determination of Absolute Configuration Using Biosynthetic and Synthetic Studies

In order to elucidate the complete structure of PD1 the correct absolute configuration and olefin geometry had to be determined for this potent SPM. Efforts from the Serhan research team on the biosynthesis of PD1 allowed the complete structural assignment of PD1 (Serhan et al., 2006). Experiments using isotopic oxygen incorporation and acid alcohol trapping products provided LC/MS-MS data that supported the involvement of an epoxide intermediate (**Figure 8**; Serhan et al., 2006).

The detailed and stepwise biosynthesis of PD1 has now been established as depicted in **Figure 8** (Serhan et al., 2006; Aursnes et al., 2015). The enzyme 15-LOX type I functions as a 17-lipoxygenase (Haeggström and Funk, 2011) and forms the 16S,17S-configured epoxide named 16S,17S-ePD. Hydrolysis of this epoxide in a regio- and stereoselective manner at the C-10 position results in the formation PD1. Water attack occurs most likely via a transient allylic carbocation species, see

**Figure 8**, since the thermodynamically less stable 11E,13E,15Z-configured triene is formed, and not the chemically more stable 11E,13E,15E triene. Moreover, the S-configuration at C17 is not altered (Serhan et al., 2006; Aursnes et al., 2015). The formation of the 17R-HpDHA stereoisomer has been observed in the presence of aspirin and recombinant isolated COX-2 enzyme (Serhan et al., 2002, 2011), that results in the formation of the 17R-epimer of PD1, coined aspirin triggered protectin D1 (AT-PD1). This biosynthetic pathway occurs most likely via a 16R,17R-configurated epoxide intermediate named ePD. Later direct evidence that 16S,17S-ePD is in fact the true intermediate in the biosynthesis of PD1 was provided by Serhan, Hansen and co-workers (Aursnes et al., 2015). These biosynthesis studies were performed using 16S,17S-ePD, stereoselectively prepared by organic synthesis, that confirmed that this epoxide was converted into PD1 in human macrophages (Aursnes et al., 2015). The double LOX product 10,17-diHDHA, named PDX, is biosynthetically produced by two sequential oxygen insertion steps followed by reduction of the 15-LOX produced hydroperoxide-intermediate, affords 10S,17S-diHDHA (Serhan et al., 2006). PDX is an isomer of PD1 and has several reported bioactions relevant for diabetes and obesity (White et al., 2014, 2015). PDX has also been subjected to other biological investigations (Masterson et al., 2015; Stein et al., 2016; Fonseca et al., 2017; Körner et al., 2018). The other isomers of PD1 investigated were reported to possess significant lower potent pro-resolving actions (Serhan et al., 2006). It has recently been



**FIGURE 9 |** Outline of Kobayashi and Ogawa's synthesis of PD1.

demonstrated that PCTR1 is also biosynthesized directly from 16S,17S-ePD (Ramon et al., 2016). Regarding the biosynthesis of the congener PD<sub>1n-3</sub>DPA the epoxide named 16S,17S-ePD<sub>n-3</sub>DPA has been shown to be an essential biosynthetic intermediate involved in the formation of PD<sub>1n-3</sub>DPA in human neutrophils (Primdahl et al., 2017). This epoxide is able to inhibit human neutrophils LTB<sub>4</sub> production and that an yet unidentified hydrolytic enzyme converts 16S,17S-ePD<sub>n-3</sub>DPA into PD<sub>1n-3</sub>DPA (Primdahl et al., 2017).

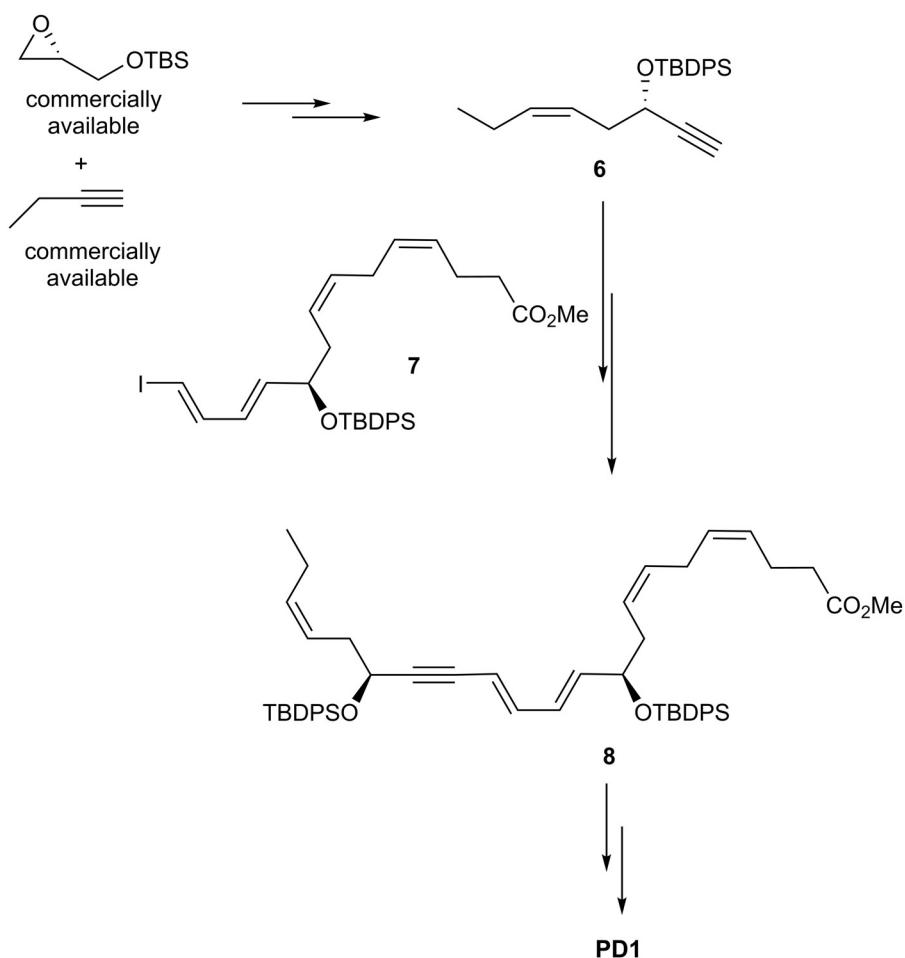
## REPORTED TOTAL SYNTHESIS OF PD1

As of today, the exact structural elucidation of SPMs using LC/MS-MS based metabololipidomics is necessary to establish the exact structure of the endogenously formed bioactive products (Chiang and Serhan, 2017), since only pico- to nanogram amounts of biosynthetic material are formed *in vivo*. Total syntheses of PD1 have been reported by four research groups (Ogawa and Kobayashi, 2011; Petasis et al., 2012; Aursnes et al., 2014b; Rodriguez and Spur, 2014), but only two groups have used synthetic and authentic material for

matching experiments using LC/MS-MS multiple reaction monitoring (MRM). We want to emphasize that such efforts are of vitally importance, since the PDs as well as the other SPMs, display very potent agonist actions toward individual GPCRs in a stereochemically defined manner (Serhan and Petasis, 2011; Chiang and Serhan, 2017). The structural elucidation and physiologic functions of the SPM receptors have recently been reviewed (Chiang and Serhan, 2017). An outline of the different total syntheses of PD1 is presented below.

### Kobayashi and Ogawa's Synthesis of PD1

Ogawa and Kobayashi (2011) disclosed the first total organic synthesis of PD1, which included a Z-selective Wittig reaction and a Suzuki-cross coupling as key steps (Figure 9). These authors prepared the iodide-aldehyde fragment **1** in several steps and then reacted **1** with the ylide of **2** in a Z-selective Wittig reaction to afford, after several other synthetic steps, the ester **3**. This ester was subjected to a Suzuki-Miyaura cross-coupling reaction with boron-compound **4** affording compound **5** with the C22 carbon skeleton of PD1 in place. Deprotection of **5**



**FIGURE 10 |** Outline of Petasis and co-workers synthesis of PD1.

was followed by basic hydrolysis that yielded PD1 (Ogawa and Kobayashi, 2011).

### The Petasis Synthesis of PD1

The first synthesis and assignment of PD1 was reported by Serhan et al. (2006). These efforts also provided synthetic stereoisomers. Biological evaluations of PD1 and its synthetic stereoisomers provided useful information on structure-functions. For the stereochemical assignment Petasis et al. (2012) reported their synthesis in details in 2012, although synthetic PD1 as well as isomers were made available for biological studies from this group much earlier (Serhan et al., 2006). The Petasis synthesis utilized a Sonogashira cross coupling reaction to make the C22 carbon skeleton (**Figure 10**). The precursor **6** was reacted with **7** using this cross-coupling reaction to yield **8**. Of note, the commercially available starting material *R*-glycidol gave rise to both **6** and **7** (Petasis et al., 2012).

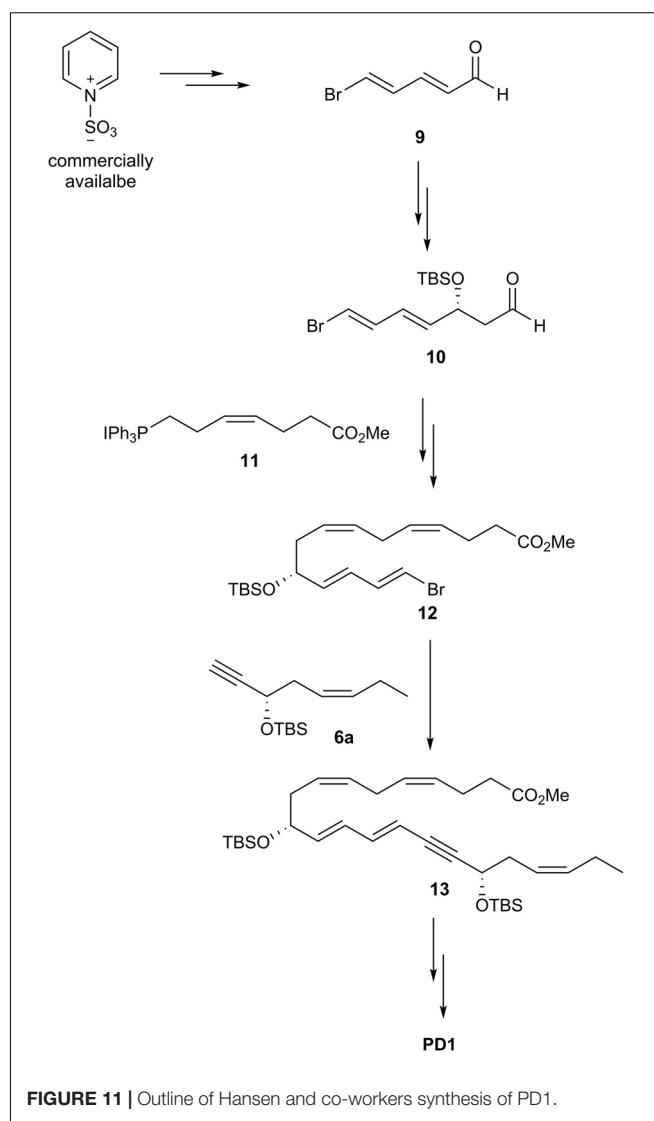
In order to obtain a Z-selective reduction of the conjugated alkyne **8** and to establish the correct Z-geometry of the double bond at C11-C12 (Serhan et al., 2006; Petasis et al., 2012), deprotection of the TBS-groups was first performed, then the authors used the Boland reduction reaction on **8** that was followed by basic hydrolysis, which furnished PD1. In addition, several isomers of PD1 were synthesized by the same research group. These isomers proved to be less potent pro-resolvents; however these studies provided useful information on structure-function relationships of PD1. The synthetic material was matched with endogenously formed PD1 and found to be identical (Serhan et al., 2006; Petasis et al., 2012).

### The Hansen Synthesis of PD1

A highly stereoselective synthesis of PD1 was published in 2014 (Aursnes et al., 2014b), mainly by using Evans-Nagao aldol-, Z-selective Wittig-, and Sonogashira-reactions (**Figure 11**). The main fragments were the terminal alkyne **6a**, the aldehyde **9** and the Wittig-salt **11**. The total synthesis of PD1 was performed in only eight linear steps from aldehyde **9**. Commercially available pyridinium-1-sulfonate was used for making aldehyde **9**. Further on, the aldehyde **9** was reacted in an Evans-Nagao aldol reaction that was followed by protection of the secondary alcohol and removal of the auxiliary to afford the intermediate **10** with high stereoselectivity. Then compound **10** was reacted with the corresponding ylide of the Wittig-salt **11** to yield the tetraene ester **12**. The alkyne **6a** was reacted with **12** in a Sonogashira cross-coupling reaction to afford **13** with the whole carbon skeleton of PD1. Deprotection of **13** and a Z-selective Lindlar reduction gave the correct Z-geometry of double bond at C15-C16. Saponification and acidic work-up furnished PD1. The synthetic material was matched with endogenously formed PD1 and found to be identical and with high chemical purity and stereochemical integrity (Aursnes et al., 2014b). The difference between alkyne **6**, used by the Petasis-group, and alkyne **6a** is the protection group.

