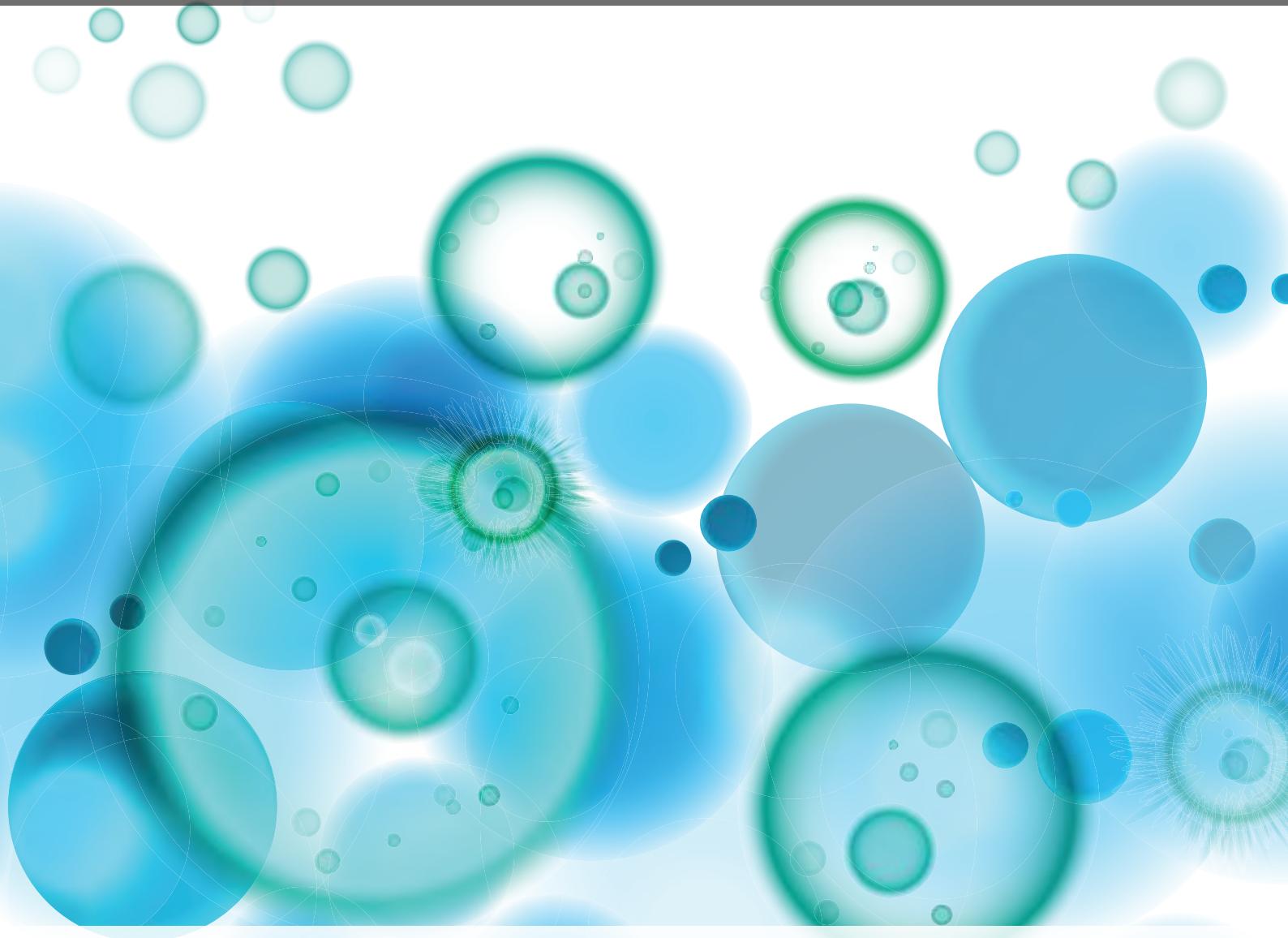
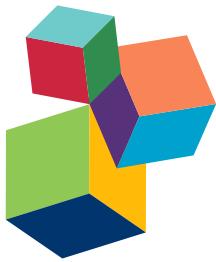


CD1- AND MR1-RESTRICTED T CELLS IN ANTIMICROBIAL IMMUNITY

EDITED BY: S.M. Mansour Haeryfar and Thierry Mallevaey
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CD1- AND MR1-RESTRICTED T CELLS IN ANTIMICROBIAL IMMUNITY

Topic Editors:

S.M. Mansour Haeryfar, Western University, Canada

Thierry Mallevaey, University of Toronto, Canada

Cell-mediated immunity to extracellular and intracellular microbes has been traditionally linked to CD4⁺ and CD8⁺ T cells that recognize pathogen-derived peptides in the context of major histocompatibility complex (MHC) class II and class I molecules, respectively. Recent progress in our understanding of early host defense mechanisms has brought ‘unconventional’, innate-like T cells into the spotlight. These are a heterogeneous population of non-MHC-restricted T cells that exhibit ‘memory-like’ properties and mount emergency responses to infection. They may directly detect and destroy infected cells, but are best known for their ability to regulate downstream effector cells including but not limited to conventional T cells. Innate-like T cells include among others CD1-restricted natural killer T (NKT) cells and MR1-restricted mucosa-associated invariant T (MAIT) cells. NKT cells recognize lipid antigens, and MAIT cells were recently demonstrated to respond to microbe-derived vitamin B metabolites. However, much remains to be learned about the antigen specificity range of these cells, their activation mode and their true potentials in immunotherapeutic applications. Like in many other areas of biology, uncertainties and controversies surrounding these cells and some of the experimental models, techniques and reagents employed to study them have brought about excitement and sometimes hot debates. This Special Topic was launched to provide updated reviews on protective and/or pathogenic roles of NKT and MAIT cells during infection. Leading experts discuss current controversies, pressing questions and the challenges that lie ahead for the advancement of this intriguing and rapidly evolving area of immunology. Unlike MHC, CD1 and MR1 display very limited polymorphism. Therefore, NKT and MAIT cells may be considered attractive targets for various diseases in diverse human populations. The potential benefits of NKT cell- and MAIT cell-based vaccination and treatment strategies in infectious diseases is an important subject that is also covered in this Topic.

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Editorial: CD1- and MR1-Restricted T Cells in Antimicrobial Immunity

S.M. Mansour Haeryfar^{1,2,3,4*} and Thierry Mallevaey⁵

¹ Department of Microbiology and Immunology, Western University, London, ON, Canada, ² Division of Clinical Immunology and Allergy, Department of Medicine, Western University, London, ON, Canada, ³ Centre for Human Immunology, Western University, London, ON, Canada, ⁴ Lawson Health Research Institute, London, ON, Canada, ⁵ Department of Immunology, University of Toronto, Toronto, ON, Canada

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The main function of the immune system is to protect the host against microbial pathogens and their deleterious products. Innate defense mechanisms quickly eliminate infectious intruders or keep them in check until highly specific adaptive responses that also give rise to immunological memory are launched. Major histocompatibility complex (MHC)-restricted T cells are key players of adaptive immunity. The remarkable diversity of their T cell receptors (TCRs) allows for specific recognition of peptides derived from virtually all protein antigens (Ags) including those harbored or even modified by the most vicious pathogens encountered over one's lifetime. Conventional T cells sense and respond to pathogen-derived peptides complexed with polymorphic MHC molecules. This is called the rule of MHC restriction (1).

Recent years have witnessed a growing appreciation for effector and regulatory functions of innate-like T cells that are restricted by non-polymorphic, MHC-like molecules. These include CD1-restricted T cells [e.g., natural killer T (NKT) cells] and MR1-restricted mucosa-associated invariant T (MAIT) cells, which are the subjects of discussion by leading experts in this Research Topic. These "unconventional" T cells may directly target infected cells, but are best known for their ability to swiftly secrete T helper 1 (Th1)-, Th2-, and/or Th17-type cytokines very early in the course of immune responses. These cytokines in turn modulate the function of numerous cell types including NK cells, macrophages, dendritic cells, conventional CD4⁺ and CD8⁺ T cells and B cells, all of which play critical roles in innate or adaptive antimicrobial immunity.

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Kendall Arthur Smith,
Weill Medical College of Cornell
University, USA

*Correspondence:

S.M. Mansour Haeryfar
mansour.haeryfar@schulich.uwo.ca

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CD1 molecules appeared around 300 million years ago. They display a high degree of conservation across vertebrates (2). However, considerable inter-species variation exists in terms of the number of CD1 isoforms expressed. In humans, the CD1 locus encodes five isoforms (i.e., CD1a–e), whereas rodents express only CD1d (3, 4). With the sole exception of CD1e, CD1 molecules are cell surface glycoproteins directly participating in lipid Ag presentation to T cells. CD1a–c, which comprise Group 1 CD1 molecules based on their genomic homology and location, have been a focus of many studies on host responses to *Mycobacterium tuberculosis* lipids (5). By contrast, certain self-lipids and exogenous glycolipids derived from a relatively wide spectrum of pathogens can be presented to NKT cells by CD1d, which is considered the Group 2 CD1 molecule. Positive selection of both type I and type II NKT cells in the thymus also requires their interaction with CD1d (6). These cell types are commonly referred to as invariant NKT (*i*NKT) and variant NKT (*v*NKT) cells, respectively. The discovery of *i*NKT cell restriction by CD1d and the ability of these cells to recognize α -galactosylceramide (α -GalCer) (7) prompted the invention of α -GalCer-loaded CD1d tetramer reagents (8, 9). This can be viewed as a technical breakthrough that has allowed for accurate identification and functional analysis of *i*NKT cells in mice and humans. Subsequently, CD1d tetramers loaded with the myelin-derived glycolipid sulfatide were generated and employed to identify a substantial fraction of *v*NKT cells (10). Of note, CD1d-restricted, sulfatide-reactive $\gamma\delta$ T cells have also been described (11, 12). To what extent CD1d-restricted $\gamma\delta$ T cells may contribute

to the resolution of infection is not clearly understood. $\gamma\delta$ T cells are not a major focus of this Topic, but have been introduced and briefly discussed (13, 14).

*i*NKT cells are perhaps the most widely studied population of CD1-restricted T cells. They are relatively infrequent in circulation and in most lymphoid and non-lymphoid tissues. However, they amass in the mouse liver and in the human omentum (15), which was dubbed the “abdominal policeman” in 1906 (16). *i*NKT cells express NK cell markers along with a canonical TCR consisting of an invariant α chain (V α 14-J α 18 in mice and V α 24-J α 18 in humans) and one of the only few β chain choices, namely mouse V β 8.2, V β 2, or V β 7 and human V β 11. Positive selection of *i*NKT cells is executed by CD1d $^+$ CD4 $^+$ CD8 $^+$ thymocytes (6). However, endogenous CD1d ligand(s) involved in *i*NKT cells’ thymic selection have been elusive. KRN7000, an exogenous glycolipid superagonist of *i*NKT cells, was first extracted from the sea sponge *Agelas mauritanicus* in a screen for novel antitumor compounds (7, 17). It has a unique α -GalCer structure and is likely to have originated from microbes forming a symbiotic relationship with *A. mauritanicus*. Up until a short time ago, α -GalCer was considered unnatural to mammals. However, a recent study documented the presence of α -anomeric glycosylceramides including α -GalCer, in minute quantities, within mammalian cells, which could serve as endogenous *i*NKT cell Ags (18). α -GalCer has been utilized as a powerful experimental tool in many mouse studies and as a therapeutic agent in several clinical trials for cancer and viral diseases (19, 20).

*i*NKT cells are among first-line emergency responders to microbes. They quickly accumulate at the sites of infection, injury or inflammation to aid in mobilization and activation of other immune cells (21). When infection alters *i*NKT cell numbers within a given tissue, it is informative to distinguish between their recruitment into the tissue and their *in situ* expansion, retention or redistribution. Intravital imaging techniques have permitted the visualization and monitoring of *i*NKT cells’ behavior in live animals (22, 23). They have revealed, for instance, that shortly after infection with *Borrelia burgdorferi*, the causative agent of Lyme disease, hepatic mouse *i*NKT cells cease to crawl along the liver sinusoids, but instead form clusters and establish firm adhesion with Kupffer cells that have engulfed the blood-borne spirochete (24). By contrast, within the joints, extravascular *i*NKT cells are not stationary and move along blood vessel walls toward *B. burgdorferi* (25). This is followed by a direct interaction with the bacterium and its elimination. There currently exists an unmet need to track and quantify CD1d-mediated presentation of pathogen-derived lipids and to investigate the characteristics of the immunological synapses formed between CD1d $^+$ Ag-presenting cells (APCs) and *i*NKT cells during infection. As such, antibodies to CD1d:glycolipid complexes and soluble *i*NKT cell TCR reagents similar to those engineered before (26, 27) may prove valuable.

Lipid Ags that can be bound to CD1d and directly detected by *i*NKT cells are present in a number of pathogenic or commensal bacteria or protozoan parasites (21, 28, 29). *Sphingomonas* spp., *Bacteroides fragilis*, *B. burgdorferi*, *Helicobacter pylori*, *Streptococcus pneumoniae*, *Streptococcus agalactiae*, *Leishmania donovani* and *Entamoeba histolytica* are examples of such microorganisms.

Viruses do not possess lipid ligands for *i*NKT cell TCRs. However, infection with some viruses (e.g., dengue virus) leads to upregulated CD1d expression consistent with *i*NKT cell activation while certain others (e.g., herpesviruses and HIV) downregulate CD1d to plausibly evade detection by *i*NKT cells (30, 31). Viral infection may also induce a shift in host cell lipid profiles, thus yielding more “antigenic” CD1d ligands (32). The presence of CD1d:self-lipid complexes is also often required for cytokine-mediated stimulation of *i*NKT cells during bacterial and viral infections. This typically occurs shortly after microbial components engage APCs’ Toll-like receptors and induce interleukin (IL)-12 and IL-18 secretion (13, 33). Finally, *i*NKT cells can be activated in a truly CD1d-independent fashion, for instance by a combination of IL-12 and IL-18 (34) or by group II superantigens of staphylococcal and streptococcal origin (35, 36).

Both protective and pathogenic roles for *i*NKT cells have been reported in infectious disease models. Perhaps even more intriguing, *i*NKT cell activation could have seemingly contradictory consequences during infection with different species of the same pathogen (e.g., *Chlamydia* spp.) (37). CD1d $^{-/-}$ and J α 18 $^{-/-}$ mice have been used extensively to address the contributions of *i*NKT cells in infection and immunity. It is noteworthy that CD1d $^{-/-}$ mice are devoid of not only *i*NKT cells but also ν NKT cells. Moreover, Bedel et al. found that the cellular deficiency of the original J α 18 $^{-/-}$ mice is more broad than initially thought and that the TCR α repertoire in these mice is shrunk by ~60% (38). Therefore, except in cases where CD1d $^{-/-}$ or J α 18 $^{-/-}$ mice have been reconstituted with *i*NKT cells as a confirmatory measure, it may be necessary to revisit earlier findings in these animals. This is now possible thanks to the recent development of a new version of J α 18 $^{-/-}$ mice with an exclusive *i*NKT cell deficiency (39) and a monoclonal antibody that can selectively deplete *i*NKT cells *in vivo* (40).

The role of *i*NKT cells in infection is dictated, at least partially, by the pro- versus anti-inflammatory dominance of the cytokines they produce. For instance, interferon (IFN)- γ -secreting *i*NKT cells contribute to the immunopathology of sepsis in the aftermath of polymicrobial infection, which can be therapeutically attenuated by Th2-skewing glycolipids (41, 42). α -GalCer analogs with Th1-polarizing properties have also been synthesized and may be considered as adjuvant candidates in preventative vaccination (43) and in immunotherapy of infectious diseases that can be potentially resolved by Th1-dominant responses (44).

ν NKT cells, as indicated by their name, have a relatively heterogeneous $\alpha\beta$ TCR repertoire (45). They are present in mice but more prominent in humans (14, 46). Unlike *i*NKT cells, ν NKT cells are unresponsive to α -GalCer. A large sub-population of ν NKT cells react with sulfatide, a self-glycolipid that is abundant within the central nervous system, liver, kidney, and pancreas. Several endogenous lipids other than sulfatide have been found to activate ν NKT cells, suggesting that ν NKT cells’ recognition mode can be both specific and somewhat promiscuous. Therefore, it is not unreasonable to assume that self-lipids released from infected or damaged cells or even microbial lipids cross-reactive with self-components may be presented by CD1d to elicit ν NKT cell responses. Potent immunomodulatory cytokines secreted by ν NKT cells mediate protection from or pathology associated with

infection (14, 46). They also enable *v*NKT cells to establish cross-talk with other cell types including but not limited to *i*NKT cells. In fact, *v*NKT and *i*NKT cells may fulfill opposing functions during infection, as exemplified by parasitic infections of mice with *Trypanosoma cruzi* (47) and *Schistosoma mansoni* (48). The limited availability of reliable reagents and tools for *v*NKT cell studies constitutes a hurdle in delineating the significance of these cells in infectious diseases (14). Sulfatide-loaded CD1d tetramers are not sufficiently stable nor do they stain all *v*NKT cells. The advent of 24 $\alpha\beta$ mice, a transgenic mouse line carrying the rearranged V α 3.2/V β 9 TCR of a *v*NKT cell hybridoma, shed light on certain aspects of *v*NKT cell biology and development (49). More recently, J α 18-deficient, IL-4 reporter (J α 18 $^{-/-}$ 4get) mice were employed to characterize *v*NKT cells at a polyclonal level (50). These mice were found to be responsive to multiple lipid Ags but not to sulfatide and several phospholipids. Therefore, until new models become available, we will continue to depend on these models and on parallel examination of CD1d $^{-/-}$ and the exclusively *i*NKT cell-deficient version of J α 18 $^{-/-}$ mice to study *v*NKT cell responses *in vivo*.