### Spur and Rodriguez Synthesis of PD1

Rodriguez and Spur (2014) reported their total synthesis of PD1 that also relied on the Sonogashira cross-coupling reaction with

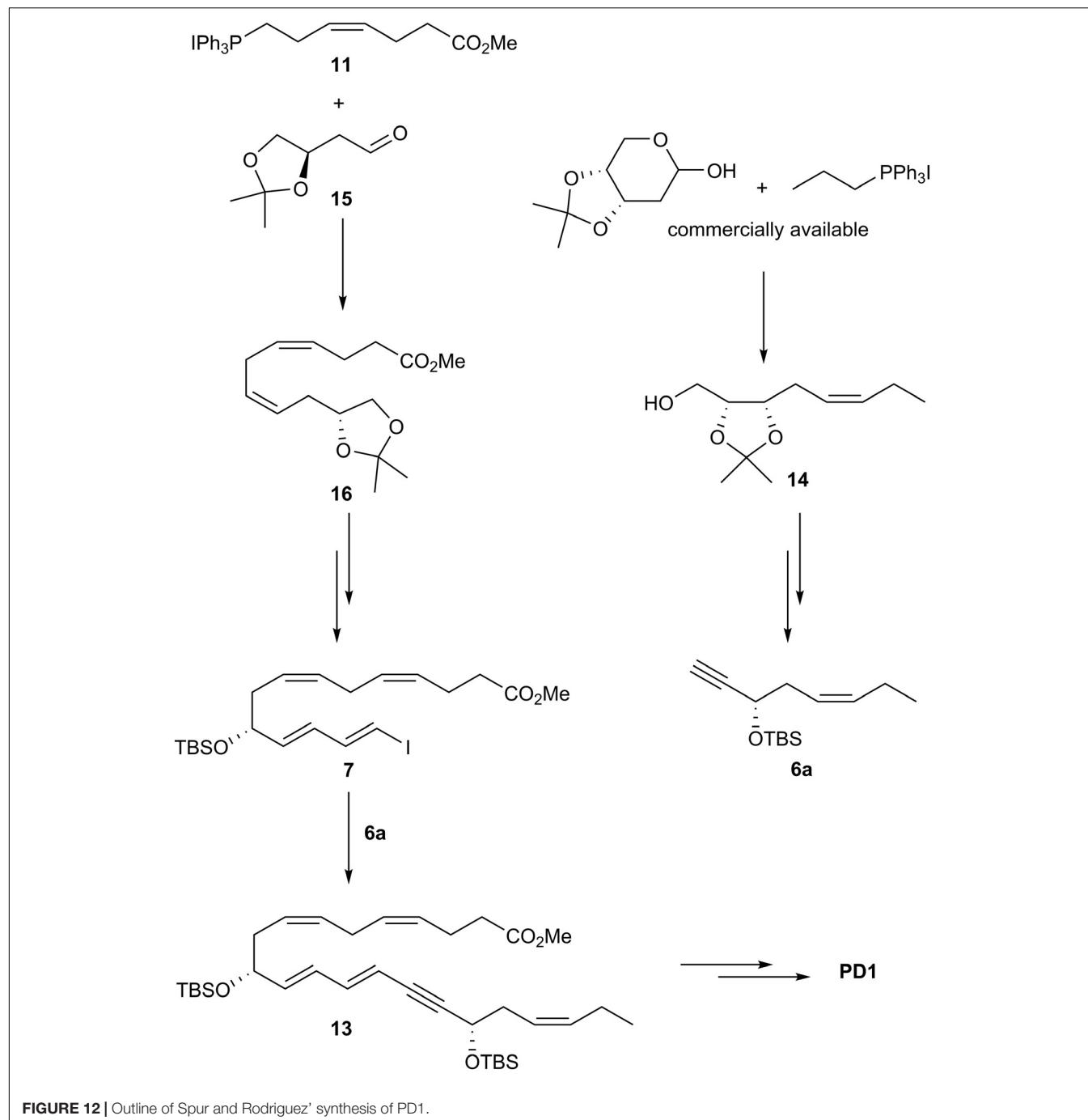


**FIGURE 11 |** Outline of Hansen and co-workers synthesis of PD1.

the terminal alkyne **6a** (**Figure 12**). Conversion of commercially available acetal-protected D-ribose gave **14** that was converted into alkyne **6a** using a different synthetic route than reported by Petasis and co-workers. The ylide of Wittig salt **11** was reacted with the aldehyde **15** in a Z-selective Wittig-reaction to give intermediate **16**. This intermediate was transformed into the vinylic iodide **7** (**Figure 12**). Finally, fragments **6a** and **7** were reacted using the Sonogashira reaction to give **13** that completed the C22 carbon-chain of PD1. The last steps included deprotection of **13** and a Z-selective alkyne reduction using the Boland protocol as well as an ester hydrolysis that gave PD1 (Rodriguez and Spur, 2014).

### Summary and Future Directions for SPM Therapy in Obesity and Diabetes

The cardinal signs of inflammation: *calor*, *rubor*, *tumor*, *dolor*, and *functio laesa* are physiologically mediated by chemical mediators, such as the PGs, and are effectively controlled by



**FIGURE 12 |** Outline of Spur and Rodriguez' synthesis of PD1.

traditional NSAIDs (Vane and Botting, 2001). These drugs give unwanted side effects. Given the increase in the inflammation-associated diseases obesity and diabetes it is paramount that new treatments and mechanisms are sought to control excessive inflammation and collateral tissue damage created by excessive PMN and their swarming in all organs (Cotran et al., 1999). Evidence for active endogenous resolution programs and novel resolution mediators holds promise for new therapeutic approaches that would not be immunosuppressive, but rather serve as immunoressolvents and pro-resolving mediator agonists

stimulating resolution (Morris et al., 2009; Serhan, 2014; Perretti, 2015; Serhan et al., 2015; Duvall and Levy, 2016; Fullerton and Gilroy, 2016; Serhan, 2017; Vik et al., 2017; Dalli and Serhan, 2018). While current treatments for inflammation can be effective, many of these can eventually become immunosuppressive, opening opportunities for infection.

The distinct properties of EPA, DHA and n-3 DPA exhibits to form pro-resolving lipid mediators may, at least in part, explain the established health effects associated with these omega-3 PUFAs. The biosynthetic pathways of these potent lipid

mediators may also explain some of the positive effects of aspirin, since COX-2 in the presence of aspirin biosynthesizes metabolically longer lasting epimers of the individual SPM (Serhan and Petasis, 2011; Serhan et al., 2011). These epimers also display potent pro-resolving and anti-inflammatory properties. The different families of SPMs display high structural complexity due to the presence of several stereogenic centers, both in the form of chiral, secondary alcohols and conjugated E- and Z-double bonds, reflecting their biochemical origins, functions and stereospecific bioactions toward individual GPCRs. Hence, acquired knowledge and distinct care must be exercised when working with SPMs. Failure of such diligent operations will not reveal the correct and exciting chemical structures or the potent bioactions that SPMs possesses as resolution agonists. Elucidating the role of PUFAs as precursors and their enzymatic oxygenated products at the cellular and molecular level in health is of current interest (Serhan, 2017). As of today, approximately 80 biologically active DHA-derived metabolites have been described with various biological roles. It is important to emphasize that as of today only the endogenous SPMs, such as the PDs, display both potent pro-resolving and anti-inflammatory bioactions *in vivo* in the low nanomolar range. Hence, the PDs are therefore among the most exciting small molecules currently under investigations toward drug development based on resolution of inflammation (Gilroy et al., 2004; Morris et al., 2009; Serhan and Petasis, 2011; Tabas and Glass, 2013; Corminboeuf and Leroy, 2014; Perretti, 2015; Serhan et al., 2015; Duvall and Levy, 2016; Serhan, 2017; Vik et al., 2017). PDs display high structural complexity due to the presence of several stereogenic centers, both in the form of chiral, secondary alcohols and conjugated E- and Z-double bonds reflecting their biochemical origins, functions and stereospecific bioactions toward individual GPCRs (Duvall and Levy, 2016). Hence, care must be exercised when working with these SPMs as resolution agonists or as pharmacological biotemplates toward drug development targeting diabetes and obesity (Tabas and Glass, 2013).

The vast majority of approved drugs have been developed to inhibit, block or antagonize specific biological pathways involved in inflammatory conditions (Vane and Botting, 2001). Hence, the inflammatory mechanisms have become central to several diseases, including obesity and diabetes. The detailed biochemical, genetic, molecular, and cellular mechanisms behind the biology of resolution of inflammation has resulted in a new paradigm in our understanding of the inflammatory process. With the appreciation and growing understanding of these intervening mechanisms drugs within “Resolution Pharmacology” will be of interest. Examples of such candidates

may be synthetic small molecular mimetics (Corminboeuf and Leroy, 2014; Vik et al., 2017) exhibiting pro-resolution and anti-inflammatory GPCR agonistic properties against obesity and diabetes (Oh and Olefsky, 2016). Moreover, activators of SPM biosynthesis or inhibitors of eicosanoid oxidoreductase (15-PG-dehydrogenase) may also be part of the potential future within the “Resolution Pharmacology” pharmacopeia. Of significance, SPMs are very potent GPCR agonists and approximately 40% of all approved drugs activate this receptor class. Based on the drug development efforts that pro-inflammatory PGs and LTs have resulted in, combined with an increasingly number of receptors identified, future drug development efforts should be facilitated (Gilroy et al., 2004; Tabas and Glass, 2013; Corminboeuf and Leroy, 2014; Duvall and Levy, 2016; Vik et al., 2017). However, such future endeavors depends, similar to past drug development successes based on pro-inflammatory mediators and their biological roles (Samuelsson, 2012), on basic biomedical research. We envision that the development of “Resolution Pharmacology” as well as exciting new findings from basic research related to SPMs will continue to evolve and enable innovative approaches for treating obesity and diabetes.

## AUTHOR CONTRIBUTIONS

All authors contributed to the writing of the manuscript and had gave approval to the final version of the manuscript.

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# Paradoxical Skin Reactions to Biologics in Patients With Rheumatologic Disorders

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Targeted immune-modulating treatment with biological agents has revolutionized the management of immune-mediated inflammatory diseases, including rheumatologic conditions. The efficacy and tolerability of biological agents, from the initial tumour necrosis factor (TNF)- $\alpha$  inhibitors to the new anti-cytokine monoclonal antibodies, have dramatically changed the natural history of debilitating conditions such as rheumatoid arthritis and seronegative spondyloarthropathies. The widening use of biologics across several rheumatologic diseases has been associated with a new class of adverse events, the so-called paradoxical reactions. These events are inflammatory immune-mediated tissue reactions, developing paradoxically during treatment of rheumatologic conditions with targeted biologics that are commonly used for treating the idiopathic counterparts of these drug-induced reactions. The skin is frequently involved, and, even if considered rare to uncommon, these cutaneous manifestations are an important cause of biologic agent discontinuation. TNF- $\alpha$  antagonist-induced psoriasis, which can manifest *de novo* or as exacerbation of a pre-existing form, is the prototypic and most frequent paradoxical skin reaction to biologics while other reactions, such as eczematous and lichenoid eruptions, hidradenitis suppurativa, pyoderma gangrenosum, Sweet's syndrome and granulomatous skin diseases, occur much more rarely. Management of these reactions consists of topical or systemic skin-directed therapies, depending on the severity and extension of the cutaneous picture, and it is generally associated with switching over to other disease-modifying regimens for treating the underlying rheumatologic condition. Here, we review in detail the current concepts and controversies on classification, pathogenesis and clinical management of this new class of cutaneous adverse events induced by biologics in rheumatologic patients.

**Keywords:** paradoxical skin reactions, biologics, rheumatological disorders, psoriasis, TNF $\alpha$ -inhibitors

**Abbreviations:** IFN, Interferon; IFN type-1, interferon-type 1; IL, interleukin; IL-10, interleukin 10; ILC, innate lymphoid cell; iTreg, induced regulatory T-cells; NDs, neutrophilic dermatoses; TGF- $\beta$ , transforming growth factor-beta; Th, T helper cell; TNF- $\alpha$ , tumor necrosis factor-alpha; TNFR2, tumor necrosis factor receptor 2; Tregs, regulatory T-cells.

## INTRODUCTION

Targeted biological agents have dramatically changed the treatment landscape of immune-mediated inflammatory diseases (IMIDs) with rheumatological conditions being at the front of this revolution. The efficacy and tolerability of targeted biological agents have determined a paradigm shift in the treatment of several rheumatologic conditions, modifying the natural history of progressive, invalidating disease, such as rheumatoid arthritis (RA) and seronegative spondyloarthropathies (SpA). While biological agents (BA) have a superior safety and tolerability profile compared to conventional disease-modifying anti-rheumatic drugs (DMARDs), they may cause different cutaneous adverse events, either of infectious, inflammatory or neoplastic origin (Hernandez et al., 2013). Furthermore, targeted treatment with BA is increasingly associated with a new class of adverse events, the so-called paradoxical immune-mediated inflammatory reactions. Paradoxical reactions (PR) are defined by the development of inflammatory immune-mediated tissue manifestations in IMID patients treated with targeted biological agents. The skin is frequently involved by this paradoxical inflammation, as in the case of plaque psoriasis developing in a rheumatological patient during treatment with TNF- $\alpha$  inhibitors (TNFi) (Viguier et al., 2009).

Cutaneous PR have been described as a class-effect of targeted BA, especially TNFi, first in rheumatologic patients and subsequently across all other indications, such as psoriasis and inflammatory bowel-disease (IBD). Reports of different, organ-specific PR are constantly increasing, as long-term use of new anti-interleukin (anti-IL-6, -IL-17, IL-12/23) and first-to-second generation TNFi biosimilars is growing (Toussirot and Aubin, 2016). Furthermore, cutaneous PR represent an intriguing immunological and clinical dilemma, whose unraveling may improve our knowledge of the pathogenesis of chronic inflammatory diseases. These puzzling cutaneous eruptions may also represent a new type of adverse drug reactions in the era of precision medicine, resulting from the interaction between targeted manipulation of cytokine-molecular networks by BA and patient's genetic predisposition (Cabaleiro et al., 2016). We review the clinical spectrum of paradoxical cutaneous inflammation induced by targeted BA in patients with rheumatological disorders, discussing the current controversies on classification, pathogenesis and clinical management.

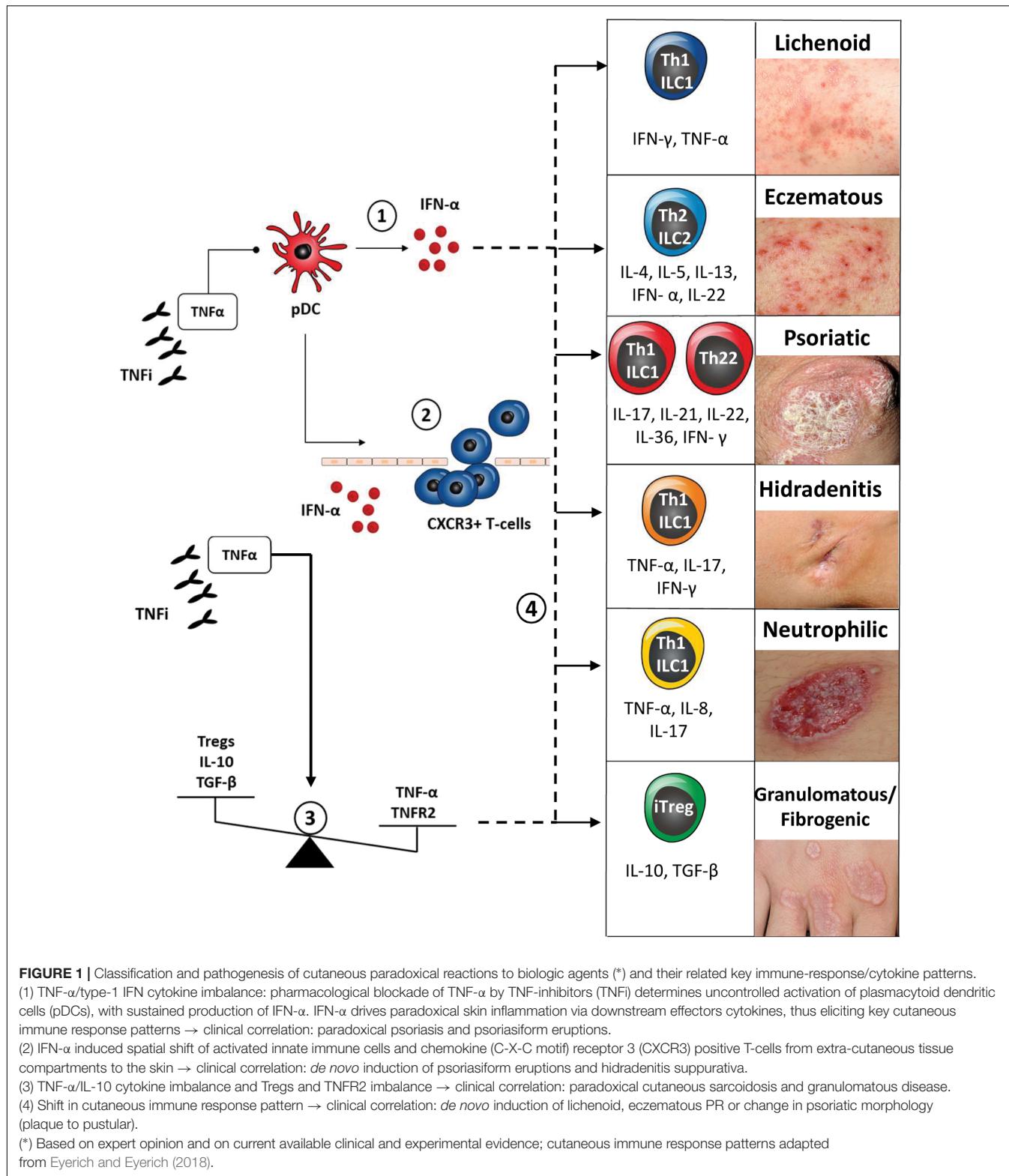
### Cutaneous Paradoxical Reactions: Definition and Scope of the Problem

Psoriasis, and its clinical variants, represents the prototypical cutaneous PR, as this was the first paradoxical reaction pattern described in rheumatologic patients treated with the first-generation BA, namely the TNFi. Therefore, most clinical and experimental studies on PR have focused on paradoxical psoriasis, providing the conceptual framework for the other cutaneous PR. The literature on epidemiology of PRs in patients treated with BA is scarce, as most of the clinical evidence derives from retrospective studies, case series and reports. The estimated

prevalence of cutaneous PR ranges from 0.6 to 5.3% in patients treated with TNFi (Sfikakis et al., 2005; Fouache et al., 2009; Ko et al., 2009; Famenini and Wu, 2013; Bae et al., 2018). In a registry-based observational study, the incidence of paradoxical psoriasis in RA patients treated with TNFi has been estimated in 1.04 per 1000 persons/years. Patients treated with TNFi presented an incidence rate ratio (IRR) of 2.0–5.94 for the onset of paradoxical psoriasis compared to patients treated with conventional DMARDs (Hernandez et al., 2013). In RA patients, the incidence of paradoxical psoriasis has been estimated in one new case for every 550 patients treated with adalimumab per year (Harrison et al., 2009). In the context of adverse drug reaction, cutaneous PRs could be classified as uncommon-to rare events. Typically, cutaneous PRs, such as paradoxical psoriasis, can be induced *de novo* in rheumatologic patients without a history of cutaneous inflammatory disease during treatment with a BA. On the other hand, cutaneous PR can be an exacerbation, with or without a change in clinical morphology, of a pre-existing cutaneous inflammatory disease in a genetically-predisposed patient. This is the case of paradoxical palmoplantar pustular psoriasis developing during TNFi in a RA patient with a history of plaque psoriasis. Key features supporting the causal relationship between a skin PR and therapy with a BA are: (a) the temporal association and (b) clinical outcome of the PR after BA withdrawal. Cutaneous PR can occur at any time during treatment with a BA, but more than 60% of cases of paradoxical psoriasis has been reported to develop within the first year of treatment (Brown et al., 2017). As observed in cutaneous drug reactions, the cessation of the triggering “culprit” BA determines clinical resolution or improvement of the skin PR. Re-treatment, or drug re-challenge, with the same BA or related BA-class has been associated with the relapse of paradoxical skin inflammation.

### CLASSIFICATION OF CUTANEOUS PARADOXICAL REACTIONS

In rheumatologic patients, cutaneous PRs induced by BA can present with different clinical aspects and extent, involving the skin, its appendages and transitional epithelial surfaces. Cutaneous PRs, as defined previously, encompass a variety of inflammatory manifestations/conditions, which can be both treated and triggered with the same cytokine-targeted BA. Cutaneous PRs reported in rheumatologic patients are summarized in **Figure 1** and include psoriasis and its spectrum of clinical phenotypes (plaque, pustular, generalized, palmoplantar, scalp, guttate, and inverse), hidradenitis suppurativa (HS), neutrophilic dermatosis (the prototypical forms pyoderma gangrenosum and Sweet's syndrome) and granulomatous skin disease (granuloma annulare, interstitial granulomatous dermatitis, necrobiosis lipoidica, and sarcoidosis). Other BA-inducible cutaneous inflammatory conditions, such as atopic dermatitis, cutaneous vasculitis, drug-induced lupus erythematosus and other allergic and hypersensitivity reactions are not strictly considered “paradoxical” because their idiopathic counterparts are not generally treated with these agents.



**FIGURE 1 |** Classification and pathogenesis of cutaneous paradoxical reactions to biologic agents (\*) and their related key immune-response/cytokine patterns.

(1) TNF- $\alpha$ /type-1 IFN cytokine imbalance: pharmacological blockade of TNF- $\alpha$  by TNF-inhibitors (TNFi) determines uncontrolled activation of plasmacytoid dendritic cells (pDCs), with sustained production of IFN- $\alpha$ . IFN- $\alpha$  drives paradoxical skin inflammation via downstream effector cytokines, thus eliciting key cutaneous immune response patterns → clinical correlation: paradoxical psoriasis and psoriasiform eruptions.

(2) IFN- $\alpha$  induced spatial shift of activated innate immune cells and chemokine (C-X-C motif) receptor 3 (CXCR3) positive T-cells from extra-cutaneous tissue compartments to the skin → clinical correlation: *de novo* induction of psoriasiform eruptions and hidradenitis suppurativa.

(3) TNF- $\alpha$ /IL-10 cytokine imbalance and Tregs and TNFR2 imbalance → clinical correlation: paradoxical cutaneous sarcoidosis and granulomatous disease.

(4) Shift in cutaneous immune response pattern → clinical correlation: *de novo* induction of lichenoid, eczematous PR or change in psoriatic morphology (plaque to pustular).

(\*) Based on expert opinion and on current available clinical and experimental evidence; cutaneous immune response patterns adapted from Eyerich and Eyerich (2018).

Classification of inflammatory skin disease is traditionally based on clinical morphology of primary and secondary skin lesions in combination with a histological description of epidermal-dermal tissue involvement and underlying

pathomechanisms (Dainichi et al., 2014). Adverse cutaneous drug reactions also share similar classification systems, with a combination of clinical descriptors of lesion morphology (psoriasiform, bullous etc.) histological pattern (spongiotic,

lichenoid/interface dermatitis, etc.) and underlying predominant immunologic/hypersensitivity mechanism (type I-IV reaction) (Isabwe et al., 2017). A recent trend in the classification of inflammatory skin disease is to integrate clinicopathological data with molecular-immunologic information, such as predominant disease cell-subset, cytokine expression patterns and molecular biomarkers (Inkeles et al., 2015; Garzorz-Stark and Lauffer, 2017). Recently, Eyerich and Eyerich (2018) summarized the cutaneous immune-response patterns (Th1-, Th2-, Th17/Th22- and Treg-cells and related cytokines) associated with specific cutaneous-tissue response patterns (lichenoid, eczematous/blistering, psoriatic, fibrogenic/granulomatous), providing a molecular-pathophysiological approach to the traditional, complex dermatological nosology. This conceptual classification can be used for the description of cutaneous PR, along with its prevalently associated clinical and immune-response patterns, according to currently published data (refer to **Figure 1**). According to its initial descriptions, cutaneous PRs can be considered almost identical to its corresponding, "classic" inflammatory skin disease in terms of clinical, histological and immunological presentation. In the following sections we will discuss some limitations of this concept, based on recent clinical and experimental studies.

In BA-treated patients, the clinical picture of cutaneous PR may vary from typical inflammatory skin lesions - clinically and histological identical to its correspondent primary, non-BA induced skin disease - to atypical inflammatory skin manifestations, with "overlapping" clinical and histological features. For example, paradoxical TNFi-induced psoriasis may present with a wide clinical spectrum, with typical erythematous-squamous or pustular lesions, clinically indistinguishable from conventional plaque or pustular psoriasis, to atypical papulo-squamous eruptions with "psoriasiform," "eczematous" or "lichenoid" lesion morphology (Succaria and Bhawan, 2017). Correlation with histological aspects of lesional skin is crucial for diagnosis of the PR type and differentiation with other cutaneous adverse events. Moreover, in most published series the spectrum of histological changes of a "psoriasiform" paradoxical eruption is quite variable, ranging from typical psoriatic pattern to lichenoid/interface dermatitis, pustular folliculitis and eosinophil-rich perivascular dermatitis pattern. Psoriatic alopecia has been recently described as a distinct clinical phenotype of anti-TNF- $\alpha$ -induced, paradoxical psoriasis, presenting with patchy, non-cicatricial alopecia due to marked inflammatory involvement of the scalp skin and hair follicles (**Figure 1**; Osório et al., 2012; George et al., 2015). Histologically, features of both conventional scalp psoriasis and alopecia areata have been observed (Doyle et al., 2011).