Mucosa-associated invariant T cells are one of the hottest topics in immunology today (51, 52). They express an invariant TCR α chain (mouse V α 19-J α 33 and human V α 7.2-J α 33) (53, 54), and undergo positive selection by MR1 $^{+}$ CD4 $^{+}$ CD8 $^{+}$ thymocytes (55). Similar to CD1, MR1 is conserved among diverse mammalian species. MAIT cells preferentially home to mucosal tissues – hence their denomination. In human, they circulate at high frequencies in the blood and also make up ~50% of the entire hepatic T cell population. MAIT cells are absent from the peripheral tissues of germ-free mice (55), indicating a strict requirement for commensal microflora in MAIT cell homeostasis. It has been hypothesized that gut dysbiosis in diseased states (e.g., type 1 and type 2 diabetes) may change MAIT cell frequencies and functions with metabolic and inflammatory repercussions (29). MAIT cells are rare in wild-type mice, and V α 19 transgenic mice were generated to circumvent the feasibility limitations of mouse studies. Although several differences have been reported between mouse and human MAIT cell compartments, recent work suggests that MAIT cells from wild-type mice resemble their human counterparts more closely than previously appreciated (56).

MR1 tetramers loaded with reduced 6-hydroxymethyl-8-D-ribityllumazine, a MAIT cell Ag, were recently developed to enable positive identification of mouse and human MAIT cells (57). The vitamin B2 (riboflavin) biosynthesis pathway supplies MAIT cell ligands (58–60). Importantly, this pathway operates in microbes that activate MAIT cells, but not in mammals. However, host-derived metabolites may potentially form adducts with intermediates of the riboflavin pathway to generate MAIT cell

neo-antigens (59). MR1 ligands are ubiquitous and harbored by many bacteria, including commensals. Therefore, how *in vivo* MAIT cell responses are controlled remains to be elucidated. Novel MR1 ligands that do not activate MAIT cells on their own but compete with bacterial and synthetic MAIT cell stimuli have been synthesized (60). This may inspire the development of MAIT cell inhibitors for experimental and therapeutic purposes.

MAIT cells can respond to numerous bacterial strains and yeasts (51, 52). These include *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Lactobacillus acidophilus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Candida glabrata*, *Candida albicans* and *Saccharomyces cerevisiae*. To test the *in vivo* significance of MAIT cells in anti-bacterial immunity, MR1 $^{-/-}$ mice have been utilized and shown to be unable to control infection with *K. pneumoniae*, *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) or *Francisella tularensis* (61–63). Last but not the least, MAIT cells can be activated by a combination of IL-12 and IL-18 in an MR1-independent manner (64), which may be important for antiviral defense.

Innate-like T cells are fast-acting and occupy strategic locations in the body. Unlike classical MHC molecules, CD1 and MR1 exhibit limited polymorphism. Therefore, it is only fitting that CD1 and MR1 ligands are considered by many as attractive targets for vaccination of genetically diverse human populations. Despite gaps in our knowledge in this exciting area, which are outlined by experts in this Topic, the availability of powerful tools, reagents and models has fueled further interest in CD1- and MR1-restricted T lymphocytes. α -GalCer has been used in clinical trials, and Th1- and Th2-promoting, disease/infection-tailored glycolipid agonists of iNKT cells may find their way into clinical practice in the future. Furthermore, it is not too far-fetched to anticipate that once the role of MAIT cells in various infectious diseases is known, their manipulation by synthetic ligands and inhibitors can be achieved and potentially used in immunotherapeutic protocols. The time is ripe for both curiosity-driven and translational studies on CD1- and MR1-restricted T cells.

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SMMH and TM served as co-editors for this Research Topic. SMMH wrote the Editorial.

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Role of innate T cells in anti-bacterial immunity

Yifang Gao^{1*} and Anthony P. Williams^{1,2}

¹ Academic Unit of Cancer Sciences, Faculty of Medicine and Institute for Life Sciences, University of Southampton and NIHR Cancer Research UK Experimental Cancer Medicine Centre, Southampton, UK, ² Wessex Investigational Sciences Hub (WISH) Laboratory, Department of Allergy, Asthma and Clinical Immunology, University Hospital Southampton NHS Foundation Trust, Southampton, UK

Innate T cells are a heterogeneous group of $\alpha\beta$ and $\gamma\delta$ T cells that respond rapidly (<2 h) upon activation. These innate T cells also share a non MHC class I or II restriction requirement for antigen recognition. Three major populations within the innate T cell group are recognized, namely, invariant NKT cells, mucosal associated invariant T cells, and gamma delta T cells. These cells recognize foreign/self-lipid presented by non-classical MHC molecules, such as CD1d, MR1, and CD1a. They are activated during the early stages of bacterial infection and act as a bridge between the innate and adaptive immune systems. In this review, we focus on the functional properties of these three innate T cell populations and how they are purposed for antimicrobial defense. Furthermore, we address the mechanisms through which their effector functions are targeted for bacterial control and compare this in human and murine systems. Lastly, we speculate on future roles of these cell types in therapeutic settings such as vaccination.

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Paul Kleinerman,
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Dieter Kabelitz,
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Germany

*Correspondence:

Yifang Gao,
Cancer Sciences Unit, Southampton
General Hospital, Somers Cancer
Research Building, Mail Point 824,
Hampshire, Southampton, UK
yg13g11@soton.ac.uk

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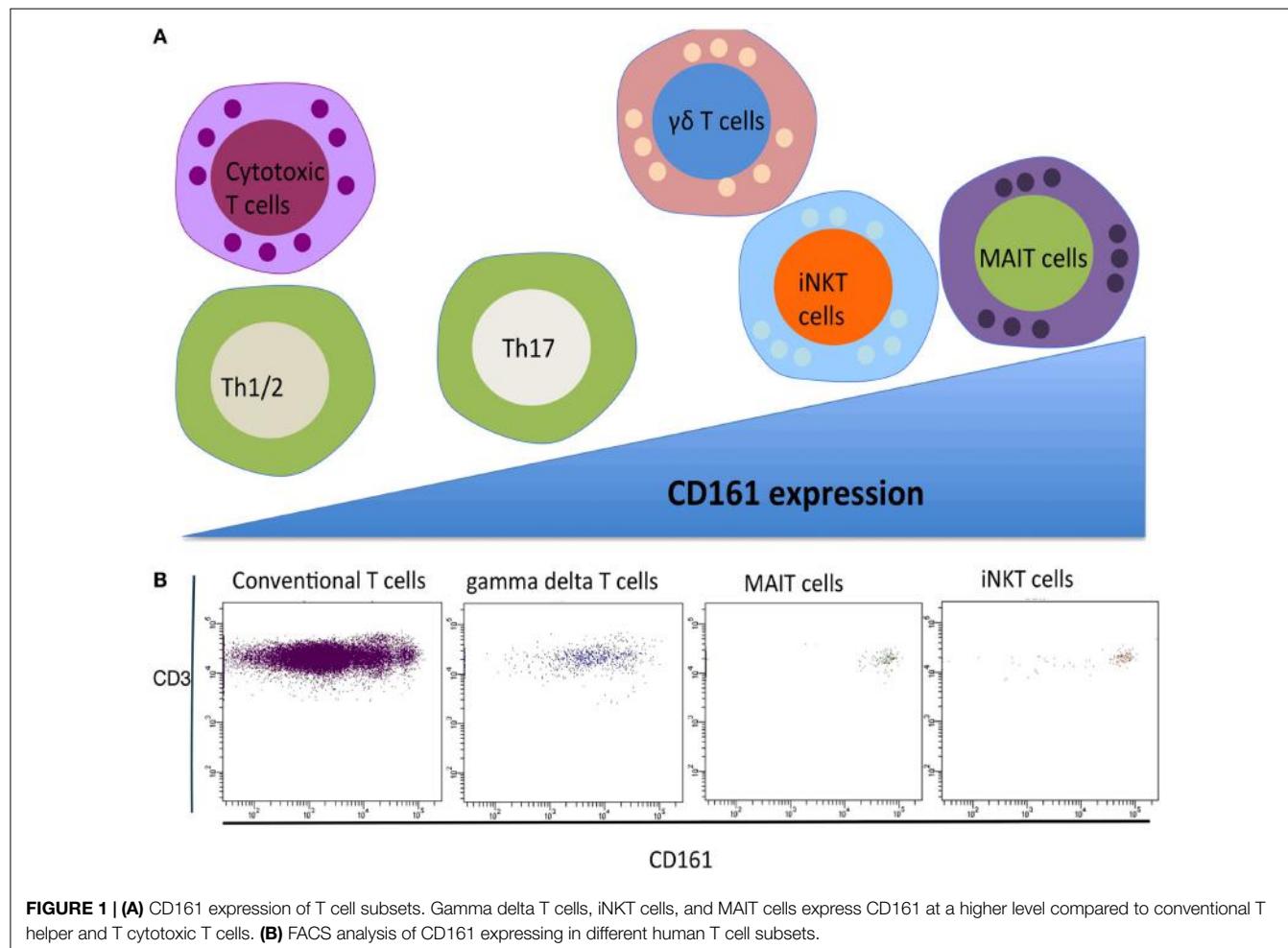
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A successful immune response to foreign pathogen requires a rapid activation of innate immunity, which directs the subsequent development of a productive adaptive immune response. Innate T cells represent a group of T lymphocytes that are able to act during the lag time while effective adaptive immune responses develop (1). Similar to conventional T cells, innate T cells undergo T cell receptor (TCR) rearrangement and thymic selection. Unlike their conventional counterparts, innate T cells rapidly recognize foreign pathogen signals and manifest immediate effector functions after activation. This allows innate T cells to perform effector immune responses much earlier than conventional T cells, and act as an additional “bridge” between innate and adaptive immune responses (2).

Classically, T cells are subdivided into two major populations based upon their TCR expression, namely alpha beta ($\alpha\beta$) T cells and gamma delta ($\gamma\delta$) T cells. Conventional $\alpha\beta$ T cells recognize a broad range of peptide antigens typically presented by Major Histocompatibility Complex (MHC I and II) complexes, enabled through their highly diverse TCR arrangement. In contrast, the $\alpha\beta$ innate T cells that have been identified display a restricted T cell repertoire characterized by the expression of an invariant or semi-invariant TCR α chain. In humans, two well-defined $\alpha\beta$ innate T cell populations have been identified in recent years, namely, mucosal-associated invariant T (MAIT) cells and invariant natural killer T (iNKT) cells. These two T cell populations together with $\gamma\delta$ T cells form the three major types of innate T cell (1). All three innate T cell populations express a C-type lectin molecule CD161. CD161 was initially identified on CD4, CD8, and $\gamma\delta$ subsets in the 1990s (3, 4). CD161 is variably expressed across human T cells, and three populations can be identified, expressing negative, intermediate, and high levels of CD161 (2). The expression of CD161 in human T cells populations is summarized in Figure 1. The level of CD161 expression is distinctive between conventional T cells and innate T cells, with MAIT cells displaying the highest levels (5).



While innate T cells are distinctive as a subpopulation of T cells, they have other distinct features, which do not overlap. They share the absence of MHC I/II peptide restriction but differ in their respective antigen presentation modalities. iNKT and MAIT cells respond to the MHC-like molecules, CD1d and MR1, respectively, while CD1c can present antigens to $\gamma\delta$ T cells. The nature of the antigens recognized by innate T cells is also diverse and broadly non-overlapping involving metabolites, bacterial products, and lipids. iNKT cells have been principally shown to respond to glycolipids, $\gamma\delta$ T cells are potently activated by (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), and MAIT cells can be activated by riboflavin metabolites – reduced 6-hydroxy methyl-8-D-ribitylumazine (rRL-6-CH₂OH), as well as folic acid metabolite, 6-formyl pterin (6FP). Finally, the sites of development, residence, and frequency within the T cell pool are distinctive and summarized in **Table 1**.

iNKT Cells

Invariant NKT cells are one of the most well-studied innate T cell populations (6–8). These cells are defined by their semi variant TCR, CD1d antigen restriction, and glycolipid recognition. Numerous studies have been undertaken with these cells following the discovery of their specific ligand, alpha-galactosylceramide, in

1997 (7, 9–11). Over the subsequent years, a range of endogenous and exogenous lipid antigens has been identified, which may change the effector responses of this innate T cell population (12–16). This cell population is also notable for its expression of previously considered NK cell specific markers such as CD161, which has subsequently been recognized on other innate T cell populations (17).

Invariant NKT cells develop in the thymus and are present at a very low number in most tissues. They are selected by CD1d, which is expressed on double-positive (CD4, CD8) thymocytes, through the recognition of endogenous lipids. In the thymus, iNKT cells acquire a memory/effector phenotype prior to exiting to the circulation. Recent studies have suggested that post-thymic education is required for iNKT cells to become fully mature and achieve functional competency (18, 19). In human peripheral blood, approximately 0.01–1% of the T lymphocytes are iNKT cells, characterized by their hallmark TCR-invariant chain V α 24-J α 18 and variant V β 11; and V α 14-J α 18 in mice with a limited number of β chains, including V β 8.2, V β 7, and V β 2 (20). Despite their presence in relatively low numbers in humans, iNKT cells can be very effective in early host defense mechanisms and are involved in a variety of disease settings (6, 21–23). A key feature of iNKT cells is their rapid release of a wide array of cytokines and chemokine following ligand activation (17). This plays an

TABLE 1 | Characteristics of innate T cells.

TCR	Human: V α 24-J α 18 Mouse: V α 4-J α 18	Human: V α 7.2-J α 33 Mouse: V α 9-J α 33	Human: V δ 1, V δ 3, V γ 9v δ 2 Mouse: V γ V δ 6.3, V γ 5V δ 1, V γ 6V δ 1
Ligand	Glycolipids, phospholipids	Vitamin B2 metabolites, transitory neo-antigens	Phosphoantigens, phycoerythrin, glycolipids
Frequency	Low (0.01–1% of T cells)	1–20% of T cells	2–10% of T cells
Location	Blood, mucosal site, and liver	Blood, gut, lung, liver	Blood, mucosal sites
Maturation	Thymus	Secondary lymphoid tissue	Thymus
Present at birth	Yes	Yes	Yes
HSCT	Yes	?	Poor

?, information not known.

important role in their early effector and regulatory properties. Our understanding of the importance of iNKT cells is largely based on disease studies undertaken in iNKT deficient mice (24). Previous studies have shown that iNKT cells play an important role in the detection of various pathogens, including *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Salmonella typhimurium*, *Mycobacterium tuberculosis*, *Listeria monocytogenes*, and *Borrelia burgdorferi* (1, 25, 26). In addition to bacterial infections, iNKT cells have also been found to play an important role in viral infections, including influenza, cytomegalovirus, and coxsackie B3 viral diseases (25, 27). Finally, they also play an important role in tumor immunity (28) and autoimmune disease (22). In human studies, a link between defects in iNKT cells may lead to susceptibility to certain infectious diseases, such as tuberculosis (29, 30), EBV (31–33), allergy (34), atherosclerosis (35), and immunodeficiency. (10, 36–38).

Role During Bacterial Infections

The presence of functional iNKT cells during bacterial sepsis has been shown in a number of different murine settings. In *S. pneumoniae* infection, a much higher level of bacteria were identified in the J α 18 knockout mice compared to the iNKT competent wild type mice, resulting in significant survival rate differences between the two strains (39). In the iNKT knockout mice, a defect was found in neutrophil recruitment to the lung together with a reduced production of neutrophil chemo-attractants, including TNF-alpha and MIP-2. A reconstitution of iNKT cells from wild-type mice to iNKT deficient mice was able to restore the production of TNF-alpha and MIP-2, leading to improved neutrophil and bacterial clearance (40). In a further bacterial infection caused by *Chlamydia pneumoniae*, accumulations of iNKT cells within the lung were visible within hours of acute infection, demonstrating IFN-gamma production at the site of infection (41). An extension of conventional bacterial challenge studies has recently been undertaken by Wong et al., which suggested that iNKT cells might play a role in control of bacterial infections associated with stroke. Compared to their WT litter mate, iNKT deficient mice were found to be more susceptible to bacterial infection post transient midcerebral artery occlusion. This was related to the ability of iNKT cells to act as a suppressor for neurotransmitter release post-stroke, which is lost in iNKT deficient mice, making them more susceptible to the bacterial infection (42). In humans, several studies have established the link between iNKT cells and *M. tuberculosis* infection with both the function and number of iNKT cells reduced in these patients (43). Two distinct pathways have been proposed for iNKT cell activation during infection. They can either be directly activated through TCR-CD1d-glycolipid recognition or indirectly through

their response to innate cytokines that are released from other innate cells.

Indirect Activation of iNKT Cells by Gram-negative Bacteria

Early secretion of IFN-gamma can be induced by iNKT cells following an encounter with both Gram-negative and Gram-positive bacteria. Innate receptors that recognize bacterial signals have a crucial role in triggering the antigen presenting cells, which subsequently direct the activation of iNKT cells (9, 44–49). The activated antigen presenting cells stimulate the iNKT cells by signaling through toll like receptors (i.e., TLR4, TLR7, and TLR9) leading to the production of IL-12, also other inflammatory cytokines. Studies by De Libero and Paget have suggested that TLR signaling through APCs are not only important for cytokine production but also the accumulation of self-lipid antigen for CD1d presentation (47, 50). A study by Darmoise et al. showed that the TLR signaling triggered the accumulation of self-lipid including iGb3 in the lysosome, leading to an enhanced iNKT cell activation (51).

Direct Activation of iNKT Cells by Gram-negative Bacteria

Another mechanism that allows iNKT cells to respond to bacterial infection occurs through the direct recognition of the glycosphingolipid in the cell wall of Gram-negative bacteria. One such example is *Sphingomonas/Novosphingobium* spp., where the glycosphingolipids present in the bacteria cell wall are alpha-galacturonylceramides and alphaglucuronylceramides (52). These glycosphingolipids contain one sugar ring and have been shown to activate iNKT cells *in vitro*, while multi-sugar ring glycosphingolipids have not been able to activate iNKT cells in co-culture. Murine studies suggested that CD1dKO mice were able to clear infections with *Sphingomonas/Novosphingobium* as well as some other LPS-negative bacteria, but at a much slower rate compared to the wild type mice (45, 53, 54). This would suggest the iNKT cells are one of the major innate cell types involved in bacterial clearance and playing a major role in the early response.

$\gamma\delta$ T Cells

$\gamma\delta$ T cells are another group of innate T cells that have been found to play an important role during bacterial infections. Unlike conventional $\alpha\beta$ T cells, $\gamma\delta$ T cells do not usually express a CD4 or CD8 lineage marker and they do not require conventional antigen presentation via MHC class molecules (55). Different subtypes of $\gamma\delta$ T cells have been described often identified by the different arrangement of their TCRs in early development. The differences in TCR arrangement directly influence their eventual principle

tissue of residence. In human, the majority of the $\gamma\delta$ T cells present in the peripheral blood express the TCR V γ 9V δ 2, whereas V δ 1 and V δ 3 TCR are primarily expressed at the mucosal surfaces. In mice, V γ 1 and V γ 4 are present in the lymphoid tissues; V γ 5 is found to be present in the skin; V γ 6 in the reproductive tract; and V γ 1, V γ 4, and V γ 6 present in the lung (56). A number of mechanisms have been described linking $\gamma\delta$ cells and bacterial infections. Similar to iNKT cells, $\gamma\delta$ are able to sense danger signals in both a TCR dependent and TCR independent way. $\gamma\delta$ T cells can be activated by Class I like molecules such as T10/T22 (in mice) and members of CD1 family; they can also be activated by MHC-unrelated molecules such as viral glycoproteins and F1-ATPase complex in human (57–59). In addition to TCR recognition, $\gamma\delta$ T cells also express pattern recognition receptor and receptor typically associated with NK cells.

$\gamma\delta$ T cells may expand in the patient's peripheral blood during bacterial infections with studies identifying up to 12% in listeriosis, 14% in tuberculosis, and 29% in brucellosis (60). Human $\gamma\delta$ T cells respond to bacterial infections by recognizing (E)-4-hydroxy-3-methyl-but-2enyl pyrophosphate (HMBPP) derived from various bacteria. $\gamma\delta$ T cells were shown to be particularly important in response to intracellular bacterial pathogens including *M. tuberculosis* and *Legionella micdadei*. In the case of *L. micdadei*, V γ 9V δ 2 T cells were found to be depleted from the circulation upon bacterial infection, followed by a sharp increase, then a slow decline over a 6-month-period (61). This dynamic change may indicate V γ 9V δ 2 might be important in contributing to the pathophysiological changes of Pontiac fever-like disease. A similar kinetic pattern is seen with *M. tuberculosis* infection, following the V γ 9V δ 2 T cell response to the metabolite IPP (62).

Early studies found that V γ 9V δ 2 T cells were the most important group that led to the eradication of bacteria (63, 64). Seminal studies identified the antigens involved in the recognition were intermediates in isoprenoid biosynthesis, namely (E)-4-hydroxy-3-methyl-but-2enyl pyrophosphate (HMBPP) (65). The level of HMBPP directly influences the magnitude of V γ 9V δ 2 T cell activation and proliferation (66). Recently, a major breakthrough in discovering the mechanisms for activating the V γ 9V δ 2 T cells was made by Bonneville and Scotet's group. They identified that a member of butyrophilin molecule family CD277 played a crucial role during $\gamma\delta$ T cells activation (67–71).

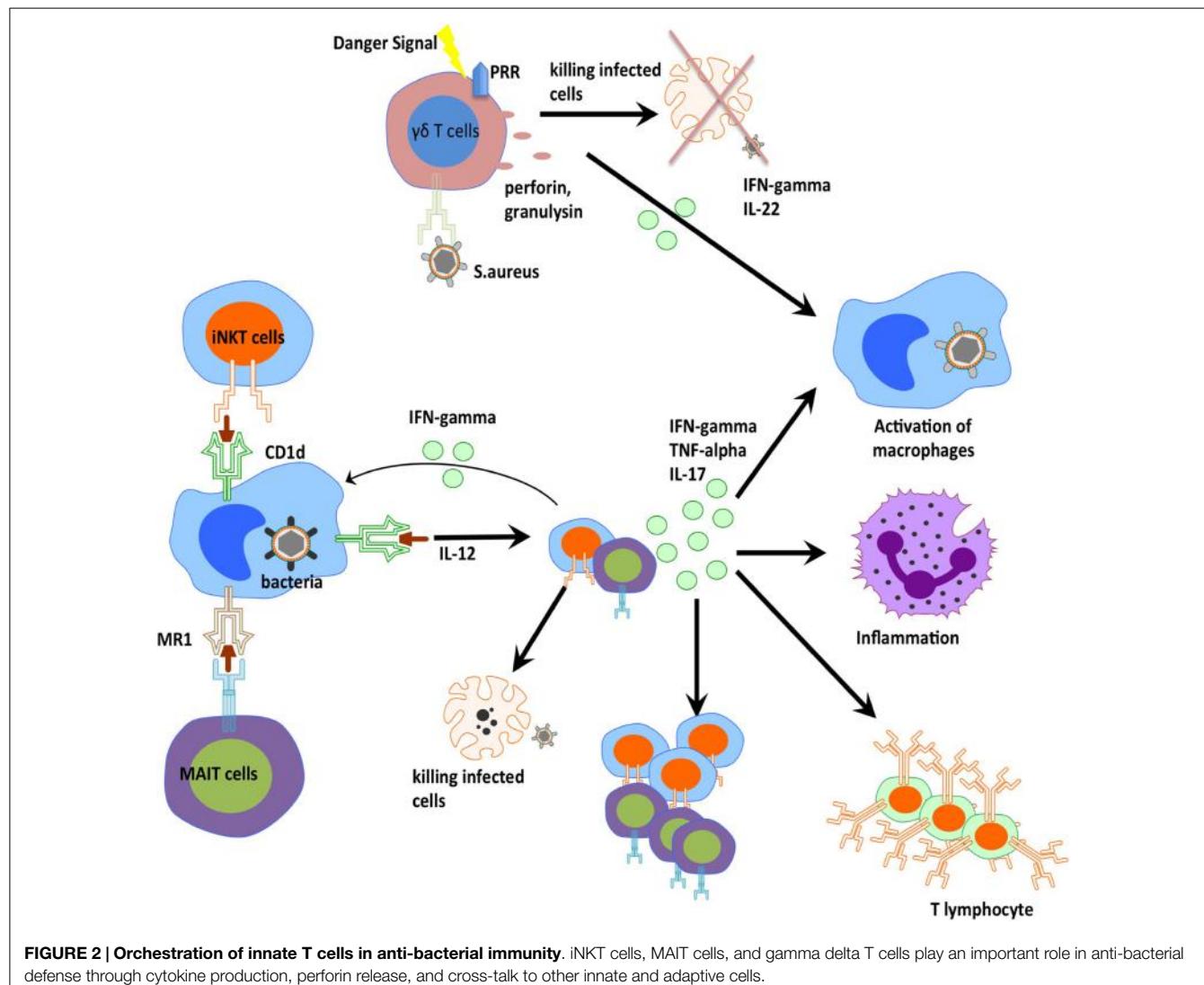
$\gamma\delta$ T cells have also been found to be able to promote self-activation through cell to cell interaction (72). However, it was demonstrated that the self-activation mechanism is not as effective as formal presentation through antigen presenting cells (73–75). One important aspect of $\gamma\delta$ T cells is that they can trigger the maturation of dendritic cells. Devilder et al. showed that V γ 9V δ 2 T cells can stimulate the maturation signal on mycobacterial infected DCs, through a Fas–Fas ligand interaction (76) and/or TCR-CD1 contact (77). Other than dendritic cells, $\gamma\delta$ T cells have also been found to be important in macrophage recruitment. During infection with *listeriosis*, $\gamma\delta$ T cells were found to be a key player in controlling the production of key macrophage chemo attractants (78). Skeen et al. also showed that macrophages failed to undergo maturation in the absence of $\gamma\delta$ T cells (79). Direct engagement of $\gamma\delta$ T cells may facilitate pathogen clearance through their production of bacteriostatic and lytic molecules, such as granulysin and defensins. During *Staphylococcus aureus*

respiratory infection, $\gamma\delta$ T cells sense the dysregulation of the mevalonate pathway within the infected cells. This leads to the activation and expansion of $\gamma\delta$ T cells, in particular, V γ 9V δ 2 T cells. The active $\gamma\delta$ T cells then produce cytokines such as IL-17, which leads to airway protection. $\gamma\delta$ T cells also play a role during *M. tuberculosis* infection, producing a variety of cytokines including IFN- γ , TNF- α , and IL-17. IFN- γ and TNF- α play are essential in host protection against *M. tuberculosis* enabling granuloma formation and disease containment.

MAIT Cells

Mucosal-associated invariant T cells are the newest members of the innate T cell family. They were first described by Tilloy et al. (80) and represent the most abundant innate T cells in humans. They express a canonical Va7.2-Ja33 chain in humans and the orthologous Va19-Ja33 in mice. The development of MAIT cells is parallel to the development of iNKT cells and both express the transcription factor ZBTB16 (81). In adults, they display an effector phenotype, whereas MAIT cells possess a naïve phenotype in cord blood. In both cord and adult blood, MAIT cells express CD161, IL-18Ra, CCR6, and about 50% of the MAIT cells express the T cells co-receptor CD8 (82–84). Recent studies also show that MAIT cells express the ABC binding cassette (ABC) B1 drug resistance transporter (85, 86). MAIT cells have a further unique antigen recognition system recognizing a MHC Class I related molecule (MR1), which is able to present bacterial derived ligand. Study by Kjær-Nielsen et al. showed that 6-formyl pterin (6-FP), a metabolite on the folic acid pathway, could stabilize the MR1 molecule but failed to activate the cells. Full activation of primary MAIT cells was achieved with ligand reduced 6-hydroxymethyl-8-d-ribityllumazine (rRL-6-CH₂OH), a riboflavin metabolite. Related products 7-hydroxy-6-methyl-8-d-ribityllumazine (RL-6-Me-7-OH) and 6,7-dimethyl-8-d-ribityllumazine (RL-6,7-diMe) have also shown similar agonistic activity for MAIT cells, leading to the rapid production of cytokines (87). In recent years, studies on MAIT cells have associated their number and function with diverse of disease settings, including bacterial infections and autoimmune disorder.

The first hint that MAIT cells have anti-bacterial activities was described in 2010, where studies by Gold et al. and Le Bourhis et al. showed that MAIT cells could recognize a range of bacteria species through MR1 (88, 89). In the study by Gold et al., MAIT cells could respond to *M. tuberculosis* even in unexposed individuals. They further showed that MAIT cells responded to *Salmonella enterica*, *Escherichia coli*, and *S. aureus* infected APC (90). Le Bourhis et al. showed that MAIT cells could MAIT cells are able to respond to a wide array of bacteria including Gram-positive Bacteria *S. aureus*, *Staphylococcus epidermidis*, *Lactobacillus acidophilus*, and Gram-negative Bacteria *E. coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, and *Mycobacterium abscessus*. Importantly, some bacterial species were shown not to activate MAIT cells, namely *Enterococcus faecalis* and *Streptococcus pyogenes*, suggesting a novel specificity. The importance of their role in bacterial defense was suggested by a study by Georgel et al. demonstrating that during the *Klebsiella pneumonia* infection, MR1 deficient mice succumbed to disseminated infection whereas the WT mice



achieved full bacteria clearance within 2 days (91). Similarly, in the Va19 transgenic mice, enhanced control of the *E.coli* and *M. abscessus* infection were observed. Further studies performed by Chua et al. and Meierovics et al. also showed that MAIT cells were needed in the early control of *Mycobacterium bovis*, BCG, and *Francisella tularensis* infection (92, 93). In humans, how MAIT cells play a role in infectious disease is less well understood. A number of studies have associated the frequency of MAIT cells in different infectious diseases (94). MAIT cell numbers were found to be lower in peripheral blood of patients with *M. tuberculosis* infection (95). Also, in a study of critically ill septic and non-septic patients, the patients with severe bacterial infections, but not viral infections, had a much lower MAIT count compared to healthy controls (96).

One of the most well-studied examples of MAIT cells in bacterial infection is during *Salmonella* infection (97, 98). Upon activation, MAIT cells produce IFN-gamma, TNF α , and IL-17. These cytokines have been shown to be critical in controlling *Salmonella* infections, with IL-17 preventing the dissemination of infection (99). MAIT cells may also play a role during *Salmonella*

infection through their early cytotoxic activity (100), although further studies are needed as MAIT cells were not able to directly kill *Salmonella* infected cell lines (94, 101).

Over the last 5–10 years, there has been advancement in the understanding and description of unconventional T cells. These studies demonstrate that unconventional T cells do indeed play an important role during bacteria infection and contribute the ability of host organism to clear and control certain bacterial infections (Figure 2). These cells are able to efficiently traffic to the sites of inflammation, and initiate rapid responses by means of cytokine production and cytotoxic activities. Further studies will elucidate the molecular details of this cellular control suggesting novel approaches to how we may harness these cells through therapeutic vaccination and pharmaceutical manipulations.

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Role of group 1 CD1-restricted T cells in infectious disease

Sarah Siddiqui, Lavanya Visvabharathy and Chyung-Ru Wang*

Department of Microbiology and Immunology, Northwestern University Feinberg School of Medicine, Chicago, IL, USA

The evolutionarily conserved CD1 family of antigen-presenting molecules presents lipid antigens rather than peptide antigens to T cells. CD1 molecules, unlike classical MHC molecules, display limited polymorphism, making CD1-restricted lipid antigens attractive vaccine targets that could be recognized in a genetically diverse human population. Group 1 CD1 (CD1a, CD1b, and CD1c)-restricted T cells have been implicated to play critical roles in a variety of autoimmune and infectious diseases. In this review, we summarize current knowledge and recent discoveries on the development of group 1 CD1-restricted T cells and their function in different infection models. In particular, we focus on (1) newly identified microbial and self-lipid antigens, (2) kinetics, phenotype, and unique properties of group 1 CD1-restricted T cells during infection, and (3) the similarities of group 1 CD1-restricted T cells to the closely related group 2 CD1-restricted T cells.

Keywords: *Mycobacterium tuberculosis*, CD1, antigen presentation, T cells, NKT cells, animal models

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Reviewed by:

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Universiteit Utrecht, Netherlands

*Correspondence:

Chyung-Ru Wang,
Department of Microbiology and
Immunology, Northwestern University
Feinberg School of Medicine, 320
East Superior Street, Searle 3-401,
Chicago, IL 60611, USA
chyung-ru-wang@northwestern.edu

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Introduction

The *CD1* gene family encodes several MHC class I-like antigen-presenting molecules, which are specialized to present lipid antigens to T cells. The lipid antigens presented by CD1 include a diverse array of lipids/glycolipids, ranging from foreign lipids unique to specific microorganisms to common mammalian self-lipids. Three major groups of CD1 isoforms have been identified in humans: group 1 CD1 (CD1a, CD1b, and CD1c), group 2 CD1 (CD1d), and group 3 CD1 (CD1e) (1). Although homologs exist in the guinea pigs and other vertebrates, mice, and rats do not express group 1 CD1 (2, 3), making the *in vivo* functional study of group 1 CD1-restricted T cells difficult until the recent development of small animal models (4–6). This review will outline group 1 CD1 antigen binding and presentation, newly identified group 1 CD1-restricted self- and microbial lipid antigens, and the interaction of group 1 CD1-expressing antigen-presenting cells (APCs) with their corresponding T cell subsets. Additionally, the development and function of group 1 CD1-restricted T cells will be discussed, with special emphasis placed on small animal models, dynamics of these T cells during *Mycobacterium tuberculosis* (*Mtb*) infection, and the contribution of microbial antigen-specific and autoreactive group 1 CD1-restricted T cells to host defense against infection. Finally, the expansion of our knowledge of group 1 CD1-restricted T cell responses allows for their comparison with a more well-studied and related subset of T cells, the group 2 CD1-restricted NKT cells. The last section will focus on comparing and contrasting the properties of group 1 vs. group 2 CD1-restricted T cells.

Expression of Group 1 CD1 Molecules

Unlike MHC class Ia, which is expressed on all nucleated cells, the expression of group 1 CD1 is mainly limited to APCs and double-positive (CD4⁺CD8⁺) cortical thymocytes (1). CD1a molecules are highly expressed on skin resident DCs, or Langerhans cells (7). CD1b is most highly expressed on

a subset of migrating lymph dendritic cells and myeloid-derived dendritic cells (8). CD1c is the most ubiquitously expressed group 1 CD1 molecule, being found on monocyte-derived DCs, B cells, and Langerhans cells under steady-state conditions (9, 10). CD1e is the only CD1 isoform that is not expressed on the surface of APCs (11). In immature DCs, CD1e is mainly localized in the Golgi apparatus and in mature DCs, it is detected in late endosomes, where it is cleaved into a functional soluble form (12). CD1e is thought to facilitate the processing and presentation of certain lipid antigens presented by CD1b (13).

Group 1 CD1 expression can also be regulated by various cytokines. Treatment of activated monocytes with GM-CSF and IL-4, which leads to dendritic cell differentiation, results in increased levels of group 1 CD1 expression (14). Group 1 CD1 expression is also altered in different disease models, implying a role for group 1 CD1-restricted T cells in the pathology and/or repression of the disease. Patients with the tuberculoid form of leprosy, a clinical pattern associated with active cellular immunity toward *Mycobacterium leprae*, show induction of CD1a, CD1b, and CD1c on DCs in dermal granulomas (15). Increased CD1b and CD1c expression has also been reported in the human skin upon *Borrelia burgdorferi* infection (16). CD1a expression on Langerhans cells has been correlated with a variety of autoimmune skin diseases, including atopic eczema (7, 17, 18) and psoriasis (19, 20). In addition, some leukemias and lymphomas express one or all group 1 CD1 molecules (21). One recent study showed that CD1c⁺ B cell leukemia precursors are efficiently targeted and lysed by CD1c autoreactive T cells, highlighting a role of group 1 CD1-restricted T cells in anti-tumor immunity (22).