Lichen planus (LP)-like or lichenoid skin eruptions are characterized by an interface dermatitis histological pattern and prominent Th1/ILC1 (type 1 innate lymphoid cell)-IFN (interferon)- $\gamma$ -biased immune-response. This skin-directed cytotoxic reaction can be triggered by microbial antigens, xenobiotics and drugs. Paradoxical, lichenoid eruptions have been reported in RA and psoriatic arthritis (PsA) patients during treatment with TNFi, variably involving the skin,

oral/genital mucosa, nails and hair-follicles (Vergara et al., 2002; Asarch et al., 2009; Garcovich et al., 2010).

Hidradenitis suppurativa is a chronic, inflammatory disease of the follicular epithelium, presenting with suppurative lesions (nodules, abscesses, pustules, fistulas, sinus-tracts, and hypertrophic scars) affecting the skin folds and anogenital area. The cutaneous immune response pattern of primary HS is characterized by Th17/ILC3 lymphocyte subset, with strong IL-1 $\beta$ , TNF- $\alpha$ , IL-17 cytokine-signature, and peripheral recruitment of IL-17-producing neutrophils and Th17-cells. Paradoxical HS has been recently reported in patients with RA or spondyloarthropathies (PsA, AS, SAPHO) during long-term treatment (mean 25 months) with TNFi and other BA (tocilizumab, rituximab) (Delobeau et al., 2016; Faivre et al., 2016). Most patients presented known risk factors for HS (smoking, overweight), but the relapse of paradoxical HS after re-treatment with involved BA supports causality.

Pustular psoriasis and neutrophilic dermatoses (pyoderma gangrenosum, Sweet syndrome) present a sterile pustule as hallmark cutaneous lesion of neutrophilic inflammation. Both "neutrophilic" inflammatory conditions share common downstream inflammatory mediators, such as TNF- $\alpha$ , IL-8, IL-17, IL12/23 and IL-36, which promote activation and migration of neutrophils in the skin. Pyoderma gangrenosum, the prototypical form of hypodermal neutrophilic, can be both treated and paradoxically triggered by almost all the TNFi (etanercept, adalimumab, infliximab, golimumab) (Vandevyvere et al., 2007; Brunasso et al., 2010; Kowalzik et al., 2013; Marzano et al., 2018; Skalkou et al., 2018).

Granulomatous skin conditions are a heterogenous group of chronic inflammatory diseases, which include also reactive or drug-induced processes. Reactive granulomatous skin eruptions have a wide spectrum of clinical morphologies with several clinical entities, such as interstitial granulomatous dermatitis (IGD) and palisaded neutrophilic and granulomatous dermatitis (PNGD). These reactive conditions can be triggered by systemic inflammatory conditions, such as connective tissue disease and the rheumatic disease, and by several drugs, such as TNFi (Deng et al., 2006). Localized and generalized forms of granuloma annulare have been reported in rheumatologic patients during treatment with TNFi (Voulgari et al., 2008). Histological evidence of dermal non-caseating granulomas is a hallmark of cutaneous sarcoidosis, which can present several clinical-morphological variants (Amber et al., 2015; Rosenbach and English, 2015). Paradoxical development of sarcoidosis-like skin lesions has been reported in rheumatologic patients treated with TNFi, especially with etanercept (Dhaille et al., 2010; Massara et al., 2010; Robicheaux Clementine et al., 2010; Lamrock and Brown, 2012; Decock et al., 2017). In sum, cutaneous PR presents a wide spectrum of clinical-histological reaction patterns.

## PATHOGENESIS OF CUTANEOUS PARADOXICAL INFLAMMATION

Since the initial descriptions, cutaneous inflammatory disease presenting *de novo* in rheumatologic patients during treatment

with potent, cytokine-targeted BA represented a clinical and immunological conundrum. Considering the molecular taxonomy of inflammatory skin disease, cutaneous PR can be explained by the complex interplay between host-specific factors (genetic predisposition) and BA-induced specific shifts in cytokine and cellular immune-response patterns (Palucka et al., 2005; Grine et al., 2015; Verwoerd et al., 2016). According to current experimental data, cutaneous paradoxical inflammation may result from different putative immune-pathogenetic mechanisms (summarized in **Figure 1**), leading to different types of clinical reactions. BA-induced immune-pathogenesis of cutaneous PRs include one or more of the following mechanisms as *primum movens*: (a) a cytokine imbalance; (b) a shift in cutaneous immune response pattern; (c) a spatial shift of activated innate or adaptive immune cells to the skin; (d) imbalance or dysfunction of regulatory T-cells.

In the case of paradoxical psoriasis, a TNFi-induced cytokine imbalance between TNF- $\alpha$  and type 1-Interferons (IFN- $\alpha$ ) has been reported as key-pathogenetic factor (Marzano et al., 2014). Lesional skin of psoriasiform PR displayed an increased tissue-expression of MxA (myxovirus-resistance protein A), i.e., type-1 interferon activity (de Gannes et al., 2007). Notably, systemic treatment with recombinant IFN- $\alpha$  and topical application of IFN- $\alpha$  inducers (imiquimod) are both able to elicit psoriatic skin lesions in clinical and experimental models (Gilliet et al., 2004). Continuous therapeutic TNF- $\alpha$  blockade thus cause a specific, cutaneous cytokine imbalance, favoring development of inflammatory plasmacytoid dendritic cells (pDCs) and increased type-1 IFN production (Conrad et al., 2018). Increased IFN- $\alpha$ -activity in turn determines abnormal trafficking and homing of pDCs and innate immune cells to the skin, in an inflammatory loop. Furthermore, in RA patients, treatment with TNFi has been associated with increased expression of the chemokine receptor CXCR3 on peripheral T-cells, potentially favoring trafficking of activated T-cells to the skin (Aeberli et al., 2005). This dysregulated, paradoxical innate immune response may then translate clinically to paradoxical psoriasis in genetically susceptible subjects.

Recent experimental studies raised several controversies on the true nature of cutaneous PR, differentiating its immune pathogenesis from their “classical,” non-paradoxical counterparts. Stoffel et al. (2018) reported both psoriasiform and eczematous PR to have a distinct lesional immune response pattern, with a common strong Th1- and type-1 IFN pattern, differing, respectively, from “classic” psoriasis and eczema controls. In the same study, psoriasiform PR presented an increased expression of type 1 IFN (IFN- $\alpha$ , IFN- $\gamma$ ) and pro-inflammatory cytokines (IL-36, IL-19, IL-20), whereas eczematous PR were associated with up-regulated Th-2 cytokines (IL-13, IL-5) and IL-22 expression. In psoriasiform PRs, the involvement of IL-1-(IL-36) and IL-17 (IL-17A) cytokine families, sustaining a pro-inflammatory loop mechanism, has been also reported in patients with IBD and psoriasis (Tillack et al., 2014; Deubelbeiss et al., 2018). Finally, the group of Gilliet et al. (2004) designed an experimental murine model for paradoxical skin inflammation to support the differences between paradoxical psoriasis and “classic” psoriasis. In this

model, induction of the “paradoxical” psoriasiform phenotype is mainly driven by type 1 IFN expression and cutaneous infiltration of pDCs due to temporal TNF- $\alpha$ /IFN imbalance. The resulting paradoxical psoriasiform skin inflammation is mostly independent from T-cells, i.e., from adaptive immune responses, which is in contrast with “classical” psoriasis (Conrad et al., 2018). BA-induced shifts of cutaneous immune response patterns may then interact with host-specific genetic risk variants for inflammatory cutaneous disease, promoting the development of PRs. Indeed, preliminary studies support the role of specific IL-23 receptor (IL-23R) gene polymorphisms to be linked to anti-TNF- $\alpha$ -induced paradoxical psoriasis (Sherlock et al., 2013).

## CLINICAL IMPLICATIONS OF PARADOXICAL REACTIONS TO BIOLOGICAL AGENTS

Cutaneous PR occurring during treatment with BA represents a clinical challenge in terms of differential diagnosis and management. Clinical management should be aimed at treating the cutaneous signs (eruption) and symptoms (pain, pruritus etc.) while maintaining control of the underlying rheumatologic condition. As discussed previously, the modification of the anti-rheumatic treatment regimen, i.e., a treatment suspension/withdrawal or therapeutic switch, is associated in most cases with an improvement or resolution of the cutaneous PR. Therefore, when approaching the rheumatologic patient with a PR, the clinician should take into account several factors, including: (a) the extent and severity of skin involved by the PR, (b) the severity and activity of the background rheumatologic condition, (c) the patient’s quality of life and comorbidities, (d) the possible loss of efficacy of the culprit BA in the case of cessation/retreatment, (e) the availability of alternative treatment options for the rheumatologic condition. Dose-reduction and discontinuation strategies of BA in rheumatologic patients should be evaluated on a per-case basis by the treating rheumatologist, as there are no definitive guidelines for the management of cutaneous PR (van Herwaarden et al., 2014). In the case of anti-TNF- $\alpha$  induced psoriasis, a practical treatment algorithm has been initially proposed by Collamer et al. (2008) and this can be adapted to other cases of cutaneous PRs as well.

In a BA-treated rheumatologic patient developing inflammatory skin lesion a high-grade of suspicion for PR should be taken. Interdisciplinary care should necessary include evaluation by a dermatologist and lesional skin biopsy, to aid clinical-histological correlations and differential diagnosis with other cutaneous adverse events. The severity of cutaneous PR can be graded with simple assessments, such as the extent of body surface area (BSA) involved, symptom-based scores (pruritus/pain intensity scores) and patient-reported outcomes (dermatology life quality index [DLQI]). The addition of a skin-directed therapy (topical or systemic) is a reasonable initial strategy to manage the cutaneous PR and to maintain the patient on treatment with the BA. Topical skin-directed therapies (topical steroids, keratolytic agents, immunomodulators, vitamin

D analogs) are a viable option for PR with mild (BSA < 5%) to moderate (BSA 5–10%) skin involvement, such as localized plaque psoriasis, lichenoid reactions or granulomatous lesions. In the case of PR with moderate-to severe skin involvement (>10% BSA), progressive course and/or high symptom-burden (QoL impairment) treatment can be escalated with the addition of UV-phototherapy or traditional systemic agents, such as methotrexate, retinoids (acitretin), cyclosporine and systemic steroids. Combination treatment regimens with systemic, skin-directed agents and ongoing BA (for example TNFi) should be carefully managed in close collaboration by the rheumatologist and the dermatology consultant.

In the case of severe PR with extensive (>10% BSA), unstable disease and/or high disease-burden, treatment with the BA should be discontinued. For example, severe plaque psoriasis, erythrodermic psoriasis, generalized pustular psoriasis or highly pruritic lichenoid or eczematous eruption would necessarily lead to discontinuation of anti-rheumatic treatment with a TNFi. According to published studies, almost 50% of patients will present an improvement or resolution of paradoxical skin lesions, following withdrawal of the BA. Another 45% of patients with anti-TNF- $\alpha$  induced psoriasis may present persistent or recurring cutaneous lesions, despite BA discontinuation. The more severe PR, such as generalized pustular psoriasis or psoriatic alopecia, can run a persistent course, only with partial improvement, after discontinuing the BA (Brown et al., 2017). Re-treatment with the same BA, after cessation of the cutaneous PR, should be evaluated on the basis of concomitant rheumatologic condition and availability of alternative treatment options. There is a substantial risk of recurrence of the cutaneous PR after re-treatment with the same BA, but there is no strong evidence in published studies (Wollina et al., 2008). Therapeutic switch of the PR-triggering BA with another BA of the same class (i.e., alternative TNFi) or of different class can be considered in moderate-to severe cutaneous PR, to control the underlying rheumatologic condition. Therapeutic switch to another BA is also indicated in the severe, paradoxical psoriasis subtypes, as

in the case of generalized pustular psoriasis. Switching to a new BA-class, for example from a TNFi to anti-IL6 treatment (tocilizumab), is a common strategy in the management of RA and has been also reported in the case of paradoxical psoriasis (Rueda-Gotor et al., 2012; Shimizu et al., 2015; Cantini et al., 2017).

## CLINICAL IMPLICATIONS OF PARADOXICAL REACTIONS TO BIOLOGICAL AGENTS

The unexpected occurrence of paradoxical inflammation during treatment with BA has emerged as a new type of drug-related adverse event, with a complex pathophysiology. The skin is one of the main organs affected by these reactions, presenting with a wide spectrum of clinical and pathological aspects. In rheumatologic patients, cutaneous PRs are frequent and clinically relevant. Adequate clinical management of these reactions is paramount to maintain control of background rheumatologic disease and to improve drug survival of BA. In some cases, therapeutic switch to another class of BA or to new, small-molecule-based disease modifying drugs is warranted to oppose paradoxical inflammation. The understanding of these new types of adverse reactions will hopefully shed light on the complex interactions between host-specific factors (genetic predisposition), immune-mediated comorbidities, immune-regulatory mechanisms and targeted immune-modulation.

## AUTHOR CONTRIBUTIONS

SG, CS, EB, MC, and AM designed and reviewed the manuscript and contributed in drafting the manuscript. GG contributed in drafting and reviewing the manuscript. All the authors approved the final version of the manuscript.