Binding and Presentation of Lipid Antigens by Group 1 CD1 Molecules

Group 1 CD1 isoforms have structurally diverse antigen-binding grooves that allow them to bind very different lipid classes. Group 1 CD1 isoforms also differ in their stability when lipids are bound in the binding groove vs. when the groove lies empty, which is consistent with their intracellular trafficking patterns and consequently the types of lipids they are exposed to (23, 24). CD1b and CD1c molecules traffic through and load lipids in the late endosome and the lysosome, and thus, are exposed to a low pH that may facilitate lipid exchange (25). However, CD1a molecules are unique in that under steady-state conditions, they do not accumulate or traffic through the late endosomes or lysosomes, and thus, are not exposed to the same low pH conditions as CD1b and -c molecules (26). Instead, modeling simulations suggest that CD1a molecules can be stably expressed on the cell surface in the absence of loaded lipid antigens (27), and other studies have shown that the CD1a binding groove can be stabilized by exogenously added lipids after being expressed on the cell surface (28).

Group 1 CD1 molecules present both microbial and self-lipid antigens to T cells. Historically, microbial antigens that were found to bind to group 1 CD1 molecules were derived from the cell wall of *Mycobacteria* species. Below, we will describe how the various group 1 CD1 molecules interact with different types of lipids, and what some of the functional consequences for T cell activation might be.

Lipid Antigen Presentation by CD1a

CD1a molecules can bind the mycobacterial lipopeptide dideoxyxymycobactin (DDM), a biosynthetic precursor of the iron-chelating siderophore mycobactin, which is essential for *Mtb* growth within macrophages (29). CD1a presentation of DDM and subsequent TCR recognition of antigen have features reminiscent of both CD1 and MHC-like binding modes in that the CD1a-restricted TCR is capable of responding in an amino-acid sequence-specific manner to the solvent-exposed portion of the DDM lipopeptide (30). DDM-specific CD1a-restricted T cells were found to secrete varying levels of IL-2 in response to different synthetic DDM analogs, with the largest amount of IL-2 being produced when the DDM antigen was in its native state (31). This showed that CD1a-restricted T cells could specifically respond to the structural components of an antigen that are responsible for virulence (31).

The crystal structure of CD1a-DDM complex showed that A' pocket of CD1a antigen-binding groove binds alkyl chains of discrete length, while the F' pocket is less rigid and allows for different chemical head groups of lipid antigens to protrude from the groove (30). The crystal structure of CD1a with the bound self-antigen sulfatide provides another example of this type of binding mode wherein the sphingosine fits into the A' pocket and the polar headgroup of sulfatide protrudes from the middle of the groove, allowing for optimal TCR recognition (32). However, despite the body of work showing that CD1a lipid antigens generally contain protruding polar headgroups and hydrophobic tails, a recent study showed that CD1a molecules could also bind and present "headless" lipid antigens to T cells (33). In fact, synthetic squalene, a lipid containing no headgroup, was able to activate the BC2 CD1a-restricted T cell line to the same extent as what was seen from challenge with sebaceous gland extract containing a mixture of CD1a autoantigens (33). This suggests that CD1a has the ability to recognize "headless" lipid antigens that lack a polar moiety available to be recognized by the TCR. A recent finding by Rossjohn's group provided a potential explanation for this (34). This study first solved the crystal structure of a TCR bound to CD1a and mechanistically showed that the TCR can directly contact CD1a in the absence of lipid interaction. This interaction is sufficient for T cell activation. However, if CD1a is loaded with non-permissive lipids, there is a disruption of the TCR-CD1a docking site (34). Therefore, CD1a autoreactivity can result from direct contact of the TCR with CD1a, but only when the surface of CD1a is not disrupted by non-permissive ligands. Furthermore, a recent study showed that bee venom-derived phospholipase 2 could activate CD1a-autoreactive T cells through the generation of small neo-antigens, such as free fatty acids and lysophospholipids, from common phosphodiacyl glycerides (35). This finding provides a potential mechanism underlying phospholipase-dependent inflammatory skin disease.

Lipid Antigen Presentation by CD1b

CD1b has the largest antigen-binding groove among the CD1 proteins described to date. As such, CD1b binds and presents the most diverse array of mycobacterial lipids, which include mycolic acid (36), glucose monomycolate (GMM) (37), glycerol monomycolate (38), phosphatidylinositol mannosides (PIMs) (39, 40),

lipoarabinomannans (LAM) (41–43), and sulfoglycolipids (44). The CD1b binding groove is composed of four interconnected pockets, which enable binding of the long hydrophobic tails of lipid antigens (45). The structure of CD1b–GMM complex showed that the A', C', F', and T' pockets in the CD1b binding groove form a deep “maze” that allows them to present large mycobacterial lipid antigens to T cells, and the close association of lipid ligands with CD1b molecules is suggestive of this complex having a long half-life (45). These studies also show that the pleiotropic binding of structurally diverse lipids to CD1b molecules can occur due to critical amino-acid residues in the CD1b binding groove having the ability to change their conformation to induce a tighter fit for different lipids (45, 46). However, the finding that CD1b molecules bind and are stabilized by such large microbial lipid ligands led to the question of how unloaded CD1b molecules retain their structure in the absence of endosomally loaded lipid. Operating from the hypothesis that endogenous self-lipids could act as stabilizers of the Ag-binding groove for CD1b in the absence of foreign ligands, Garcia-Alles et al. showed that CD1b associates with phosphatidylcholine (PC) and a long 40+ carbon spacer molecule prior to endosomal lipid loading (47). The combined number of carbons shared between PC and the spacer molecule is greater than the C65–C70 capacity of the CD1b binding groove, but this “overloading” of the groove may actually facilitate lipid exchange in the endolysosome (47).

Mycolic acids were the first CD1b-restricted lipids identified (36). Mycolic acids are the major *Mtb* cell wall lipid components and are crucial for *Mtb* virulence (48). Studies in untreated human *Mtb* patients showed that many patients contained IFN- γ producing, mycolic acid-specific CD1b-restricted T cells in their PBMCs (49), suggesting that mycolic acids are immunodominant CD1b-restricted antigens relevant during *Mtb* infection. While free mycolic acids can activate some CD1b-restricted T cells; one recent study suggested that mycolic acids may act as scaffolds for other lipid antigens, with the tight association of the long hydrophobic tails in the CD1b binding pockets leading to proper positioning of various lipid antigenic headgroup for TCR recognition (38).

Diacylated sulfoglycolipids (Ac₂SGL) are produced by virulent *Mtb* strains upon infection of the human host (44, 50). Studies using human CD1b-restricted T cell lines specific for Ac₂SGL showed that the aliphatic tail region is responsible for anchoring the molecule deep in the CD1b antigen-binding groove, while the trehalose headgroup is responsible for TCR recognition (50). However, the antigen specificity of particular T cell lines could be influenced by the length and isomerization of the hydrophobic tail region of Ac₂SGL (50). This corroborates the study described above, which showed that the long hydrophobic lipid tails of CD1b lipid antigens, including Ac₂SGLs, can act to position the antigenic headgroups optimally for TCR recognition (38, 47).

Mycobacterium tuberculosis PIMs and their polyglycosylated extensions lipomannans (LM) and LAM (51) are components of the *Mtb* cell envelope (52). Many forms of LM and LAM are presented by CD1b (42, 43). Biochemical and functional characterization of structurally diverse forms of LM showed that the pathogenic *Mtb* strain H37RV contained LM moieties with longer and more branched mannan polymer domains than those from the avirulent strain *Mycobacterium smegmatis* (43). Thus, it is possible

that virulent *Mtb* strains evolved to use their LM lipids as immune evasion factors, as CD1b-LM complexes from H37Rv were less potent activators of CD1b-restricted T cell responses than those from *M. smegmatis* (43).

Lipid Antigen Presentation by CD1c

The only known *Mtb* antigen presented by CD1c is phosphomycocetide (PM), a lipid that contains a single fully saturated alkyl chain with methyl branches at every fourth carbon (53, 54). The production of *Mtb* mannose-1- β -phosphomycocetide (MPM) occurs via enzymatic catalysis by Pks12, which is only found in slow-growing pathogenic strains of *Mtb* (55), suggesting that MPM is a potential virulence factor in *Mtb* pathogenesis. Recent studies showed that PM, a putative biosynthetic precursor for MPM can also bind to CD1c and activate CD1c-restricted T cells (56).

CD1c molecules have a unique antigen-binding mode that allows for presentation of diverse antigens, such as highly branched lipid species to T cells (57). Whereas lipid antigens bind in both the A' and F' pockets in CD1a and CD1b molecules (32, 45), MPM binds only to the A' pocket in CD1c molecules, allowing for the branched side chains to protrude out from the groove (57). Like CD1a, CD1c can also present lipopeptide antigens to T cells (58). In addition, CD1c has been shown to bind sulfatide and other diacylated lipids similar to CD1a and CD1b (50, 59), suggesting that the CD1c binding groove has evolved to present a diverse array of lipid species.

A recent paper also helped shed light on a potential role for autoreactive CD1c-restricted T cells (22). Autoreactive CD1c-restricted T cells were found to recognize methyl-lysophosphotidic acid, a novel class of self-lipids, which accumulate in leukemia cells (22). While these autoreactive CD1c-restricted T cells poorly recognize non-tumor CD1c-expressing cells, they killed CD1c⁺ acute leukemia cells and protected immunodeficient mice against CD1c⁺ human leukemia cells (22). This study suggests that autoreactive CD1c-restricted T cells may play a role in anti-tumor immunity (22). However, their role in anti-microbial immunity remains unknown.

Kinetics, Phenotype, and Unique Properties of Group 1 CD1-Restricted T Cells

Much of the information about group 1 CD1-restricted T cells comes from *in vitro* assays using human T cell clones. Recent discoveries in tetramer development and improved cell isolation technology have greatly enhanced the study of group 1 CD1 molecules in humans, though animal models are critical to fully understand the dynamic biology of group 1 CD1-restricted T cells. A recent study using rhesus macaques identified a GMM-specific T cell population in BCG-immunized monkeys (60). However, unlike humans, GMM-specific T cells were found to be CD1c restricted (61). Though interesting, following up on such findings in primates can be challenging due to cost constraints. As an alternative to studying non-human primates, small animal models using transgenic mice have proven invaluable for the study of group 1 CD1-restricted T cells. In the following sections, we will

review recent discoveries about group 1 CD1-restricted T cells and the role they play in host immunity *in vivo*.

Small Animal Models to Study the *In Vivo* Function of Group 1 CD1-Restricted T Cells

There have been a number of small animal models used to study the *in vivo* function of group 1 CD1-restricted T cell responses during *Mtb* infection, including the guinea pig model and humanized mouse model. Based on comparison with human CD1 isoforms, the nine guinea pig CD1 genes encode four CD1b-like, three CD1c-like, one CD1d-like, and one CD1e-like molecule (2, 62). Immunization of guinea pig with mycobacterial lipids has been shown to elicit antigen-specific proliferation and cytolytic capacity of group 1 CD1-restricted T cells (63). In addition, *Mtb* lipid-vaccinated guinea pigs exhibit reduced lung pathology after subsequent *Mtb* challenge (64). However, it has been difficult to show that group 1 CD1-restricted T cells mediate these protective effects due to limited experimental tools and reagents. Moreover, guinea pigs lack CD1a and have multiple isoforms of the CD1b and CD1c genes, suggesting that the group 1 CD1-restricted T cells response may be different in guinea pigs and humans.

A humanized mouse model developed by Gumperz's group was observed to develop a functional CD1 compartment (6). This model involved grafting immunodeficient mice with human fetal thymus, liver, and CD34⁺ hematopoietic stem cells (6). While these mice were observed to express group 1 CD1 molecules, CD1a tissue expression patterns differed from humans (6). For instance, CD1a-expressing cells, which are normally abundant in human skin, are largely absent in humanized mice. It remains to be seen whether group 1 CD1-restricted T cell responses can be elicited in these humanized mice upon immunization or during infection.

A transgenic mouse model that expresses the human group 1 CD1 genes under their endogenous human promoters has been generated (4). Human group 1 CD1 transgenic (hCD1Tg) mice express CD1a, CD1b, and CD1c in a pattern similar to that seen in humans and support the development of group 1 CD1-restricted T cells (4). These T cells from hCD1Tg mice also share many phenotypic and functional characteristics with CD1-restricted T cells found in humans (4). These data demonstrate the viability of hCD1Tg mice as an animal model to study the *in vivo* function of group 1 CD1-restricted T cells. Indeed, both infection with *Mtb* and immunization with *Mtb* lipids elicit group 1 CD1-restricted T cell responses in hCD1Tg mice (4).

Group 1 CD1-Restricted T Cell Responses During *Mtb* Infection

The presentation of mycobacterial lipid antigens by group 1 CD1 molecules has been well characterized. However, our understanding of the phenotype and function of responding T cells was mostly limited to *in vitro* assays with T cell clones until recently. Nevertheless, *in vitro* studies have demonstrated that group 1 CD1-restricted T cells are cytotoxic and produce IFN- γ and TNF- α upon encountering mycobacterial antigens (65, 66). Moreover, group 1 CD1-restricted *Mtb* lipid antigen-specific T cells are found in higher frequencies in individuals exposed to *Mtb* compared with a control population, suggesting

that *Mtb*-specific CD1-restricted T cells are activated following infection with *Mtb* (38, 44, 65–67). The discovery of CD1-tetramer technology has made it easier to study T cell reactivity by enabling the direct isolation of antigen-specific T cells from the blood and tissue (56, 68). Moreover, the establishment of a group 1 CD1 transgenic mouse model has enabled the study of tissue distribution, activation kinetics, and phenotype of group 1 CD1-restricted T cells.

CD1a dextramers loaded with the mycobacterial lipopeptide DDM stained CD1a-restricted T cells in individuals with active TB and tuberculin-positive individuals, suggesting an expansion had occurred upon exposure to *Mtb* (69). CD1a/DDM dextramer⁺ T cells were found to be either CD4⁺ or CD8⁺, and produced IFN- γ and TNF- α in response to CD1a-expressing APCs treated with DDM. Studies with CD1b/GMM tetramers revealed the expansion of CD1b/GMM-specific T cells in individuals infected with *Mtb*, but not healthy individuals (68). These cells were found to be CD4⁺ TCR $\alpha\beta^+$ and appeared to have a conserved TCR repertoire. CD1b/GMM-specific T cells were later separated into two types based on their avidity to CD1b/GMM. GMM-specific T cells with a high avidity to CD1b/GMM termed GEM (Germline-encoded, mycolyl-reactive) T cells expressed highly conserved TCRs composed of a TRAV1-2 (V α 7.2) V segment rearranged with TRAJ9, with limited CDR3 α diversity (70). GEM T cells were found at a very low frequency in *Mtb* naïve individuals. A second population with an intermediate avidity to CD1b/GMM was also observed and this population showed diverse TCR usage (71). Recently, Moody and colleagues described that CD1c-restricted T cells respond to glycosylated and unglycosylated forms of mycoketide presented by DCs or B cells (56). However, in cell-free systems, CD1c-restricted T cells responded only to unglycosylated forms of mycoketide (PM). Moreover, PM-loaded CD1c tetramers could detect antigen-specific T cells from PBMC of donors with latent TB. A comprehensive molecular analysis of TCR recognition of CD1c-mediated PM/MPM presentation revealed that some T cell clones recognize both PM and MPM while others are specific to either PM or MPM (72).

Studies infecting group 1 CD1-expressing hCD1Tg mice with BCG or *Mtb* revealed that the kinetics of group 1 CD1-restricted T cell responses were different from group 2 CD1d-restricted iNKT cells (4). Group 1 CD1-restricted T cells were detected at week 3 or 4 post infection, suggesting that they follow similar activation kinetics as peptide-specific conventional T cells. This study provided the first direct *in vivo* evidence that group 1 CD1-restricted T cell responses are induced by *Mtb* infection (4). Immunizing mice with BMDCs pulsed with *Mtb* lipid antigens followed by secondary challenge accelerated group 1 CD1-restricted T cell responses, indicating that group 1 CD1-restricted T cells can mount a memory response (4). Taken together, these studies support the potential use of group 1 CD1-restricted *Mtb* lipid antigens as components of subunit vaccines.

Autoreactive Group 1 CD1-Restricted T Cells

Early reports of T cell recognition of group 1 CD1 molecules suggested that many CD1-restricted T cells are autoreactive (73, 74). A recent analysis of T cell clones from human PBMC showed that CD1a self-reactive T cells are present at high frequencies in the

blood of healthy individuals (75). CD1a-restricted autoreactive T cells expressed diverse TCRs and skin homing receptors CCR4, CCR6, CCR10, and secreted IL-22. Given the properties of CD1a-autoreactive T cells and the fact that CD1a recognizes skin lipid antigens and is highly expressed on Langerhans cells implies that these T cells play a role in dermal immunity (33, 75). Another study investigated that the frequency and effector phenotype of autoreactive group 1 CD1-restricted T cells in human blood and cord blood using C1R transfectants expressing CD1a, CD1b, and CD1c (76). They found that the frequencies of group 1 CD1 autoreactive T cells were in the range of 1/10–1/300 circulating T cells. CD1a- and CD1c-restricted T cells comprised the most abundant autoreactive T cells. These group 1 CD1 autoreactive T cells had a diverse phenotype and exhibited mostly Th1 and Th0 functional activities (76).

Most of the CTLs derived from hCD1Tg mice are autoreactive (4). While the endogenous lipid antigens that activate these CTLs were not identified in this study, these clones were found to lyse hCD1Tg DC and group 1 CD1-expressing cells from humans. This suggests that the self-antigens recognized by these CTLs are conserved between mice and humans. Autoreactive T cells can also be detected from *Mtb*-infected mice. A transgenic mouse model, HJ1Tg, expressing an autoreactive CD1b-restricted TCR has been generated to study the development and function of group 1 CD1 autoreactive T cells (5). Similar to CD1d-restricted iNKT cells, HJ1 T cells exhibited an activated phenotype (CD44⁺CD69⁺CD122⁺) and a subset of HJ1 T cells expressed NK1.1 and was enriched in the liver (5). This finding raises the possibility that autoreactive group 1 CD1-restricted T cells may also reside within the NKT cell compartment of the human liver. HJ1 T cells were cytolytic and secreted proinflammatory cytokines (IFN-γ, IL-17A, and TNF-α) in response to CD1b-expressing DCs. TLR agonists (Pam3Cys and LPS) enhanced the autoreactivity of HJ1 T cells by stimulating DCs to produce IL12/IL23. Furthermore, HJ1 T cells were activated early during *Listeria* infection and played an immunoprotective role (5). The potential role of group 1 CD1 autoreactive T cells in other infections remains to be defined.

Developmental Requirements of Group 1 CD1-Restricted T Cells

The development of group 1 CD1-restricted T cells has not been well-studied due to a lack of a suitable small animal model, but some progress has been made in this regard. Studies using HJ1Tg mice showed that CD1b-expressing hematopoietic cells are necessary and sufficient to mediate the positive selection of HJ1 T cells (5). Cortical thymocytes are most likely the main cell type involved in the selection of HJ1 T cells as CD1b is not expressed on mature thymocytes. HJ1 T cells that developed in the hCD1Tg background expressed similar levels of the transcription factor PLZF as iNKT cells. PLZF is expressed by several non-conventional T cell subsets that display an activated phenotype and is responsible for their innate-like effector program (77–80). However, studies of group 1 CD1 autoreactive T cells in humans showed that autoreactive group 1 CD1-restricted T cells were present in both naive (CD45RA⁺) and effector/memory (CD45RO⁺) compartments (75, 76). It is unclear whether TCR-CD1 avidity and the nature of the selecting lipid antigen(s) may play a role in determining the phenotype of

group 1 CD1 autoreactive T cells. Moreover, the developmental selection program for microbial lipid antigen-specific group 1 CD1-restricted T cells has not yet been investigated.

Group 1 vs. Group 2 CD1-Restricted T Cells: Some Similarities, Many Differences

Group 1 and group 2 CD1 molecules share many similarities beyond the presentation of lipid antigens to T cells, including molecular assembly mechanisms and sharing self-lipid moieties that stabilize the CD1 binding groove in the absence of foreign antigen (81, 82). However, many differences also exist regarding the functional capacity of their cognate T cells and APC-T cell interactions. The following sections will focus on comparing and contrasting group 1 and group 2 CD1-restricted T cells in terms of TCR diversity, lipid recognition, cytokine production, and APC-T cell interactions. Table 1 highlights the properties of these two groups of CD1-restricted T cells.

TCR Diversity in CD1-Restricted T Cells

CD1d-restricted NKT cells can be divided into two subsets based on TCR diversity (83). Type I NKT cells, also known as invariant NKT (iNKT) cells, express an invariant TCR α chain. Murine type I NKT cells express an invariant Vα14-Jα18 TCR α chain paired with a limited set of TCR β chains, including Vβ8.2, Vβ7, and Vβ2 (83). Like murine type I NKT cells, human type I NKT cells express the invariant Vα24-Jα18 TCR α chain paired with predominantly the Vβ11 chain (84). All type I NKT cells recognize the marine sponge-derived glycolipid, α-galactosylceramide (α-GalCer) (85). Other populations of CD1d-restricted NKT cells that respond to lipid antigens are broadly classified as type II NKT cells, which exhibit more TCR sequence diversity compared to type I NKT cells (86). Type II NKT cells do not respond to α-GalCer, and therefore, cannot be identified using α-GalCer/CD1d tetramers (86). Several studies have suggested that group 1 CD1-restricted T cells have diverse TCR usage (4, 33, 74, 76). However, CD1b-restricted GEM T cells were recently described to express highly conserved TCRs composed of a TRAV1-2V segment rearranged with TRAJ9, with limited CDR3α diversity (70).

Lipid Antigen Recognition by Group 1 and Group 2 CD1-Restricted T Cells

Both group 1 and group 2 CD1 have been shown to present self-lipid antigens to CD1 autoreactive T cells. However, each member of the CD1 family binds distinct self-lipids. CD1d binds to sphingomyelin, gangliosides (e.g., GD3), globo/isoglobosides, phospholipids, plasmalogens, and lysophospholipids (82, 87–93). However, it remains unclear which of these lipids stimulate iNKT cells under physiological conditions and which contribute to the activation of iNKT cells during microbial infection (94). Gangliosides (e.g., GM1, GD1) were also described to bind to CD1b and activate CD1b-restricted T cells (95). Diacylglycerophosphocholines and diacylglycerophosphoinositols were shown to be bound by CD1c (87). However, it is unclear whether these self-lipids can be recognized by CD1c-restricted T cells. Self-lipids bound by CD1a molecules include squalene, wax esters, and triacylglycerides derived from the skin (33). Interestingly, sulfatide can bind to all

TABLE 1 | Comparison of group 1 and group 2 CD1-restricted T cells.

	Group 1 CD1-restricted T cells		Group 2 CD1-restricted T cells	
Types	Microbial antigen-specific	Autoreactive	Type I (iNKT)	Type II (vNKT)
Restricted by	CD1a, CD1b, CD1c		CD1d	
Antigens recognized	<i>Mtb</i> lipid antigens	CD1a – squalene, wax esters, triacylglycerides, sulfatide CD1b – gangliosides, sulfatide CD1c – methy-lysophosphatidic acid, sulfatide	α -GalCer analogs, glycosphingolipids, α -Gal-diacylglycerols, phospholipids, gangliosides, isoglobosides	β -GluCer, sulfatide, lysosulfatide, lysophospholipids
Antigen capture	CD1a – cell surface and early endosomes CD1b – late endosomes and lysosomes CD1c – endosomes		Endosomes and lysosomes	
Cell type that mediates selection	Unknown	Hematopoietic cells	Hematopoietic cells	
Subset	CD4+, CD8+, DN	CD4+, CD8+, DN	Human-CD4+, CD8+, DN Mouse-CD4+, DN	CD4+, CD8+, DN
TCR usage	Diverse TCR $\alpha\beta$ chains, V α 7.2-V β 9 (GEM T cells)	Diverse TCR $\alpha\beta$ chains	Human – V α 24- J α 18; V β 11 Mouse – V α 14- J α 18; V β 8.2, V β 7, V β 2,	Diverse TCR $\alpha\beta$ chains (mouse – V α 3.2, V α 8, V β 8 bias)
Effector functions	Cytotoxic, produce IFN γ , TNF α	Cytotoxic, produce IFN γ , TNF α , IL-17	Cytotoxic, produce Th1, Th2, Th17 cytokines	Cytotoxic, produce Th1, Th2 cytokines

group 1 and group 2 CD1 proteins (CD1a, CD1b, CD1c, CD1d) and activate CD1-restricted T cells (59, 96).

Most studies examining microbial antigen presentation by group 1 CD1 molecules have focused on *Mtb*, but CD1d-restricted iNKT cells have been shown to bind microbial antigens from a variety of pathogens. Both CD1d and CD1b molecules bind and present PIM moieties from *Mtb*, though they bind different isoforms (40, 97). In addition, diacylglycerol lipid species from pathogens including *B. burgdorferi* and *Streptococcus pneumoniae* have been identified as CD1d-restricted iNKT cell ligands, with physiologically relevant effects on T cell responses during infection (98, 99). No comparable diversity in microbial ligands has been shown to exist in group 1 CD1-mediated antigen presentation. However, the biochemical nature of the group 1 CD1 antigen-binding groove suggests that diverse microbial antigens can be presented.

Effector Functions of Group 1 and Group 2 CD1-Restricted T Cells

Group 1 and group 2 CD1-restricted T cells display antigen-specific cytotoxicity in the context of sterile cancer and infectious disease. Like group 1 CD1 autoreactive T cells, type I and type II NKT cells are capable of directly lysing tumor cells (100–103). Upon activation, type I NKT cells can produce both Th1 (IFN- γ , TNF- α) and Th2 (IL-4, IL-5, IL-13) cytokines, and this dual functionality enables them to play both pathogenic and immunoregulatory roles in various disease settings (104). Type I NKT cells were also shown to produce anti-inflammatory cytokines, such as IL-10 and TGF- β . Similar to type I NKT cells, type II NKT cells are also capable of producing multiple cytokines in response to polyclonal TCR stimulation, including IFN- γ , IL-4, GM-CSF, and IL-13 (105). These findings show that group 2 CD1-restricted NKT cells can produce a variety of Th1, Th2, and regulatory cytokines in response to different environmental cues.

Group 1 CD1-restricted T cells have also been shown to produce multiple cytokines in the context of infection, inflammation, and

cancer. Autoreactive group 1 CD1-restricted T cell lines produce IFN- γ , TNF- α , IL-17, and IL-22 in response to self-antigenic stimulation (5). Both *in vitro* and *in vivo* assays demonstrated that group 1 CD1-restricted T cells produce IFN- γ and TNF- α upon encountering mycobacterial antigens (4, 65, 66). Unlike group 2 CD1-restricted T cells, most group 1 CD1-restricted T cells do not appear to produce Th2 and/or regulatory cytokines in response to antigenic stimulation. However, to date, all the studies examining group 1 CD1-restricted T cells do so in *Mtb* infection settings. Further experiments need to be conducted to ascertain whether group 1 CD1-restricted T cells produce Th2 and/or regulatory cytokines under other infectious or inflammatory conditions.

Antigen-Presenting Cell-T Cell Interactions

Although all APCs expressing CD1d have the potential to present lipid antigens to NKT cells, studies depleting specific types of APCs have revealed that APC subsets differ in their capacity to prime type I NKT cells with lipid antigens (106). A recent study demonstrated that CD8 α $^+$ DEC-205 $^+$ dendritic cells were the main cell type responsible for capturing and presenting multiple forms of α -GalCer and stimulated type I NKT cell responses *in vivo* (106). However, it is unclear which APC subset is responsible for presenting *Mtb* antigens to group 1 CD1-restricted T cells *in vivo*. Macrophages are the primary host cells for *Mtb* but do not express group 1 CD1 molecules. It was demonstrated that group 1 CD1-expressing DCs take up apoptotic vesicles from bystander infected cells and presented them to human CD8 $^+$ T cells in an *in vitro* co-culture experiment (107). Thus, group 1 CD1-expressing DCs may similarly cross-present *Mtb* lipid antigens to T cells during infection (**Figure 1**).

Most of the studies involving CD1d have been performed in murine models. However, as humans possess both group 1 CD1 and group 2 CD1 molecules, it is possible that presence of group 1 CD1 molecules may affect CD1d antigen presentation. While there is some specialization in the antigen presentation functions of CD1

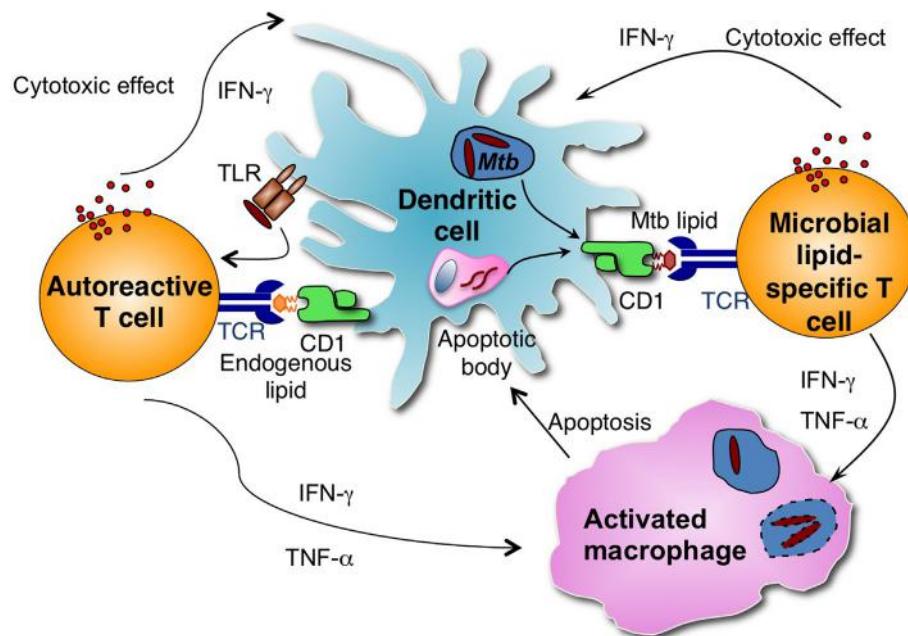


FIGURE 1 | Group 1 CD1-restricted T cells consist of autoreactive and microbial antigen-specific T cells. Microbial lipid-specific T cells are activated by microbial lipids that are loaded on group 1 CD1 molecules. Autoreactive T cells are activated in combination by proinflammatory cytokines produced on TLR engagement and self-lipid antigens. During *Mtb* infection, group 1 CD1-expressing DCs can either be directly infected by *Mtb*

or take up apoptotic vesicles from bystander infected cells and present *Mtb*-derived antigens to group 1 CD1-restricted *Mtb* lipid-specific T cells. Activated group 1 CD1-restricted T cells are cytotoxic and can directly lyse *Mtb*-infected cells. Activated group 1 CD1-restricted T cells also produce IFN- γ and TNF- α , which can activate infected macrophages and help to control *Mtb* growth.

molecules, whereby they sample lipids from different intracellular compartments, sulfatide, a glycosphingolipid, is capable of promiscuously binding to CD1a, CD1b, CD1c, and CD1d (59). As CD1c tends to be co-expressed with CD1d on blood dendritic cells and a fraction of B cells, a recent study evaluated how the presence of CD1c influences the activation of CD1d-restricted iNKT cells (108). This study demonstrated that CD1c is able to present α -GalCer as a weak agonist to human iNKT cells, and the presence of CD1c enhances α -GalCer-dependent activation of iNKT cells by CD1d (108).

Role of Group 1 and Group 2 CD1-Restricted T Cells During Infection

The studies described above support the likely role of group 1 CD1-restricted T cells in protective immunity against *Mtb* infection. However, the mechanisms by which they control *Mtb* infection remain to be elucidated. Aside from *Mtb*, the role of group 1 CD1-restricted T cells in other microbial infection has not been addressed. In contrast, the role of CD1d-restricted iNKT cells in host defense against various pathogens has been extensively studied (109). Studies examining iNKT cell dynamics in mice infected intravenously with BCG showed an early expansion of iNKT cells in the lung with a peak at day 8 after infection, followed by a subsequent decline likely due to programmed cell death (110, 111). Other studies have suggested that iNKT cells may play a role in the formation of granulomas in response to mycobacterial cell wall components (112, 113). Furthermore, *in vitro* experiments have shown that iNKT cells possess the ability to kill *Mtb*-infected APCs through the production

of GM-CSF (114). However, *Mtb* infection of CD1d $^{-/-}$ or J α 18 $^{-/-}$ mice with *Mtb* resulted in no significant difference in mortality or bacterial burdens in the lung, suggesting that iNKT cells may play a redundant role in the control of *Mtb* (115–117).

Conclusion

We highlight several recent studies that have contributed to our understanding of group 1 CD1 antigen presentation and the role group 1 CD1-restricted T cells play during *Mtb* infection. Further studies are needed to characterize *Mtb* lipid-specific T cell memory responses and evaluate whether *Mtb* lipid antigens can effectively be used in a subunit vaccine. Future studies also need to expand on the role of autoreactive group 1 CD1-restricted T cells in *Mtb* infections, as it is unknown whether they have similar activation kinetics as antigen-specific T cells and how they affect host immunity to infection. Moreover, it remains unclear whether group 1 CD1 molecules present lipids from other microbial species and whether they play a role in other inflammatory diseases, presenting fertile ground for future investigation.

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The response of CD1d-restricted invariant NKT cells to microbial pathogens and their products

Luc Van Kaer*, Vrajesh V. Parekh and Lan Wu

Department of Pathology, Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, TN, USA

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S. M. Mansour Haeryfar,
Western University, Canada

Reviewed by:

Tomasz Zal,
University of Texas MD Anderson
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Northwestern University, USA

*Correspondence:

Luc Van Kaer,
Department of Pathology,
Microbiology and Immunology,
Vanderbilt University School of
Medicine, Room AA5206A, Medical
Center North, 1161 21st Avenue
South, Nashville, TN 37232, USA
luc.van.kaer@vanderbilt.edu

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Invariant natural killer T (iNKT) cells become activated during a wide variety of infections. This includes organisms lacking cognate CD1d-binding glycolipid antigens recognized by the semi-invariant T cell receptor of iNKT cells. Additional studies have shown that iNKT cells also become activated *in vivo* in response to microbial products such as bacterial lipopolysaccharide, a potent inducer of cytokine production in antigen-presenting cells (APCs). Other studies have shown that iNKT cells are highly responsive to stimulation by cytokines such as interleukin-12. These findings have led to the concept that microbial pathogens can activate iNKT cells either directly via glycolipids or indirectly by inducing cytokine production in APCs. iNKT cells activated in this manner produce multiple cytokines that can influence the outcome of infection, usually in favor of the host, although potent iNKT cell activation may contribute to an uncontrolled cytokine storm and sepsis. One aspect of the response of iNKT cells to microbial pathogens is that it is short-lived and followed by an extended time period of unresponsiveness to reactivation. This refractory period may represent a means to avoid chronic activation and cytokine production by iNKT cells, thus protecting the host against some of the negative effects of iNKT cell activation, but potentially putting the host at risk for secondary infections. These effects of microbial pathogens and their products on iNKT cells are not only important for understanding the role of these cells in immune responses against infections but also for the development of iNKT cell-based therapies.