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# Platelet-Derived Microparticles From Obese Individuals: Characterization of Number, Size, Proteomics, and Crosstalk With Cancer and Endothelial Cells

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**Rationale:** Obesity is a risk factor for atherosclerosis and various cancers. However, the mechanisms are not yet completely clarified.

**Objectives:** We aimed to verify whether the microparticles (MPs) released from thrombin-activated platelets differed in obese and non-obese women for number, size, and proteomics cargo and the capacity to modulate *in vitro* the expression of (i) genes related to the epithelial to mesenchymal transition (EMT) and the endothelial to mesenchymal transition (EndMT), and (ii) cyclooxygenase (COX)-2 involved in the production of angiogenic and inflammatory mediators.

**Methods and Results:** MPs were obtained from thrombin activated platelets of four obese and their matched non-obese women. MPs were analyzed by cytofluorimeter and protein content by liquid chromatography-mass spectrometry. MPs from obese women were not different in number but showed increased heterogeneity in size. In obese individuals, MPs containing mitochondria (mitoMPs) expressed lower CD41 levels and increased phosphatidylserine associated with enhanced Factor V representing a signature of a prothrombotic state. Proteomics analysis identified 44 proteins downregulated and three upregulated in MPs obtained from obese vs. non-obese women. A reduction in the proteins of the α-granular membrane and those involved in mitophagy and antioxidant defenses-granular membrane was detected in the MPs of obese individuals. MPs released from platelets of obese individuals were more prone to induce the expression of marker genes of EMT and EndMT when incubated with human colorectal cancer cells (HT29) and human cardiac microvascular endothelial cells (HCMVEC), respectively. A protein, highly enhanced in obese MPs, was the pro-platelet

basic protein with pro-inflammatory and tumorigenic actions. Exclusively MPs from obese women induced COX-2 in HCMEC.

**Conclusion:** Platelet-derived MPs of obese women showed higher heterogeneity in size and contained different levels of proteins relevant to thrombosis and tumorigenesis. MPs from obese individuals presented enhanced capacity to cause changes in the expression of EMT and EndMT marker genes and to induce COX-2. These effects might contribute to the increased risk for the development of thrombosis and multiple malignancies in obesity.

**Clinical Trial Registration:** [www.ClinicalTrials.gov](http://www.ClinicalTrials.gov), identifier NCT01581801.

**Keywords:** microparticles, platelets, obesity, proteomics, cellular cross-talk

## INTRODUCTION

The activation of platelets in response to tissue damage is an early event in the reparative process (Gawaz et al., 2005). However, in some circumstances, the cascade of biological processes involved in tissue healing can be affected, thus translating into the development of a chronic inflammatory state which promotes the development and progression of numerous disorders, including atherothrombosis and colorectal cancer (CRC) (Gawaz et al., 2005; Patrignani and Patrono, 2016). Since platelets up-take proteins and genetic material from plasma (Best et al., 2015), the platelet phenotype is influenced by the individual clinical condition.

Platelets release small membrane-bound microparticles (MPs) containing bioactive proteins and genetic material which can be delivered to recipient cells, including immune, endothelial, epithelial and cancer cells (Dovizio et al., 2018); through this mechanism cells acquire novel phenotypes and functions which may promote the development of pathological states (Dovizio et al., 2018).

Lifestyle factors, such as western style dietary habits, and lack of physical activities associated with overweight and obesity, are risk factors for various types of cancer (Basen-Engquist and Chang, 2011). Excess body fat is potentially a modifiable cancer risk factor (Basen-Engquist and Chang, 2011). However, the biological mechanisms underlying the relationship between obesity and cancer have not been completely elucidated yet. We hypothesize that platelet-derived MPs and their proteomic content are altered in obesity, thus promoting cancer.

This study aimed to characterize the number, size, and proteome of MPs generated *in vitro* in response to thrombin from platelets of obese women and their matched lean controls. Moreover, we performed experiments *in vitro* to explore the capacity of platelet-derived MPs of both groups to influence the expression of marker genes of epithelial- and endothelial-mesenchymal transition (EMT and EndMT, respectively), in the HT29 human colorectal adenocarcinoma cells and human cardiac microvascular endothelial cells (HCMEC). The effect of MPs of both groups on endothelial cyclooxygenase (COX)-2 expression, a pro-angiogenic and inflammatory pathway (Wang and DuBois, 2004), was also evaluated.

## MATERIALS AND METHODS

### Subjects

We studied four obese and four non-obese women. Demographic and clinical characteristics of the two groups are reported in **Table 1**. All individuals were enrolled at the Unit of Obesity disorders, Policlinico Gemelli, Catholic University of Rome (Italy). Obesity was defined as a BMI (Body Mass Index; calculated as weight in kilograms divided by the square of height in meters) of 30 and above. The two groups had comparable age ( $43.50 \pm 5.33$  and  $43.25 \pm 4.35$  years, mean  $\pm$  SD, respectively) and did not present hypertension, diabetes mellitus or dyslipidemia (**Table 1**). They did not use any medication. The two groups differed for the BMI ( $49.50 \pm 1.12$  and  $21.89 \pm 1.01$ , respectively,  $P < 0.01$ ) (**Table 1**). The experimental protocol was approved by the Ethics Committee of Policlinico Gemelli (Catholic University, Rome, Italy) (Clinicaltrials.gov Registration number NCT01581801). This study was carried out following the recommendations of the Declaration of Helsinki and the approved guidelines from the Ethics Committee of Policlinico Gemelli. After signing the informed consent, all individuals underwent blood collection.

**TABLE 1 |** Demographic and clinical characteristics of healthy and obese individuals.

	Healthy subjects	Obese individuals	P-values <sup>a</sup>
Number	4	4	
Sex, female (%)	4 (100%)	4 (100%)	
Age (years)	$43.25 \pm 4.35$	$43.50 \pm 5.33$	$>0.05$
BMI (kg/m <sup>2</sup> )	$21.89 \pm 1.01$	$49.50 \pm 1.12$	$<0.0001$
Diabetes, n (%)	0 (0.00)	0 (0.00)	
Hypertension, n (%)	0 (0.00)	0 (0.00)	
Epatic steatosis, n (%)	0 (0.00)	0 (0.00)	
Total cholesterol	$184.00 \pm 10.35$	$175.80 \pm 8.34$	$>0.05$
HDL mg/dL	$64.25 \pm 1.03$	$50.00 \pm 5.80$	$>0.05$
LDL mg/dL	$106.30 \pm 8.08$	$100.00 \pm 11.32$	$>0.05$
Glucose mg/dL	$85.75 \pm 2.394$	$95.25 \pm 3.794$	$>0.05$
Drugs, n (%)	0 (0.00)	0 (0.00)	

Data are expressed as mean  $\pm$  SD. <sup>a</sup>By Fisher's Exact Test or Unpaired Student's t-test, as appropriate.

## Platelet Microparticle (MP) Preparation

Washed platelets were obtained, as previously described (Dovizio et al., 2013; Vasina et al., 2013), and analyzed for the contamination of leukocytes [identified for their positivity to Syto16 fluorescent nucleic acid stain (Thermo Fisher Scientific, Milan, Italy) and CD45 (using mAb from BD Biosciences, Milan, Italy)] and erythrocytes [recognized for the surface expression of CD235a (using mAB from BD Biosciences)] by flow cytometry. Platelets were stimulated with thrombin (1 U/ml, Sigma-Aldrich) for 30 min at 37°C to generate MPs, as previously described (Vasina et al., 2013). Platelet MPs were characterized using a flow cytometer with mAb against CD41 (CD41-PerCP-Cy5.5, BD Biosciences) and the presence of whole platelets in the suspension was ruled out. Platelets and MPs were analyzed by FacsVerse cytometer (BD Biosciences), and data were examined using FACSuite v 1.0.5 (BD Biosciences) software.

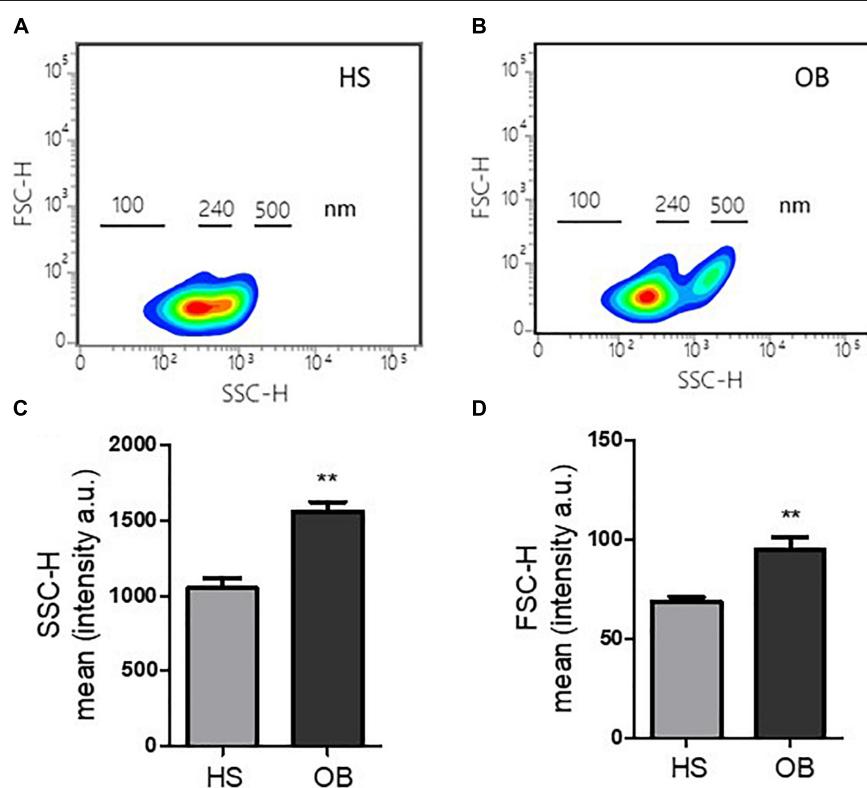
## Flow Cytometry Analysis of Platelet MPs

After resuspension of MP pellet in Annexin buffer (BD Biosciences), platelet MPs were labeled with MitoStatus-APC (Thermo-Fisher)/Phalloidin (Sigma-Aldrich, Milan, Italy)/CD41-PerCP-Cy5.5 (BD Biosciences)/AnnexV-V500 (BD Biosciences), as reported in the manufacturer's instructions, and counted by flow cytometry. MPs were gated based on their size, and the scatter properties were analyzed by running

Megamix Plus beads (Biocytex, Marseille, France) at the same photomultiplier (PMT) voltages used for MP detection. Phalloidin negative events (of total MPs or MitoStatus positive MPs) were analyzed for CD41 expression. CD41+ events were then evaluated for their positivity to AnnexinV.

## Assessment of MP Protein Content by Liquid Chromatography-Mass Spectrometry (LC-MS/MS)

Samples were prepared as previously described (Parsons et al., 2018). Protein concentration was assessed by Bradford protein assay (Bio-Rad, Hercules, CA, United States) using bovine serum albumin (BSA) (Sigma-Aldrich) as standard for the calibration curve and for each sample 30 µg proteins were precipitated with 95% acetone (4:1 acetone: sample volume) overnight. LC-MS/MS analysis of proteins was performed, as previously reported (Parsons et al., 2018). Briefly, dried protein pellets were resuspended in 8 M Urea/ 25 mM Tris-HCl, pH 8.2, at 37°C with gentle agitation. Disulfide bonds were reduced with 5 mM DTT and protected with 15 mM iodoacetamide. Proteins were first digested with Lys-C (1:100; Promega, Madison, WI, United States) followed by digestion with trypsin (1:100; Promega). Peptides were purified using ZipTipC18 pipette tips according to manufacturer instructions (Millipore, Billerica, MA, United States) and resuspended in