Keywords: invariant natural killer T cells, CD1d, glycolipid antigens, microbial pathogens, microbial products, immunological unresponsiveness, immunotherapy

Introduction

The immune response to microbial pathogens is orchestrated by reciprocal interactions between various components and cells of the innate and adaptive immune systems. While cells of the innate immune system recognize foreign invaders via conserved receptors that bind molecular patterns contained within a variety of microorganisms, cells of the adaptive immune system recognize foreign invaders via highly diverse antigen receptors that exhibit substantial pathogen-specificity. A key aspect of the adaptive immune system is its capacity to remember prior encounters with the same antigen, a property that forms the basis for the efficacy of vaccines. In addition to immune cells that can be clearly labeled as belonging to the innate or adaptive arms of the immune system, studies over the past few decades have identified several lymphocyte subsets that express antigen-specific receptors, yet exhibit many characteristics typical of cells of the innate immune system. This family of cells includes both B and T lineage cells and is often referred to as innate-like B and T lymphocytes (1). Innate-like B cells include B-1a and B-1b B cells, subsets of regulatory B (B_{reg}) cells, marginal

zone (MZ) B cells, and innate response activator (IRA) cells. Innate-like T cells include subsets of $\gamma\delta$ T cells, mucosal T cells expressing CD8 $\alpha\alpha$ homodimers, mucosal-associated invariant T (MAIT) cells, and natural killer T (NKT) cells. Each of these cell types expresses a limited repertoire of antigen-specific receptors, responds rapidly to antigenic stimulation, and is unable to induce long-lasting immunity. These cells cannot be easily categorized as innate or adaptive, and have therefore been referred to as "inbetweeners" (2). Several of these cell types reside at mucosal surfaces, body cavities, or entry points of lymphoid organs, where they are one of the first cell types to interact with pathogens, thus playing a sentinel function in the immune system. These cells, through recognition of non-specific innate immune signals and production of immunomodulatory cytokines, interact with and influence the function of multiple cell types in the innate and adaptive branches of the immune system, and thus shape subsequent inflammatory responses and impact disease outcomes. Such innate effector functions permit these cells to respond rapidly during the early stage of immune and inflammatory responses and serve as a bridge to adaptive immunity.

In this review article, we focus on NKT cells, and particularly the subset of NKT cells called invariant natural killer T (iNKT) cells. These cells play a critical role in the immune response against a variety of microbial pathogens, a topic that is described in a number of excellent review articles (3–5). Here, we focus on the mechanisms of iNKT cell activation by microbial pathogens and the dynamics of the ensuing iNKT cell response.

General Properties and Functions of iNKT Cells

Natural killer T cells are a subset of T lymphocytes that recognize lipid and glycolipid antigens when bound with the major histocompatibility complex (MHC) class I-related protein CD1d (6–9). Because the nomenclature of NKT cells and related cell types is confusing, we refer the reader to an opinion article on this topic (10). Two subsets of NKT cells have been identified: type 1 or iNKT cells express a semi-invariant T cell receptor (TCR), whereas type 2 or variant NKT (vNKT) cells express more diverse, yet oligoclonal TCRs (10).

Murine iNKT cells express V α 14-J α 18 chains paired with either V β 8.2, -7, or -2 chains, and human iNKT cells express homologous V α 24-J α 18 chains paired with V β 11. These cells also express a variety of receptors such as NK1.1 (expressed in some mouse strains) and members of the Ly49 family that are characteristic of the natural killer (NK) cell lineage. iNKT cells also express surface markers such as CD25, CD44, and CD69, which are characteristic of activated and memory T cells. The majority of iNKT cells also express the co-receptor CD4, and a small subset of human (but not mouse) iNKT cells expresses CD8 α . iNKT cells are most abundant in spleen, liver, thymus, and bone marrow, and are also found in lymph nodes, peripheral blood, adipose tissue, skin, and mucosal surfaces in the intestine and lungs. In humans, iNKT cells are less abundant than in mice and their prevalence varies widely among different individuals, for reasons that remain unclear.

Following their activation iNKT cells can quickly elicit an effector response, including rapid cytokine production and cytotoxicity, making them a very crucial component of the immune response (11). Activation of iNKT cells with a cognate ligand induces secretion of a wide variety of cytokines, chemokines, and colony-stimulating factors. During this activation process, iNKT cells also interact with other cells of the immune system, resulting in their activation, recruitment, and/or differentiation (12). While iNKT cells can simultaneously produce multiple cytokines, it is now clear that subsets of iNKT cells producing distinct cytokines and with distinct effector functions exist. This includes Tbet $^+$ NKT1 cells producing IFN- γ , GATA3 $^+$ NKT2 cells producing IL-4, ROR γ T $^+$ NKT17 cells producing IL-17A, IL-21, and IL-22 (13), and Bcl6 $^+$ follicular helper NKT (NKT $_{FH}$) cells producing IL-21 (14). iNKT cells with immunosuppressive functions have also been identified, including regulatory NKT10 cells producing IL-10 (15), E4BP4 $^+$ regulatory iNKT cells in adipose tissue producing IL-2 and IL-10 (16), and Foxp3 $^+$ regulatory iNKT cells (17). Whether the latter cell types represent separate subsets of regulatory iNKT cells remains unclear (18).

Because of their ability to produce such a mixture of cytokines and to interact with a variety of other cells of the immune system, iNKT cells can either promote or suppress immune responses in different disease conditions (11, 19). They confer natural immunity to cancer (20), provide protective immunity to various infectious agents (3–5), generally play a suppressive role during autoimmune responses (18) and graft-vs.-host disease (21), and contribute to the development of allergic airway reactivity (22), contact hypersensitivity (23), experimental hepatitis (24), atherosclerosis (25), and obesity-associated insulin resistance (26). Because iNKT cells display such a wide variety of versatile functions, they have been referred to as the "Swiss army knife of the immune system" (27).

In keeping with their immunoregulatory functions, numerous studies have explored the therapeutic activities of iNKT cells in a variety of diseases (6–9, 11, 12). Many of these studies have been performed with the prototypical iNKT cell antigen α -galactosylceramide (α -GalCer), a potent iNKT cell agonist that was originally isolated from a marine sponge (28). α -GalCer and related glycolipids have potent anti-metastatic activities (29), hasten clearance of some microbial pathogens (3–5), enhance the efficacy of vaccines (30, 31), prevent graft-vs.-host disease (21), and protect against autoimmunity in experimental models for type 1 diabetes (32), multiple sclerosis (33), arthritis (34), and systemic lupus erythematosus (35).

Mechanisms of iNKT Cell Activation by Microbial Pathogens

Invariant natural killer T cells become activated in response to challenge by a variety of microorganisms, including bacteria, viruses, fungi, and protozoa (5). While some of these microorganisms contain glycolipid or phospholipid antigens that can bind with CD1d to activate the iNKT cell TCR, most microorganisms activate iNKT cells independently of cognate antigens. iNKT cells are highly responsive to stimulation by certain types of cytokines, which may be induced in antigen-presenting cells

(APCs) via engagement of pathogen recognition receptors (PRRs) with pathogen-associated molecular patterns (PAMPs). Activation of iNKT cells via superantigens has also been reported. We will briefly discuss these distinct modes of iNKT cell activation in the following sections.

iNKT Cell Activation by Microbial Lipid Antigens

A number of microorganisms, especially bacteria, contain lipid antigens that can activate iNKT cells (**Figure 1A**). *Sphingomonas* species, which include organisms that are ubiquitous in the environment, produce glycosphingolipids with α -linked glucuronic or galacturonic acid (36–38), and *Borrelia burgdorferi* (39) and *Streptococcus pneumoniae* (40) contain diacylglycerols with α -linked glucosyl or galactosyl moieties that are recognized by the iNKT cell TCR. Other documented or proposed iNKT cell antigens include phosphatidylinositol mannoside from *Mycobacterium bovis* (41), a cholesterol ester with an α -linked glucoside from *Helicobacter pylori* (42), an α -GalCer from the common gut bacterium *Bacteroides fragilis* (43), lipophosphoglycans from the protozoan parasites *Leishmania donovani* (44) and *Entamoeba histolytica* (45), and the glycosphingolipid asperamide B from the fungal pathogen *Aspergillus fumigatus* (46). While most of these antigens activate all iNKT cells, some likely activate only a subset of iNKT cells (5). Interestingly, one study showed that *B. fragilis* contains, in addition to an iNKT cell-activating α -GalCer, an inhibitory α -GalCer (Bf717) that regulates the homeostasis of host intestinal iNKT cells (47).

Some of the microbial antigens, especially those derived from *Sphingomonas* bacteria, bear structural similarity with α -GalCer, the prototypical iNKT cell antigen obtained from the marine sponge *Agelas mauritianus* (28). This finding led to speculation that α -GalCer might, in fact, be derived from bacteria, possibly *Sphingomonas* species, that colonize the sponge. As *Sphingomonas*

bacteria are ubiquitous in the environment, including soil and the ocean, this is a likely yet unproven explanation for the rather strange capacity of sponge-derived products to activate a small subset of cells in the mammalian immune system.

While purified or synthetic versions of microbial antigens can potently activate iNKT cells both *in vitro* and *in vivo*, the contribution of these antigens to the response of iNKT cells to intact microorganisms is less clear. Instead, the available evidence suggests that innate cytokine-driven signals, rather than microbial antigens, are the main drivers of iNKT cell activation during microbial infection (5).

Cytokine-Driven iNKT Cell Activation During Microbial Infection

A major difference between conventional T cells and iNKT cells is that the latter but not the former are highly responsive to innate and cytokine-driven signals (5). iNKT cells constitutively express a number of cytokine receptors, most notably the receptors for IL-12 (48) and IL-18 (49). Consistent with their innate effector functions, stimulation of iNKT cells with IL-12 or IL-18 can induce IFN- γ production by these cells. In this context, iNKT cells have been shown to play a critical role in the anti-tumor activities of IL-12 (50). IL-12 has been implicated in the capacity of many microorganisms to activate iNKT cells. This phenomenon was initially described for *Salmonella typhimurium*, a Gram-negative bacterium that lacks cognate iNKT cell antigens (51). APCs cultured with this organism were able to induce IFN- γ production by iNKT cells, which was blocked by addition of neutralizing anti-IL-12 antibodies. In addition to the intact microorganisms, *S. typhimurium* lipopolysaccharide (LPS) similarly activated iNKT cells in an IL-12-dependent manner, suggesting a critical role for toll-like receptor (TLR) activation in the APCs. These findings lead to the concept that microbes lacking cognate antigens can activate iNKT cells in a manner that involves TLR signaling in APCs, production of IL-12 by the APCs, and IL-12R signaling in iNKT cells (**Figure 1B**). This concept has been tested and extended to iNKT cell activation by a variety of microorganisms, including viruses, bacteria, fungi, and protozoa (5). It was shown that TLR ligands for either cell surface or endosomal TLRs may be involved and, in the case of fungi, β -glucans that signal through Dectin-1 on APCs can similarly activate iNKT cells (52). While IL-12 played a critical role in iNKT cell activation induced by many microbes, IL-18 was the dominant APC-derived cytokine responsible for iNKT cell activation to LPS derived from *Escherichia coli* (53), and type 1 interferons played a dominant role in the activation of iNKT by the TLR-9 agonist CpG (54). Based on these findings, a general model has emerged for the activation of iNKT cells by microorganisms that lack cognate antigens (**Figure 1B**): PAMPs activate APCs (predominantly DCs) to produce pro-inflammatory cytokines, which, in turn, activate iNKT cells. As already mentioned, additional evidence suggests that this might also be the dominant pathway for iNKT cell activation by many microbes that contain iNKT cell antigens (55).

An unusual mode of cytokine-driven iNKT cell activation was observed for hepatitis B virus (HBV) (56). HBV induces secretory phospholipases in infected hepatocytes that convert phosphatidylethanolamine to lysophospholipids. The

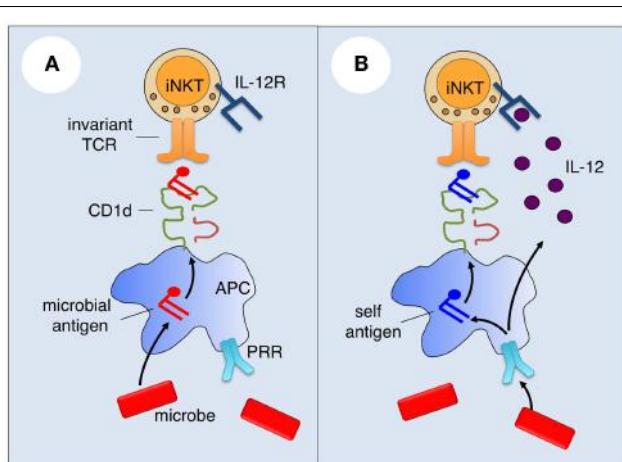


FIGURE 1 | Mechanisms of microbial iNKT cell activation. (A) For microbes that contain iNKT cell antigens, such antigens may be sampled by antigen-presenting cells (APCs) and loaded onto CD1d for presentation and activation of iNKT cells. **(B)** Microbes lacking or containing iNKT cell antigens can activate iNKT cells by innate cytokine-driven mechanisms. Microbial products may engage pattern recognition receptors (PRRs) on APCs to induce cytokines such as IL-12 that bind with cytokine receptors on iNKT cells, and the production of endogenous iNKT cell antigens.

lysophospholipids bind CD1d to activate type 2 NKT (vNKT) cells that in turn induce IL-12 production by APCs to indirectly activate iNKT cells. These findings are consistent with prior studies providing evidence that activation of vNKT cells with cognate antigens can lead to the trans-activation of iNKT cells (57).

A topic of some debate is whether iNKT cells themselves express functional TLRs and, thus, might be activated directly by PAMPs independently of APCs (5, 58). One study showed that TCR engagement on iNKT cells can induce TLR expression, which was able to enhance iNKT cell activation following TLR stimulation (59). Whether direct TLR engagement on iNKT cells contributes to their activation during microbial infections remains to be explored.

During the original studies with *S. typhimurium*, it was found that iNKT cell activation in the *in vitro* cultures could be partially blocked with anti-CD1d antibodies, suggesting a role for TCR engagement on iNKT cells (51). Similar observations were made for a number of other microbes (5, 60). These findings suggested that microbes activate iNKT cells in a manner that involves both cytokine receptor- and TCR-mediated signaling (Figure 1B). However, activation of iNKT cells by some microbes such as murine cytomegalovirus (MCMV) (61) and by microbial products such as *E. coli* LPS (53), did not appear to require TCR signaling, suggesting that cytokine signaling is sufficient to activate iNKT cells during infections.

While some cytokines such as type 1 interferons can induce CD1d expression (62), microbial infection is not always associated with an increase in CD1d expression on APCs. In fact, several microbes interfere with CD1d expression, presumably in an attempt to avoid iNKT cell responses (63). Nevertheless, induction of CD1d expression on APCs might contribute to iNKT cell activation during certain infections.

An appealing hypothesis emerging from these studies was that microbes might induce endogenous lipid antigens for iNKT cells (Figure 1B). This possibility was supported by the finding that microbial products can induce enzymes involved in glycosphingolipid synthesis and that inhibitors of this pathway can suppress iNKT cell activation by certain microbial products (64). Much debate has focused on the nature of the relevant glycolipid(s) involved. While iNKT cells potently react with α -linked but not β -linked glycolipids, production of glycosphingolipids in mammals has long been assumed to be limited to β -linked anomers. Nevertheless, additional studies identified the β -linked glycosphingolipid isoglobotrihexosylceramide (iGb3) as a weak self-antigen that was also suggested to be involved in iNKT cell activation in response to microbial products (37, 65). Subsequent studies cast doubt on this possibility and instead provided evidence that β -linked glucosylceramides (β -GluCer) that accumulate in mammalian cells in response to microbial products are the relevant self-antigens that synergize with APC-derived cytokines during the activation of iNKT cells by microbial products (66). The latter studies were predominantly performed with synthetic versions of β -GluCer, which, as it turned out, contained minuscule amounts of α -anomeric GluCer (67, 68). Careful studies with iNKT cell-stimulating glycosphingolipids enriched from mammalian cells eventually led to the conclusion that mammalian cells produce small amounts of α -linked glycosphingolipids such as α -GalCers

and α -GluCers that can activate iNKT cells (67, 68). However, the enzymatic pathways involved in the synthesis of these antigens remain to be identified.

Most of the studies implicating a role of CD1d and self-antigens in the capacity of microbes or their products to activate iNKT cells were performed *in vitro*. The contribution of TCR engagement to *in vivo* iNKT cell activation by microbes therefore remained unclear. Surprisingly, using a reporter mouse that can detect TCR signaling, a recent study showed that *S. typhimurium* and several TLR ligands were able to activate iNKT cells in a TCR-independent manner (69). Therefore, these findings indicate that many microbes can activate iNKT cells in the absence of TCR signaling.

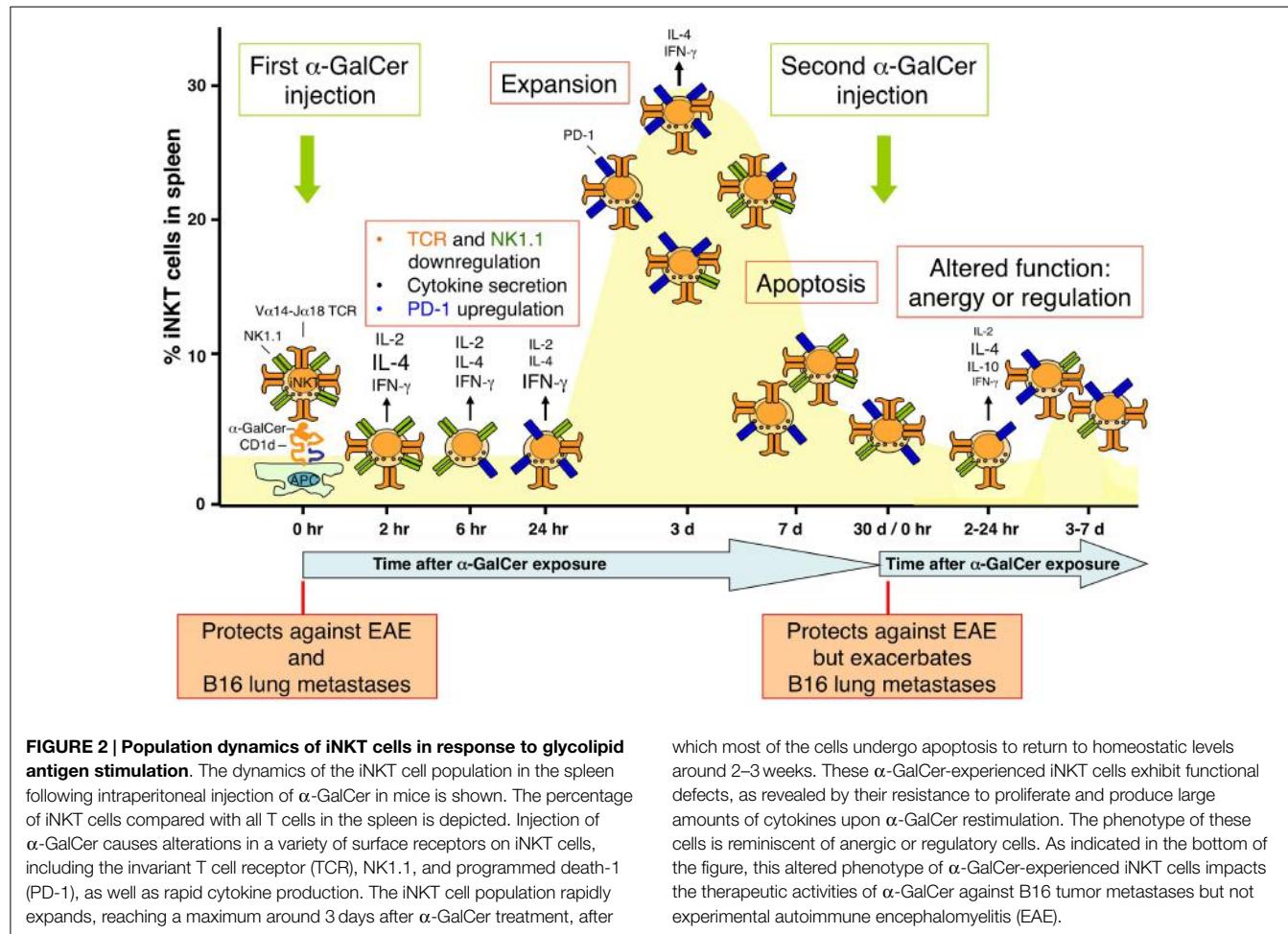
Superantigen-Mediated iNKT Cell Activation

Superantigens are microbial toxins that cause non-specific activation of T cells by engaging MHC class II molecules and the variable region of the β -chain of the TCR. *Staphylococcal* enterotoxin B (SEB) interacts with V β 8, which is expressed by a majority of iNKT cells. SEB was able to activate V β 8-expressing iNKT cells in a CD1d-independent manner (70, 71).

The *in vivo* Response of iNKT Cells to Glycolipid Antigens

Most studies that have investigated the *in vivo* response of iNKT cells have focused on synthetic glycolipid antigens, most notably KRN7000, an optimized version of the original sponge-derived α -GalCer. These studies have revealed that the *in vivo* response of iNKT cells to an intraperitoneal injection of α -GalCer is characterized by the following series of events (Figure 2) (72):

1. Prompt activation and cytokine production: α -GalCer is presented to iNKT cells predominantly by CD8 α -expressing DCs and potentially some macrophages. iNKT cell activation involves induction of a variety of activation markers (e.g., CD69, CD25, and ICOS), as well as cytokine production, with an initial burst of IL-4 (as soon as 1 h after treatment with a peak at 4 h) followed by IFN- γ (peaks at 24 h). However, this cytokine production gradually diminishes to very low levels at 3 days after treatment (11).
2. Cross-talk with other cell types: α -GalCer-activated iNKT cells engage in extensive cross-talk with other immune cell types (11). This includes activation, induction of cytokine production (most notably IL-12), and differentiation of DCs and macrophages, modulation of neutrophils, recruitment and modulation of the suppressive activities of myeloid-derived suppressor cells, profound activation and induction of IFN- γ production by NK cells, modulation of B cell and antibody responses, and modulation of CD8 and CD4 T cell responses. Most studies have provided evidence that α -GalCer treatment promotes Th2-dominant immunity. These effects form the basis of the immunomodulatory and therapeutic properties of α -GalCer and other iNKT cell antigens (12).
3. TCR downregulation: quickly following their activation by α -GalCer, iNKT cells profoundly downregulate their TCR (73). This is due to agonist-mediated inhibition of TCR recycling to the cell surface and makes these cells nearly undetectable by



- staining with anti-CD3 antibodies, anti-TCR antibodies, and CD1d-tetramers for a short time period, around 12–30 h after treatment.
- NK1.1 downregulation: NK1.1 downregulation starts around 24 h after treatment, making it hard to accurately detect iNKT cells by anti-NK1.1 antibodies for an extended time period (19, 73). NK1.1 expression slowly returns to normal levels, but only about half of these cells express NK1.1 at 1 month after treatment.
 - Induction of the programmed death-1 receptor: programmed death-1 (PD-1) is an inhibitory member of the CD28 family of co-stimulatory molecules by interacting with its ligands PD-L1 and PD-L2. PD-1 has received a lot of interest in the tumor immunology field as a potent immune checkpoint whose blockade can unleash anti-tumor responses. PD-1 expression by iNKT cells is evident as early as 2–3 days after α -GalCer treatment and is sustained for up to 2 months (74–76).
 - Population expansion: iNKT cells expand in spleen and to a lesser extent in peripheral blood, bone marrow, and liver (73, 77). Expansion is maximal around 3 days after α -GalCer treatment and reaches levels about 10- to 15-fold over the starting population in spleen.
 - Apoptosis and return to homeostatic levels: following their expansion, most iNKT cells undergo apoptosis and the iNKT

cell population returns to relatively normal homeostatic levels around 2–3 weeks after their expansion (73, 78). Apoptosis of iNKT cells involves the pro-apoptotic Bcl-2 family member Bim (77) and Fas/FasL interactions (79).

- Acquisition of a hyporesponsive phenotype: as revealed by a blunted response to α -GalCer re-injection after the initial α -GalCer treatment, α -GalCer-experienced iNKT cells become unresponsive to α -GalCer restimulation (19, 78). This hyporesponsiveness was observed in terms of reduced iNKT cell activation (lack of induction of activation markers), blunted proliferation and cytokine production (IFN- γ production was more profoundly blunted than IL-4 production), and reduced capacity to activate other cell types such as DCs and NK cells. This hyporesponsive phenotype was evident between 3 days and up to 2 months after the original α -GalCer treatment. This hyporesponsive phenotype of iNKT cells was largely intrinsic to these cells and has been referred to as iNKT cell anergy. The induction and to a lesser extent the maintenance of this anergic phenotype involves PD-1/PD-L interactions (74–76, 80), as well as the egr2/3 transcription factors (80), which induce the E3 ligase Cbl-b that monoubiquitinates the CARMA1 signaling molecule in the NF- κ B signaling pathway (81). An alternative explanation for the long-term effects of α -GalCer on iNKT cells proposed more recently is that the iNKT cells

in α -GalCer-experienced mice adopt a regulatory phenotype with production of IL-10 (i.e., NKT10 cells) (15). Regardless of the mechanism involved, it has been established that α -GalCer-experienced iNKT cells exhibit impaired anti-tumor responses but retain their capacity to protect mice against experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis (19).

In addition to α -GalCer, the response of iNKT cells to a variety of other glycolipids has been investigated (11, 12). Many of these studies have focused on the therapeutic properties of iNKT cells and have identified glycolipid antigens that induce biased cytokine responses in iNKT cells in attempt to enhance either their anti-tumor activities or their protective effects against autoimmune or inflammatory diseases. Additional studies have explored methods to prevent or overcome the altered phenotype of α -GalCer-experienced iNKT cells. This has been accomplished by delivering α -GalCer in the context of strong co-stimulation such as α -GalCer-loaded DCs (19, 82), via intradermal, intranasal, or oral rather than systemic administration (83, 84), nanoparticles (85), recombinant CD1d molecules (86), or PD-1/PD-L blockade (74, 75, 87). Additionally, glycolipids that can potently activate iNKT cells, yet largely lack the long-term effects on iNKT cells associated with α -GalCer, have also been developed (88).

These methodologies to prevent induction of iNKT cell functional impairments are particularly important for developing improved iNKT cell-based therapies. Clinical studies with human subjects have shown long-term effects of free glycolipid treatment on human iNKT cells (89). Repeated free α -GalCer treatment resulted in increasingly weaker biological responses, which was consistent with the acquisition of iNKT cell dysfunction upon α -GalCer stimulation. Interestingly, delivery of α -GalCer in the context of DCs was able to avoid the induction of iNKT cell dysfunction in human cancer patients (90). In this context, it is also worth noting that several preclinical studies have shown that the anti-metastatic activities of α -GalCer synergize with those of PD-1/PD-L blockade (75, 87). Thus, the therapeutic activities of iNKT cells may be enhanced by methods that prevent the induction of activation-induced iNKT cell dysfunction.

The *in vivo* Response of iNKT Cells to Microbes

Our knowledge regarding the *in vivo* response of iNKT cells to glycolipid antigens has been employed as a framework to explore the response of these cells to microbes. Most microbes and many of their products can activate iNKT cells to express a variety of activation markers and to induce cytokine production, with wide effects on other immune cell types and the outcome of the infection (5). While α -GalCer induces both IL-4 and IFN- γ production by iNKT cells, microbes typically induce little IL-4 production, which is consistent with the notion that most microbes activate iNKT cells in an innate cytokine-driven manner and that IL-12 promotes an IFN- γ -biased cytokine profile in these cells. When investigated, PD-1 upregulation was not observed, but sustained NK1.1 downregulation was common. In sharp contrast with α -GalCer, microbes or their products rarely induce systemic iNKT cell expansion *in vivo* (as discussed below, *M. bovis* is an

exception), and this is true even for microbes containing iNKT cell antigens. Nevertheless, an accumulation of iNKT cells has been observed in some infected organs, such the lungs of mice infected with *Cryptococcus neoformans* (91) and the liver of mice infected with malaria parasites (92). A phenomenon observed for some microbes, including systemic infection with lymphocytic choriomeningitis virus (LCMV) (93) and *L. monocytogenes* (94, 95), is partial or complete iNKT cell depletion, which may last for several weeks.

Systemic exposure to a number of microorganisms, including *E. coli*, *S. aureus*, *S. typhimurium*, *L. monocytogenes*, and *M. bovis*, has long-term effects on iNKT cell kinetics and functions, resulting in a hyporesponsive phenotype reminiscent of that observed following α -GalCer treatment (94–96). A similar phenotype was observed for mice treated with TLR agonists such as LPS and flagellin (95, 96). Induction of this hyporesponsive phenotype required IL-12 expression (95), which itself does not induce iNKT cell dysfunction and is not required for α -GalCer-induced iNKT cell hyporesponsiveness. Furthermore, microbe-induced iNKT cell dysfunction involved both iNKT cell-intrinsic and -extrinsic mechanisms and was independent of the PD-1/PD-L pathway. While α -GalCer-experienced iNKT cells exhibited more profound defects in IFN- γ than IL-4 cytokine production, the opposite was true for the functional alterations of iNKT cells in response to microbes. Thus, the mechanisms involved in the induction of iNKT cell dysfunction mediated by glycolipids and microbes appear to be distinct. Whether specific regulatory iNKT cell subsets expand during microbial infections has not been explored. iNKT cells from mice systemically exposed to *E. coli* exhibited impairments in their therapeutic activities against metastatic tumors, but not in their capacity to protect mice against EAE (95). The latter finding might have important implications when considering iNKT cell-based therapies, as they suggest that the functions and therapeutic activities of iNKT cells in patients are influenced by recent infections.

A few studies have investigated the response of human iNKT cells to microbial pathogens. HIV infection substantially decreases iNKT cell numbers and functions, and this depletion was most profound for the CD4 $^{+}$ subset (97–99). The reduced numbers of iNKT cells may be due to a combination of HIV infection and induction of apoptosis (97, 99). Interestingly, the residual iNKT cells in infected individuals exhibited impaired ability to proliferate and produce IFN- γ in response to α -GalCer stimulation, and expressed elevated levels of PD-1 (100). Blocking experiments indicated that these functional defects were largely PD-1-independent (100). A similar although less profound reduction in iNKT cell numbers was observed in patients with active *M. tuberculosis* infection (101). The poor response of iNKT cells from these patients to α -GalCer was found to be due to increased iNKT cell apoptosis and iNKT cell dysfunction. The latter was associated with an elevated expression of PD-1, and blockade of PD-1 signaling was able to enhance the response to α -GalCer (101). iNKT cells were found to be activated during acute dengue virus infection, and the level of activation was associated with disease severity (102). These cells also exhibited reduced functional responses to subsequent α -GalCer stimulation but mechanisms were not explored (102). These studies suggest that at least some

of the findings obtained in mice may also apply to infections in humans.

The response of iNKT cells to microbial pathogens makes sense from the standpoint of host-pathogen interactions. The effector functions of iNKT cells play a critical role by influencing the behavior of cells of the innate arm of the immune system and to assist in the initiation and differentiation of adaptive immune responses. Thus, iNKT cells predominantly contribute to early immune responses and their capacity to produce cytokines should therefore largely be limited to a relatively short time window early in an infection. Overactivation of iNKT cells is known to cause severe immunopathology such as liver damage (103, 104). As iNKT cells produce large bursts of cytokines with potent pro-inflammatory properties, the cytokine production potential of these cells needs to be tightly controlled to avoid the generation of a cytokine storm or a chronic inflammatory response. This may be accomplished by inducing apoptosis or functional impairments in these cells. One potential disadvantage of this strategy is that it might put the host at risk for developing secondary infections with organisms that depend on iNKT cells for protective immunity.

As responses of iNKT cells to distinct types of microbes are quite divergent, we briefly discuss below the response of iNKT cells to select microbial organisms.

Listeria monocytogenes

Intravenous inoculation of *L. monocytogenes* resulted in rapid induction (within 1 day) of the activation marker CD69 on iNKT cells and these cells produced IFN- γ but not IL-4 (94, 95). This activation resulted in a gradual reduction in the number of iNKT cells in spleen and liver. This reduction in the prevalence of CD1d/ α -GalCer-tetramer⁺ cells was not just due to activation-induced downregulation of TCR expression. These cells recovered by week 4 in the liver but not spleen. NK1.1 was downregulated for an extended time period and closely mirrored its expression following α -GalCer treatment. iNKT cells in *L. monocytogenes*-experienced mice showed reduced responses to α -GalCer in terms of CD69 induction, expansion, cytokine production (with more profound effects on IL-4 than IFN- γ), and trans-activation of DCs and NK cells. These reduced responses were not just due to lower numbers of iNKT cells but involved iNKT cell dysfunction, which was present for at least 1 month after infection. iNKT cells have been shown to play a protective role in the immune response against *L. monocytogenes* (105).

Mycobacterium bovis

Intravenous inoculation of *M. bovis* vaccine strain bacillus Calmette-Guérin (BCG) causes an increase in CD69 expression on iNKT cells by day 7, which was further increased at day 14 in both spleen and liver (96). NK1.1 became markedly downregulated for an extended time period. Alterations in PD-1 or CTLA-4 expression by iNKT cells were not detected. Numbers of iNKT cells increased following infection, expanding approximately twofold in the spleen and fivefold in the liver at 7 days after infection. Numbers of iNKT cells subsequently contracted, reaching pre-infection levels around 2–3 weeks after infection. iNKT cell death was associated with an increase in Fas expression on these cells. iNKT cells were able to produce IFN- γ quickly following infection,

reaching a peak at day 7, but the capacity to produce IFN- γ quickly waned thereafter. iNKT cells from infected animals also became resistant to CD69 upregulation in response to α -GalCer treatment. This refractory period lasted for approximately 1 month. While iNKT cell-deficient mice eliminated BCG as efficiently as wild-type mice, these animals had more granulomas in liver and lung, with signs of caseation, large cellular infiltrates, and some multinucleated macrophages, which were not seen in wild-type animals (106). These findings therefore suggested an anti-inflammatory role for iNKT cells during BCG infection.

Lymphocytic Choriomeningitis Virus

Intraperitoneal infection of mice with the Armstrong strain of LCMV caused a selective, long-term loss of iNKT cells in both spleen and liver (93, 107). This apparent loss of iNKT cells was not just due to downregulation of TCR expression. It was observed as soon as 3 days after infection, was most profound around 10 days, and lasted up to 3 months. The dying cells expressed active caspase 3, indicating apoptosis, but this process was independent of Fas/FasL interactions. While the reasons for this sustained loss of iNKT cells remains unclear, it has been suggested to be due to either activation-induced cell death or direct virus infection. Wild-type and CD1d-deficient animals cleared LCMV at similar levels but splenocytes from CD1d-deficient animals produced significantly higher amounts of cytokines (IL-2, IL-4, and IFN- γ), suggesting that iNKT cells suppress the magnitude of the acute antiviral immune response against LCMV (108).

Interaction of iNKT Cells with the Microbiota

A microbiota is a group of microorganisms that resides in a specific environment. The human host engages in mutualistic relationships with commensal microbes that reside in different parts of the body, especially the gastrointestinal tract. Recent studies have provided evidence that iNKT cells are influenced by the microbiota and that, conversely, iNKT cells can shape the composition of the microbiota (109). Germ-free animals were shown to contain increased numbers of mature, functionally competent iNKT cells in the gut and lung as compared with specific pathogen-free mice (110, 111). These alterations in iNKT cells made germ-free mice more susceptible to tissue damage and inflammation in mouse models of asthma and inflammatory bowel disease. These effects of microbiota on iNKT cells were not limited to mucosal surfaces, as splenic iNKT cells from germ-free animals exhibited reduced expression of activation markers and reduced capacity to produce cytokines. Similar findings were made for fetal human iNKT cells (112), which develop in the absence of microbiota. Fetal small intestinal iNKT cells were phenotypically and functionally mature, whereas their splenic counterparts exhibited reduced expression of activation markers. These findings provide the intriguing possibility to manipulate iNKT cell numbers and functions via the microbiota. The mouse studies have shown that iNKT cells can be reprogrammed in this manner during neonatal but not adult life.

The finding that iNKT cell numbers and functions are influenced by the gut microbiota provides a potential explanation for

some of the divergent studies that have been published in the iNKT cell field. For example, pathogenic (113), neutral (114–117), and suppressive (118, 119) roles of iNKT cells in the pathogenesis of obesity-associated inflammation and insulin resistance have been reported (26). This might be caused by differences in the endogenous microbiota in the animal facilities where the different studies were performed.