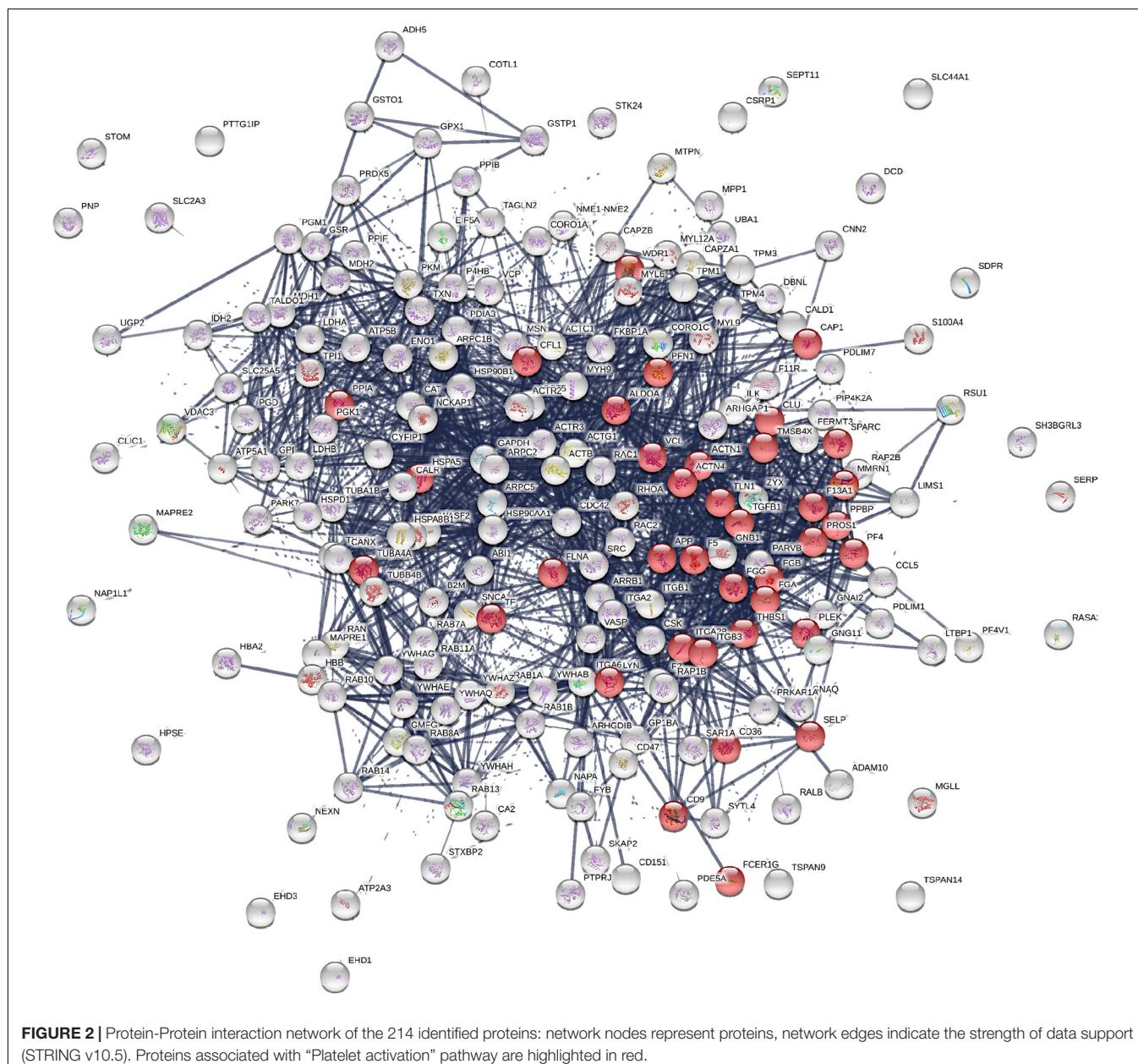
**FIGURE 1 |** Features of MPs released from platelets of obese and non-obese women. **(A,B)** Density plots of forward scatter height (FSC-H) vs. side scatter height (SSC-H) of a typical MP suspension from non-obese (HS) and obese (OB) individuals, and size distribution. **(C,D)** Fluorescence intensity of FSC-H and SSC-H parameters reported as arbitrary unit (a.u.) ( $n = 4$  for each group); \*\* $P < 0.01$  vs. HS.

1% formic acid. Approximately 2 µg of purified peptides were injected per LC-MS/MS analysis using an Ultimate3000 nano-LC system coupled to a hybrid quadrupole-orbitrap mass spectrometer (Q Exactive, Thermo Fisher Scientific). Peptides were separated by an increasing acetonitrile gradient from 2 to 33 % in a linear LC gradient of 40 min on a C18 reverse phase chromatography column packed with 2.4 µm particle size, 300 Å pore size C18 material (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) to a length of 120 mm in a column with a 75 µm ID, using a flow rate of 250 nL/min. All data were acquired with the mass spectrometer operating in an automatic data-dependent acquisition mode (DDA, shotgun). A full MS service scan at a resolution of 70,000, AGC target 3e6 and a range of *m/z* 350–1600 was followed by up to 12 subsequent MS/MS scan with

a resolution of 17,500, AGC target 2e4, isolation window *m/z* 1.6 and a first fix mass of *m/z* 100. Dynamic exclusion was set to 40 s.

The MS data have been submitted to the PRIDE proteomics identification database<sup>1</sup> under accession numbers PXD011563. Downstream analysis of proteomic data was performed by Perseus software (version 1.6.0.7). LFQ intensities of three technical replicates were averaged, and only the proteins present in at least 50% of the samples in one group (healthy donors and obese subjects) were considered identified. Proteins found to be differentially expressed between groups (*P*-value <0.05, FDR 0.01) were subjected to pathway mapping analysis and were distributed into categories according to

<sup>1</sup>[www.ebi.ac.uk/pride](http://www.ebi.ac.uk/pride)

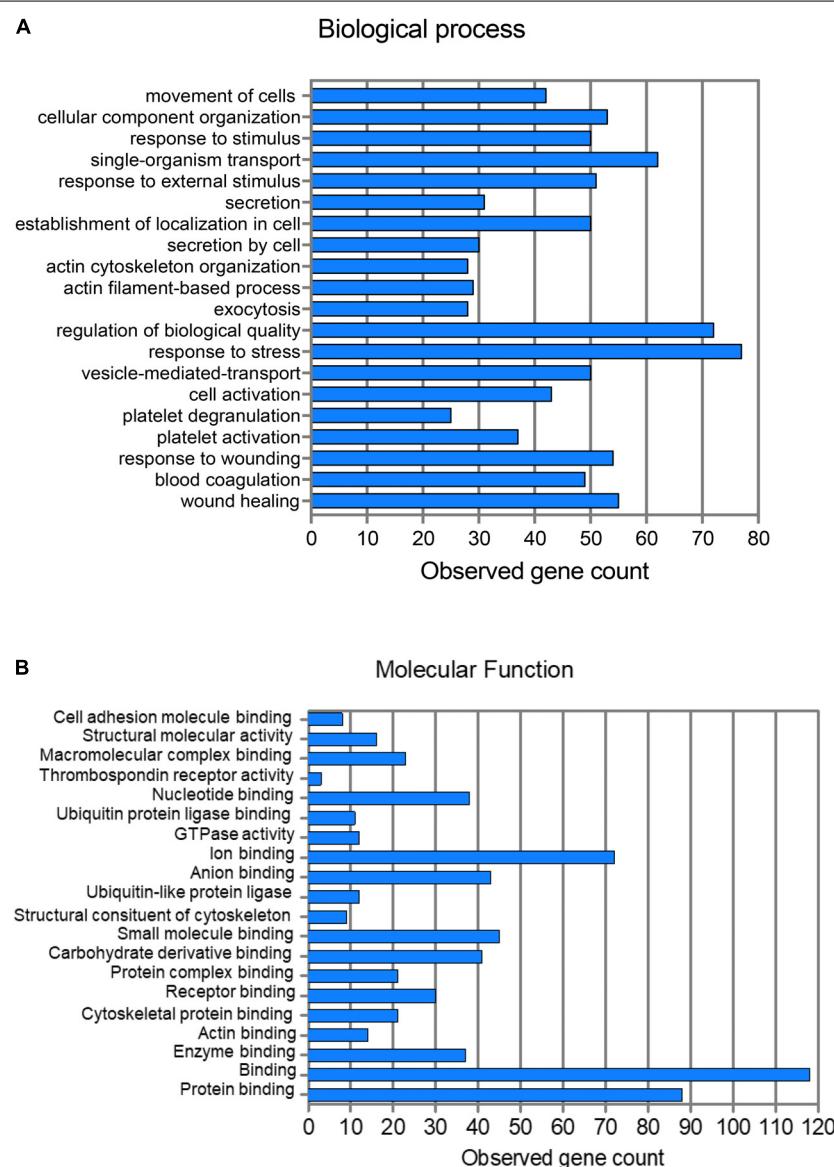


their cellular component, molecular function, and biological process using Ingenuity Pathway Analysis (IPA) [QIAGEN (Redwood City, CA)] or STRING Database (Version 10.5). The molecular activation prediction (MAP) algorithm in IPA was used to predict the upstream and downstream effects of activation and inhibition of associated network functions. STRING<sup>2</sup> was also used to generate protein-protein interaction networks, and the KEGG pathway enrichment analysis tool in PANTHER classification system was also applied to these networks. Finally, the STRING was used to validate IPA findings and provide unique perspectives based on each tool.

<sup>2</sup>www.string-db.org

## Effects of Platelet-Derived MPs on the Expression of Target Genes in Cancer and Endothelial Cells

The human colon carcinoma cell line HT29 and human cardiac microvascular endothelial cells (HCMEC) were purchased by ATCC (Milan, Italy) and Lonza (Milan, Italy), respectively, and cultured following the manufactory's instructions. HT29 or HCMEC cells ( $0.25 \times 10^6$ ) were incubated for 24 h with MPs ( $0.25 \times 10^8$ ) generated from thrombin activated platelets of obese and non-obese individuals. MPs were assessed for the capacity to cause changes in the expression of marker genes of EMT and EndMT when incubated with HT29 cells and HCMEC, respectively (Dovizio et al., 2013). Finally, MPs were assessed for the capacity to induce endothelial COX-2 expression



**FIGURE 3 |** Pathway analysis of 214 proteins showing the top 20 biological processes (A) and molecular functions (B).

(Dovizio et al., 2013). mRNA levels were evaluated by qPCR as previously described (Dovizio et al., 2013).

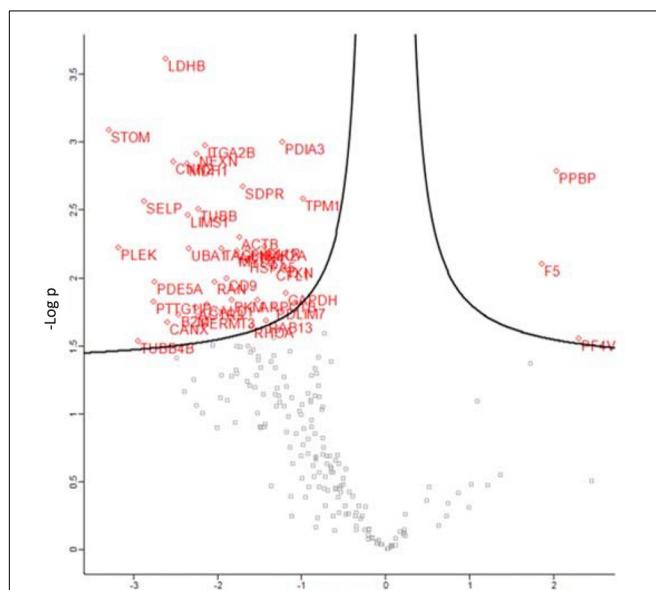
## Statistical Analysis

All data are reported as mean  $\pm$  SD unless otherwise stated. Statistical analysis was performed by using GraphPad Prism Software (version 5.00 for Windows; GraphPad, San Diego, CA, United States). Student's *t*-test was used to compare the means of two independent groups to each other; instead, one-way analysis of variance followed by Newman-Keuls post-test was used to compare the means of more than two independent groups. Thus, were considered statistically significant *P*-values  $<0.05$ .

## RESULTS

Washed platelets were isolated from the whole blood (Dovizio et al., 2013) of four obese individuals and as many non-obese controls. The cellular suspension contained predominantly platelets [98.24  $\pm$  1.10% (mean  $\pm$  SD)]. MPs, released from washed platelets activated with thrombin (1 IU/mL), were collected. The total platelet-derived MP count per  $\mu$ L, assessed by cytofluorimeter, was not significantly different in non-obese and obese individuals (19608  $\pm$  9236 vs. 24259  $\pm$  5796, respectively).

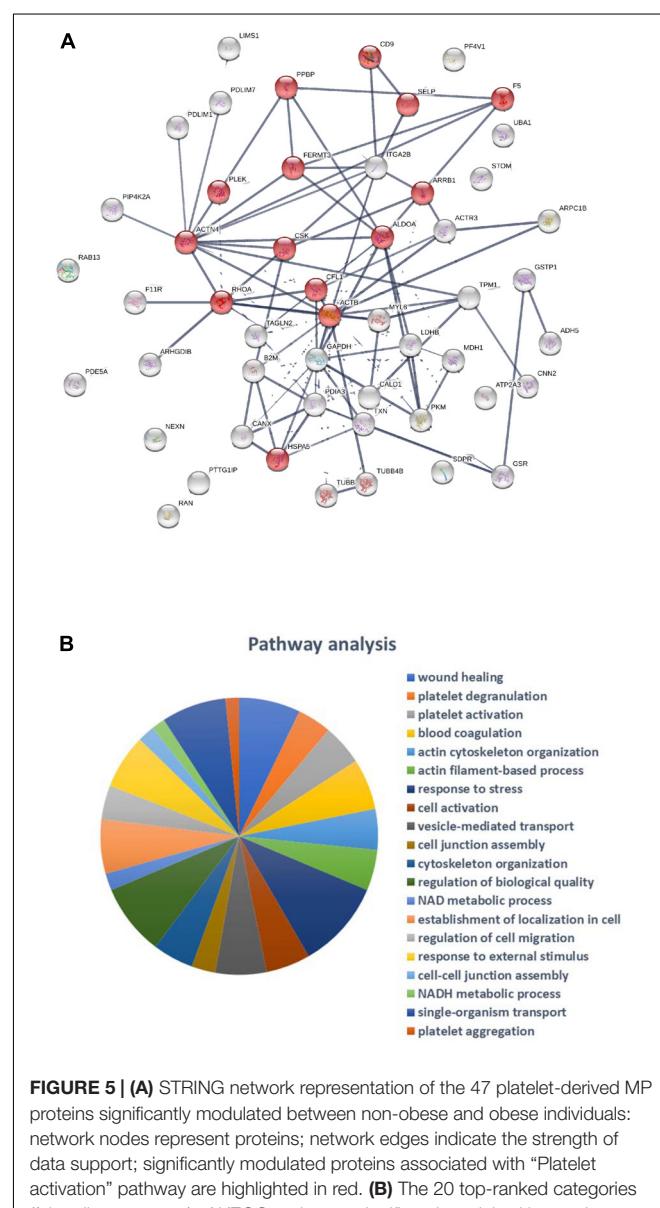
We studied the biophysical light scatter properties of MPs obtained in the two groups using cytofluorimeter. The density plot of side scatter (SSC) vs. forward scatter (FSC) of a typical MP suspension from non-obese and obese individuals (Figures 1A,B, respectively) showed a different size distribution between the



**FIGURE 4 |** Volcano plot displaying the 47 differential expressed proteins between obese (OB) and healthy control (HC) platelet-derived MPs; the y-axis corresponds to the mean expression value of  $\log_{10}$  (*p*-value), and the x-axis displays the difference values (OB-HC), the red dots represent the differentially expressed proteins (*P*  $< 0.05$ ), and the gray dots represent the proteins whose expression levels did not reach statistical significance (*P*  $> 0.05$ ).

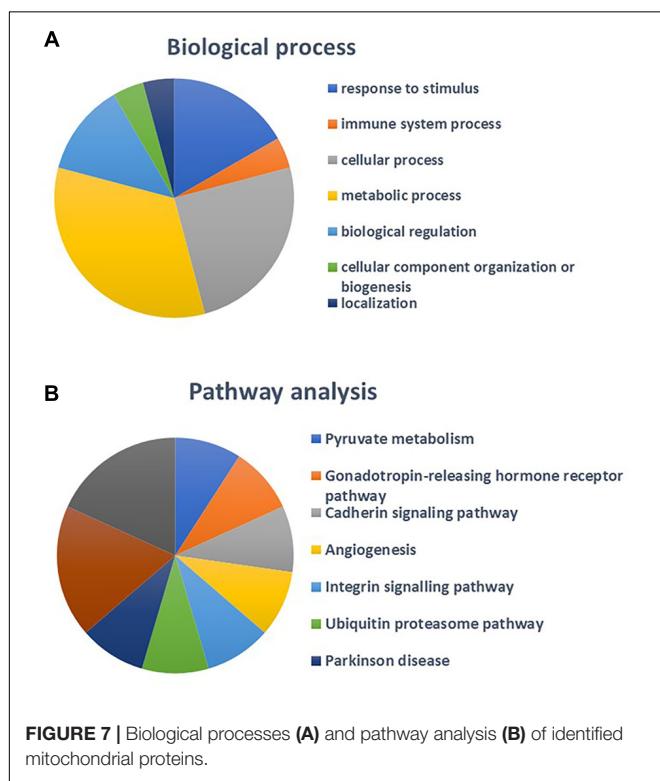
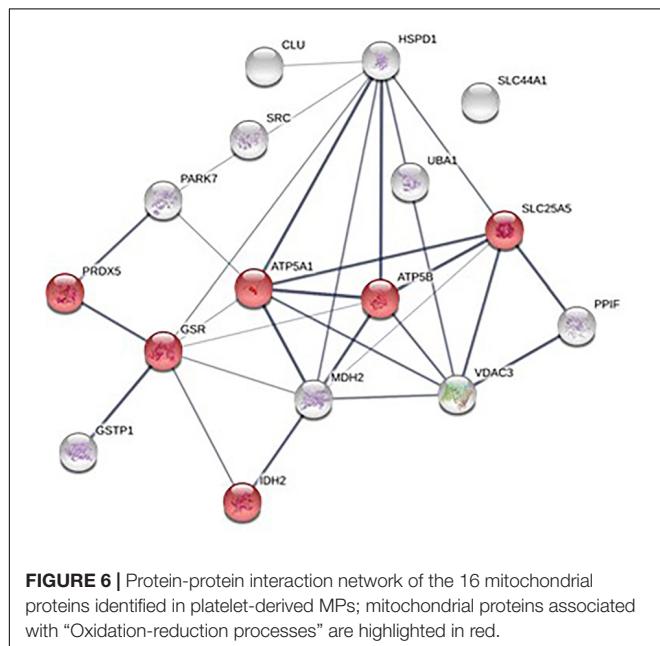
two groups. Interestingly, in the obese individuals, MPs with size  $>240$  nm were detected (Figures 1A,B). Platelet MPs from obese individuals had a significantly higher SSC and FSC signal intensity than non-obese individuals (Figures 1C,D, respectively).

We characterized the proteomic profile of MPs generated from thrombin activated platelets of non-obese and obese individuals. Thus, proteins from MPs were digested and analyzed by LC-MS/MS. In total, we identified 214 proteins in MPs. In Supplementary Table 1, the list of proteins identified in MPs is reported. Statistical analysis identified 47 proteins significantly modulated between the two groups (44 were downregulated while three were upregulated in MPs of obese vs. non-obese) (Supplementary Table 2). A further three proteins were detected



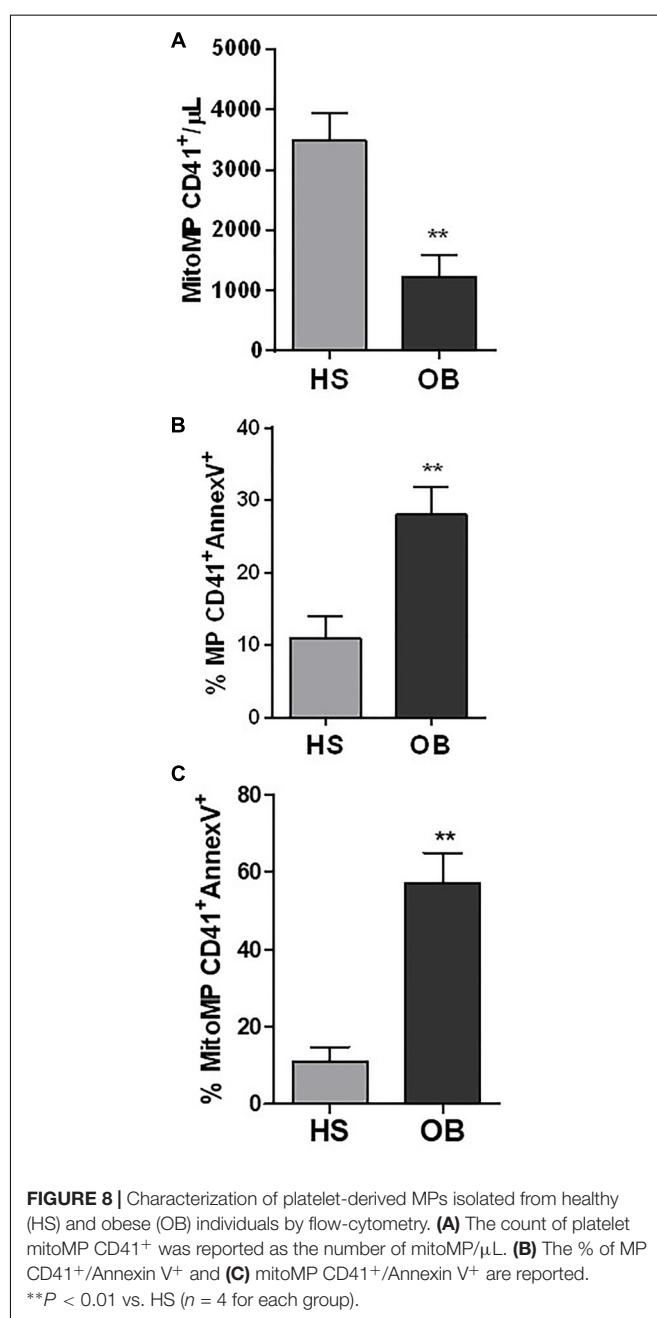
**FIGURE 5 | (A)** STRING network representation of the 47 platelet-derived MP proteins significantly modulated between non-obese and obese individuals: network nodes represent proteins; network edges indicate the strength of data support; significantly modulated proteins associated with "Platelet activation" pathway are highlighted in red. **(B)** The 20 top-ranked categories (false discovery rate) of KEGG pathways significantly enriched in our dataset.

only in MPs released from thrombin-stimulated platelets of obese individuals [ubiquitin like modifier activating enzyme 1 (UBA1), glutathione reductase, mitochondrial (GSR) and tyrosine-protein kinase (CSK)], while two proteins were present only in the MPs from non-obese individuals [calnexin (CANX) and cGMP-specific 3,5-cyclic phosphodiesterase (PDE5A)] (**Supplementary Table 2**).



A network analysis of all proteins was determined using the STRING database (**Figure 2**). Biological process and molecular function terms associated with the MP proteins are reported in **Figures 3A,B**, respectively. Many biological processes were associated with platelet activation and degranulation (**Figure 3A**).

Classification of the 47 modulated proteins was performed by STRING database and KEGG pathway enrichment analysis. The results showed that the proteins mapped to platelet functions, such as platelet activation and degranulation, and blood coagulation, but also to the regulation of cell migration, wound healing and vesicle-mediated transport (**Figures 4, 5A,B**). Moreover, 20 top-ranked categories of KEGG pathways



significantly enriched in our dataset were associated with different platelet functions (**Figure 5B**).

The number of MPs positive for mitochondria (mitoMPs) generated from thrombin-activated platelets was comparable in both groups ( $4384 \pm 1497$  and  $5867 \pm 4441$  number/ $\mu\text{L}$ , respectively). They represented the  $23.30 \pm 16.41$  and  $25.38 \pm 8.08$  %, respectively, of total MP population. Interestingly, 16 mitochondrial proteins were identified in our proteomic analysis of platelet-derived MPs (**Supplementary Table 1**). Pathway analysis performed using STRING database showed that the identified mitochondrial proteins mapped to regulation of integrin signaling pathway, angiogenesis, and pyruvate metabolism (**Figures 6, 7A,B**). Among the mitochondrial proteins, two were detected only in obese MPs (UBA1, GSR) whereas one was downregulated in MPs from obese vs. non-obese individuals (glutathione S-transferase pi 1, GSTP1) (**Supplementary Table 2**).

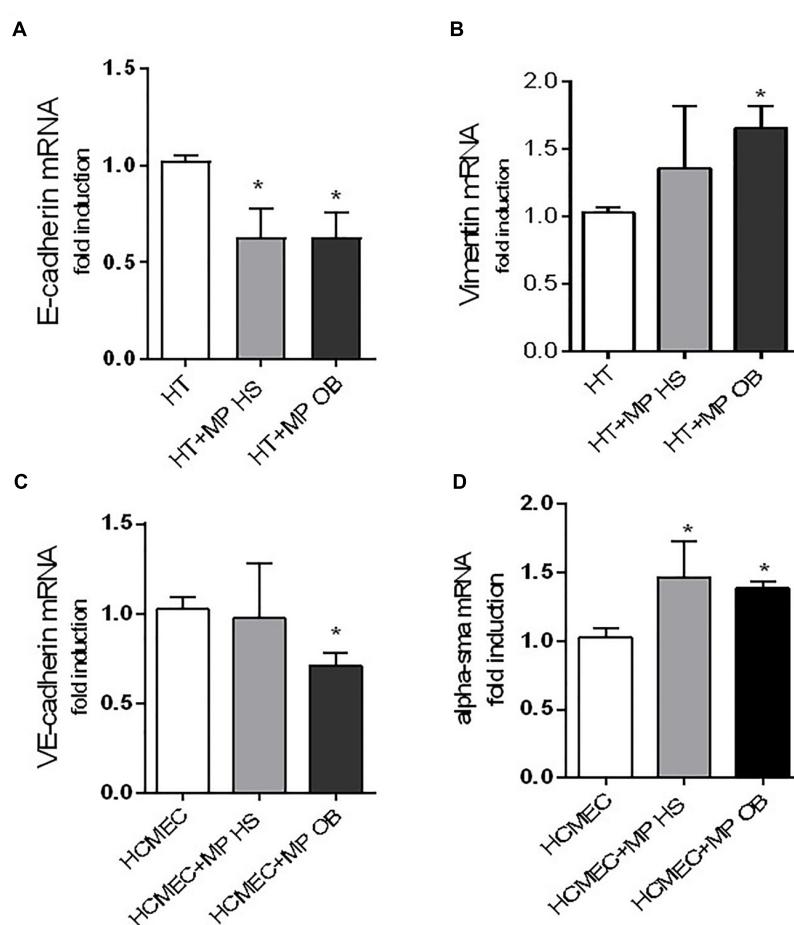
It is noteworthy the fact that two major glycolytic enzymes were among the 44 reduced proteins, lactate dehydrogenase B (LDHB, a subunit of lactate dehydrogenase enzyme;  $P = 0.00024$ ;

fold-change, 0.162) and pyruvate kinase muscle isozyme (PKM) ( $P = 0.0145$ ; fold-change, 0.281) (**Supplementary Table 2**).

In obese MPs, reduced levels of P-selectin (gene name, SELP) ( $P = 0.003$ , fold-change, 0.136) and CD41 (i.e., integrin subunit alpha 2b; gene name ITGA2B) ( $P = 0.001$ ; fold-change, 0.225) were found (**Supplementary Table 2**).

Using flow cytometry, the number of mtoMP CD41 $^{+}$  were lower in obese ( $1231 \pm 727.9$  number/ $\mu\text{L}$ ) vs. non-obese individuals ( $3486 \pm 1021$  number/ $\mu\text{L}$ ) (**Figure 8A**) ( $P < 0.01$ ).

It is known that platelet-derived MPs can expose phosphatidylserine (PS) which in turn binds annexin V and that the annexin V-PS bond represents a true reflection of MP procoagulant activity (Connor et al., 2009). In non-obese and obese individuals, % of total MP CD41 $^{+}$  which binds annexin V was  $10.95 \pm 6.73$  vs.  $27.97 \pm 7.54$ %, respectively ( $P < 0.01$ ) (**Figure 8B**). MitoMP CD41 $^{+}$ annexinV $^{+}$  were  $10.88 \pm 9.56$  and  $56.99 \pm 16.01$ %, respectively ( $P < 0.01$ ) (**Figure 8C**). These results are consistent with the proteomic data showing that Factor V was upregulated in obese MPs vs. non-obese MPs ( $P = 0.008$ , fold-change, 3.635) (**Supplementary Table 2**).



**FIGURE 9 |** Effects of MPs on the expression of gene markers of EMT and EndMT. Co-culture experiments between colon adenocarcinoma cells HT29 ( $0.25 \times 10^6$ ) (**A,B**) or human coronary microvascular endothelial cells (HCMEC) ( $0.25 \times 10^6$ ) (**C,D**) and MPs ( $0.25 \times 10^8$ ) from obese (OB) and healthy (HS) individuals for 24 h were reported. Gene expression was evaluated by qPCR and normalized to those of GAPDH as control and expressed as fold-change. Data are reported as mean  $\pm$  SEM ( $n = 4$ , for each experimental condition); \* $P < 0.05$  vs. HT29 cultured alone or HCMEC cultured alone.

Also, we characterized the property of MPs generated from obese and non-obese thrombin-activated platelets for their property to alter the expression of molecular markers involved in EMT, a key process mediating the progression of malignant tumors (Kalluri and Weinberg, 2009). As shown in **Figure 9A**, platelet MPs of both groups incubated with HT29 cells caused a significant downregulation of expression levels of E-cadherin, a typical epithelial marker. This effect was associated with an increase in the mesenchymal marker vimentin, which was significant only with MPs isolated from obese platelets (**Figure 9B**).

Microparticles of both groups were studied for the capacity to alter the expression profile of marker genes of EndMT in HCMC. Only MPs obtained from obese individuals caused a significant downregulation of VE-cadherin in HCMC (a typical endothelial marker) (**Figure 9C**). In contrast,  $\alpha$ -SMA was significantly upregulated by MPs derived from both groups (**Figure 9D**).

IPA analysis of the 47 modulated proteins showed that some processes enriched in our proteomic analysis might regulate apoptosis and cell death signaling (**Figure 10A**).

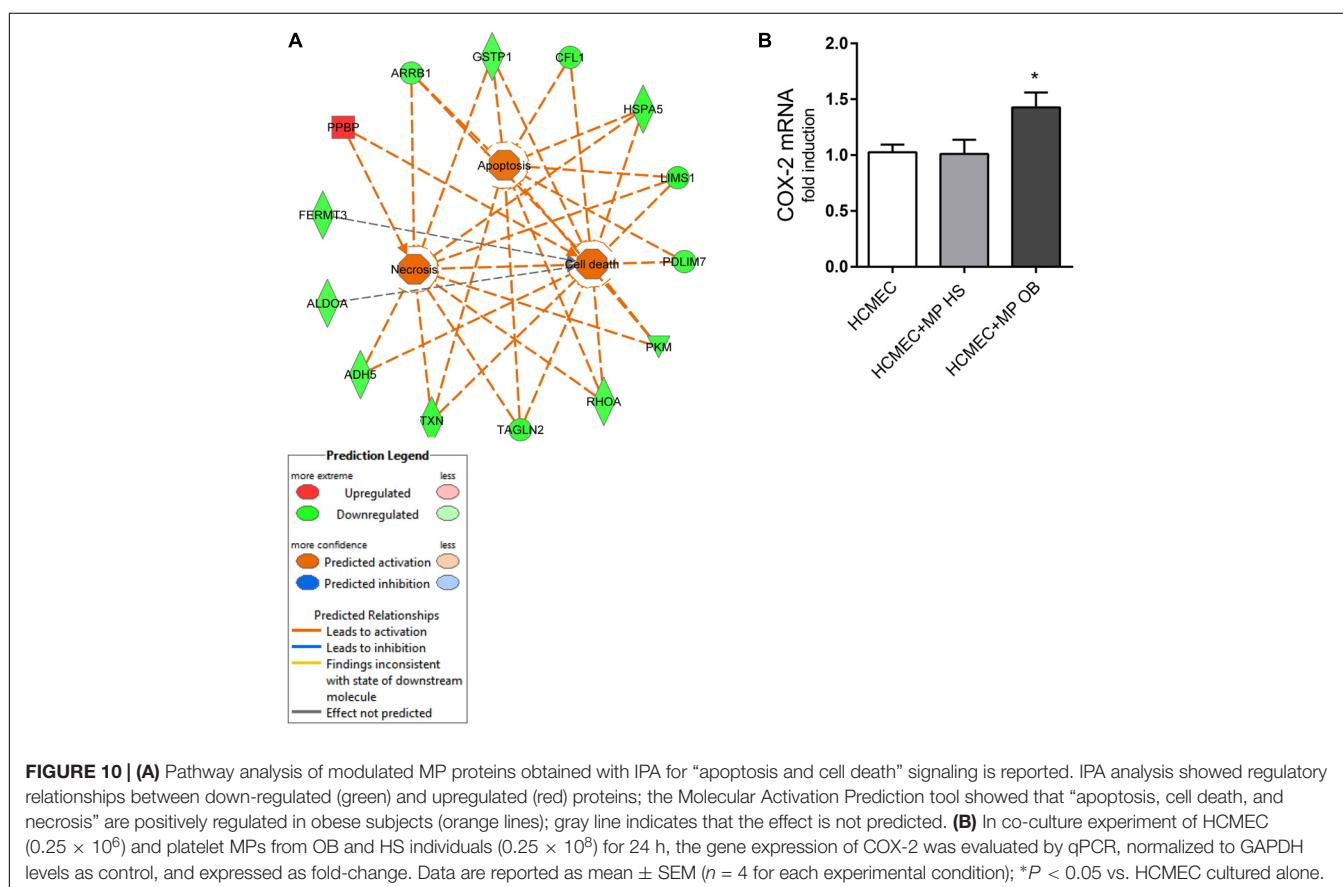
Finally, we assessed the effect of MPs to induce the pro-inflammatory and pro-angiogenic gene COX-2 (Wang and DuBois, 2004) in endothelial cells (**Figure 10B**). Platelet MPs from obese individuals induced COX-2 while MPs from non-obese did not.

## DISCUSSION

In the present study, we aimed to verify whether obesity influences the number, size and proteome of MPs generated *in vitro* from platelets in response to thrombin. Moreover, we studied the capacity of platelet-derived MPs to influence the expression of marker genes of EMT and EndMT and COX-2 *in vitro*.

We found that MPs, released from activated platelets of obese individuals, were not different in number as compared with non-obese controls, but were characterized by greater heterogeneity in size distribution. However, in obese women, the count of mitoMPs positive for CD41 was significantly lower. This finding can be explained by the fact that in obesity a strong platelet activation associated with enhanced oxidative stress occurs (Santilli et al., 2011). These events may lead to the alteration of mitochondrial functions and proteolytic cleavage of proteins.

Proteomics data showed reduced levels of pyruvate kinase (PKM) in obese MPs, which is a regulator of mitophagy (i.e., the process of the removal of damaged mitochondria) via enhanced pyruvate formation (Park et al., 2015). A defect in platelet mitophagy response has been described in diabetes (Lee et al., 2016) and may lead to increased thrombosis in response to oxidative stress. The mitochondrial protein, GSTP1 was also



reduced in obese MPs vs. non-obese MPs. This protein plays an important role in antioxidant defenses (Meiers, 2010).

Platelet MPs obtained from obese individuals had reduced levels of the  $\alpha$ -granular transmembrane (TM) proteins P-selectin (SELP) and stomatin (gene name, STOM). Two other TM proteins found on  $\alpha$ -granule membranes had decreased expression on obese MPs, i.e., alpha-IIb (gene name, ITGA2B) and CD9 antigen (gene name, CD9). These changes may reflect an alteration in membrane fluidity in obesity leading to a biological modification in platelet and MP membranes (Tangorra et al., 1988; Cazzola et al., 2011). Interestingly, another transmembrane protein the junctional adhesion molecule A (gene name, F11R), which functions as an endogenous inhibitor of platelet function (Naik et al., 2011), was also reduced in obese MPs.

Microparticles from platelets of obese individuals presented three proteins that were not detectable in non-obese MPs. Among them, there is UBA1 which catalyzes the first step in ubiquitin conjugation to mark cellular proteins for degradation through the ubiquitin-proteasome system (Ciechanover and Schwartz, 1998). Its presence in obese MPs might play a role in the reduced levels of many proteins detected vs. MPs of non-obese individuals.

Soluble megakaryocyte-derived  $\alpha$ -granule components were increased in obese MPs, including coagulation Factor V (gene name, F5) and pro-platelet basic protein (gene name, PPBP). Factor V functions as a membrane-bound cofactor and plays an essential role in hemostasis through its profound influence on the production of thrombin (Camire, 2010). Enhanced content of Factor V, together with increased exposure of membrane PS in the MP of obese individuals, may account for the pro-thrombotic risk associated with obesity. Interestingly, in MPs of obese individuals, high levels of PPBP (also known as CXCL7) were found. PPBP is the precursor of platelet basic protein (PBP), a platelet-derived growth factor stored in platelet  $\alpha$ -granules and is a potent chemoattractant and activator of neutrophils. CXCL7 mediates different effects through its G-protein-coupled receptors CXCR-1 and CXCR-2, which activate the ERK and PI3 kinase pathways (Grépin et al., 2014). The activation of these receptors expressed in HT29 cells (Desurmont et al., 2015) and endothelial cells (Grépin et al., 2014) might contribute to EMT and EndMT and cellular migration.

Another protein highly upregulated in MPs from obese individuals is a variant form of platelet factor 4 (PF4 variant 1/CXCL4L1, gene name, PF4V1) (**Supplementary Table 2**). It is a potent inhibitor of angiogenesis (Sarabi et al., 2011) and may induce random endothelial cell migration (Sarabi et al., 2011) thus possibly contributing to EndMT.

Platelet-derived MPs from obese, but not from non-obese, individuals, induced COX-2 expression in HCMEC. This effect

might play a role in the obesity promotion of cancer, since COX-2-dependent prostaglandin (PG) E<sub>2</sub> induces angiogenesis at the earliest stage of tumor development (Wang and DuBois, 2004).

The limitations of this study are: (i) the small number of individuals analyzed; (ii) only women were studied; (iii) the effect of MPs on the expression of EMT marker genes was evaluated on HT29 cell line, not on primary cancer cells derived from patients; (iv) the impact of MPs on functional assays of EMT and EndMT was not studied.

The strength of this study is the development of a proteomics approach to determine the composition of MPs generated from activated platelets. Also, this study provides data on the variability of MP generation from activated platelets, such as number and size, in obese and non-obese individuals. This information will be helpful to design larger clinical studies, in this setting.

In conclusion, our results suggest that and the assessment of proteomics signature of MPs, generated from thrombin-activated platelets, can be suitable for monitoring the efficacy of lifestyle, pharmacologic, and surgical options in obesity. However, larger studies should be performed to validate our findings.

## AUTHOR CONTRIBUTIONS

PP, PBM, and GM conceptualized and designed the study. RG, MD, SM, PBS, HE, HC, PL, MM, and AC performed the data acquisition, analysis, or interpretation of data. PP, PBM, MD, and RG drafted the manuscript. AB and FN critically revised the manuscript for important intellectual content. All authors provided approval for publication of the content, and agreed 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.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2019.00007/full#supplementary-material>

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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.

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