Mechanisms responsible for the effects of the microbiota on iNKT cell numbers and functions remain unclear but may include recognition of microbe-associated molecular patterns, microbial metabolites, and microbial iNKT cell antigens. With regard to the latter possibility, a provocative study showed that monocolonization of germ-free mice with *B. fragilis* can restore the colonic iNKT cell levels (47). As mentioned above, *B. fragilis* contains α -GalCers that can either activate (43) or inhibit (47) iNKT cells. The inhibitory α -GalCer (Bf717) from this organism was able to limit CD1d-dependent colonic iNKT cell proliferation in germ-free mice (47).

Summary and Outstanding Questions

The studies discussed here have revealed that iNKT cells become activated during infection by different types of pathogenic as well as commensal microorganisms. While some microorganisms contain iNKT cell ligands, their contribution to iNKT cell activation to the intact organism remains unclear. Innate cytokine-driven pathways play a dominant role in microbial iNKT cell activation. iNKT cells often become activated and produce cytokines early after microbial infection and may transiently expand, contract, or maintain their population size. A common feature of the response of iNKT cells to microbial infection is that it induces long-term unresponsiveness to TCR stimulation.

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Despite this progress in our understanding of the response of iNKT cells to microbes, a number of outstanding questions remain to be answered:

- What is the contribution of microbial iNKT cell antigens to the immune response of iNKT cells to microbial pathogens?
- Do endogenous iNKT cell antigens play a role in the response of iNKT cells to microbial pathogens *in vivo*?
- What are the molecular mechanisms responsible for the long-term effects of distinct microbes and their products on the numbers and functions of iNKT cells?
- Can commensal microorganisms or their glycolipid antigens be employed to reprogram iNKT cell functions in humans?
- How do the mechanisms responsible for microbial iNKT cell activation relate to those that mediate iNKT cell activation during sterile inflammation?
- How similar are the mechanisms that control iNKT cell activation in response to microbes in mice and humans, and how do they differ?
- How can this information be employed for the development of improved iNKT cell-based therapies?

These questions will provide rich and fertile avenues for future investigations.

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Recognition of microbial glycolipids by natural killer T cells

Dirk M. Zajonc* and Enrico Girardi†

Division of Cell Biology, La Jolla Institute for Allergy and Immunology, La Jolla, CA, USA

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Edited by:

Thierry Mallevaey,
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Reviewed by:

Sho Yamasaki,
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Monash University, Australia

***Correspondence:**

Dirk M. Zajonc,
Division of Cell Biology, La Jolla
Institute for Allergy and Immunology,
9420 Athena Circle, La Jolla,
CA 92037, USA
dzajonc@iitai.org

†Present address:

Enrico Girardi,
CeMM, Research Center for
Molecular Medicine of the Austrian
Academy of Sciences,
Vienna, Austria

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T cells can recognize microbial antigens when presented by dedicated antigen-presenting molecules. While peptides are presented by classical members of the major histocompatibility complex (MHC) family (MHC I and II), lipids, glycolipids, and lipopeptides can be presented by the non-classical MHC member, CD1. The best studied subset of lipid-reactive T cells are type I natural killer T (iNKT) cells that recognize a variety of different antigens when presented by the non-classical MHC homolog CD1d. iNKT cells have been shown to be important for the protection against various microbial pathogens, including *B. burgdorferi*, the causative agents of Lyme disease, and *S. pneumoniae*, which causes pneumococcal meningitis and community-acquired pneumonia. Both pathogens carry microbial glycolipids that can trigger the T cell antigen receptor (TCR), leading to iNKT cell activation. iNKT cells have an evolutionary conserved TCR alpha chain, yet retain the ability to recognize structurally diverse glycolipids. They do so using a conserved recognition mode, in which the TCR enforces a conserved binding orientation on CD1d. TCR binding is accompanied by structural changes within the TCR binding site of CD1d, as well as the glycolipid antigen itself. In addition to direct recognition of microbial antigens, iNKT cells can also be activated by a combination of cytokines (IL-12/IL-18) and TCR stimulation. Many microbes carry TLR antigens, and microbial infections can lead to TLR activation. The subsequent cytokine response in turn lower the threshold of TCR-mediated iNKT cell activation, especially when weak microbial or even self-antigens are presented during the cause of the infection. In summary, iNKT cells can be directly activated through TCR triggering of strong antigens, while cytokines produced by the innate immune response may be necessary for TCR triggering and iNKT cell activation in the presence of weak antigens. Here, we will review the molecular basis of iNKT cell recognition of glycolipids, with an emphasis on microbial glycolipids.

Keywords: microbes, glycolipids, antigen-presentation, CD1d, TCR, NKT cells

Introduction

Microbial antigens can be recognized by various receptors of both the innate and adaptive immune system. While many receptors, especially innate immune receptors, bind their antigen in free form, T cell antigen receptors (TCRs), which are exclusively expressed on T cells, have evolved to recognize antigens when presented by dedicated antigen-presenting molecules (1). This feature distinguishes them from soluble immunoglobulins (Igs), which can bind to virtually any molecule in solution, even though, the Ig fragment that is responsible for antigen binding (Fab) is structurally very similar to the TCR. While exceptions to this rule exist, such as gamma delta ($\gamma\delta$) TCRs, which can bind

antigens with or without antigen-presenting molecules (2), for the purpose of this review, we will exclusively focus on cells carrying the more well-characterized $\alpha\beta$ TCRs.

T lymphocytes are key cells of the adaptive immune system. They recognize infection, initiate, and regulate immune responses, especially by controlling the activation of bystander immune cells (3). The hallmark of T cell activation is the direct binding of the TCR to an antigen that is presented by major histocompatibility complex (MHC)-encoded molecules. While classical MHC class I or II molecules are important for peptide presentation, non-classical MHC molecules, especially CD1, are required for glycolipid presentation to T cells (4–8). Other non-peptidic antigens, such as microbial vitamin B metabolites, can also be recognized by T cells when they are presented by the non-classical MHC I molecule, MR1 (9, 10).

The CD1 Family

First identified in the late 1980s in the group of Cesar Milstein, CD1 is a group of MHC class I-like antigen-presenting molecules (11). CD1 proteins exhibit little or no polymorphism, in stark contrast to the MHC-encoded antigen-presenting molecules. However, the number of expressed CD1d genes varies widely by species. Humans express five functional isotypes (CD1a–e) (12), with CD1e being the only member that does not directly present antigens to T cells (13). Mice express only CD1d, while other species, such as guinea pigs, express multiple forms of the same isotype, CD1b (14). CD1 molecules are conserved throughout vertebrate evolution (15) and have been identified to present lipids, lipopeptides, and glycolipids. While we will exclusively focus on the presentation and T cell recognition of glycolipids, it should be noted that the CD1-mediated presentation of phospholipids and lipopeptides is also well characterized (16–20).

CD1 Structure

CD1 overall resembles MHC I molecules, where the heavy chain (MHC or CD1) non-covalently associates with β_2 -microglobulin (β_2m) (4). The heavy chain can further be divided into three domains. The N-terminal $\alpha 1$ and $\alpha 2$ domains together form the antigen-binding site, while the $\alpha 3$ -domain pairs with $\beta 2m$ to support the $\alpha 1$ – $\alpha 2$ platform. The CD1 binding groove is formed by two anti-parallel α -helices that sit atop an anti-parallel β -sheet platform. CD1 has evolved a hydrophobic antigen-binding groove, which is deeper than that of MHC I and well suited for the binding and presentation of hydrophobic molecules, such as lipids (4). For in-depth information about the structural details of different CD1 isoforms and species, see Ref. (21–32). Each CD1 protein has adopted isoform and species-specific binding pockets that differ in shape and size; however, all mammalian CD1 binding grooves contain the two major pockets, A' and F'. While the A' pocket is larger, donut shaped, and deeply buried, the F' pocket is more open and accessible to the solvent. Each hydrophobic pocket generally binds one alkyl chain of a dual alkyl chain glycolipid, while the carbohydrate portion is located at the CD1d surface for TCR interaction. TCR recognition requires the proper presentation of the glycolipids by CD1d and TCR

binding of the exposed carbohydrate epitope in conjunction with CD1d. The lipid backbone itself can also be in contact with the TCR to varying degrees; however, its main role is to anchor and orient the carbohydrate for T cell recognition (33). Previously, we assumed that the CD1d-binding groove is rather rigid and that each lipid will have to find the right fit inside both pockets. However, we now know that especially at the surfaces above both A' and F' pockets, subtle structural changes can be induced upon lipid and/or TCR binding (26, 33–35). Lipid-induced structural changes in mouse CD1d have only been observed by using synthetic glycolipids and whether natural lipids exist that have the same effect is currently unknown. However, lipid-induced structural changes especially around the F' pocket greatly influence iNKT cell activation (33, 36) as this is the primary binding site for the TCR (37, 38).

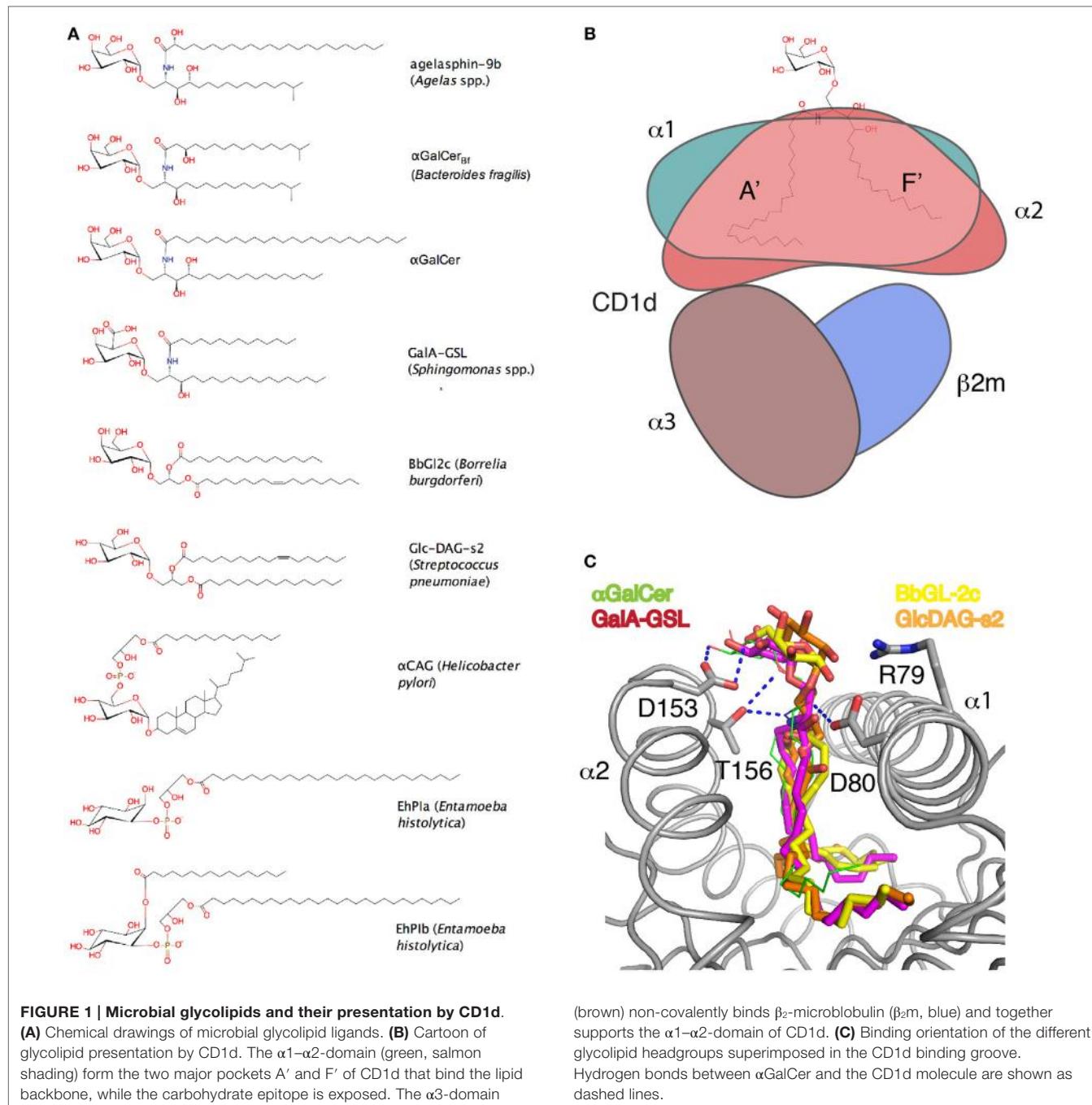
Lipid-Reactive T Cells

Lipid-reactive T lymphocytes are a minor population compared to peptide reactive T cells but have been reported to influence the outcome of the immune response (3). Lipid-reactive T cell, especially those expressing an $\alpha\beta$ TCR can be divided further based on their CD1 restriction and antigen-reactivity. Human group I CD1 (CD1a–c)-restricted T cells are generally considered diverse in their TCR repertoire and have often been reported to recognize mycobacterial antigens as well as self-antigens (39–46). Group II CD1 (CD1d)-restricted T cells, also called NKT cells, on the other hand, can further be classified into type I (V α 14i, iNKT) and type II NKT cells based on their TCR expression. Type I NKT cells are characterized by their evolutionarily conserved TCR α chain rearrangement [TRAV11–TRAJ18 (V α 14J α 18) in mice and TRAV10–TRAJ18 (V α 24J α 18) in human] and their reactivity toward the prototypical antigen α -galactosylceramide (α GalCer) (Figure 1), while type II NKT cells do not have a common antigen and represent all the remaining CD1d-reactive T cells that do not react to α GalCer (6, 47, 48). Recently, a population of CD1b-restricted T cells, called germline-encoded mycolyl lipid-reactive (GEM) T cells has been identified that similar to type I NKT cells, use a more restricted TCR $\alpha\beta$ repertoire (predominantly TRAV1–2–TRAJ9) to bind mycobacterial antigens with high affinity (49). Type I NKT cells in particular have been identified as important in the protection against various microbial pathogens through direct recognition of microbial glycolipids.

Microbial Activation of Type I NKT Cells

Cytokine-Mediated Activation With and Without TCR Stimulus

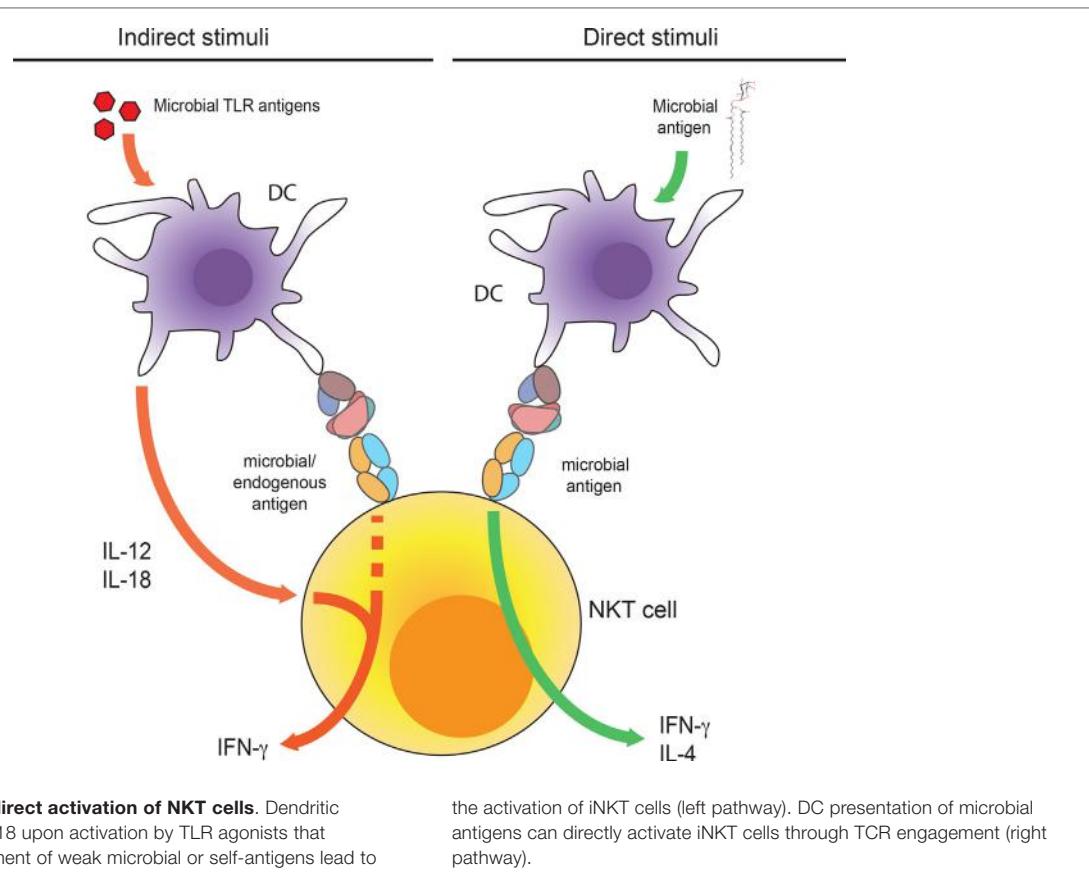
Type I NKT cells have been shown to participate in protection of mice from a variety of microbial parasites (3, 50, 51). While type I NKT cells expand upon infection, this is not necessarily due to the direct recognition of microbial antigens by the TCR (52). Furthermore, type I NKT cells can be activated *in vitro* and *in vivo* directly by cytokines, such as IL-12 plus IL-18 (53), or IL-12 alone, even in the apparent absence of a TCR signal (54) (Figure 2). Mouse cytomegalovirus infection leads to the activation of type I



NKT cells in a CD1d-independent but IL-12-dependent manner, hinting to a protective role of type I NKT cells in viral infection (55, 56). Even in the case of bacterial infection, where foreign, microbial antigens are present, type I NKT cells can be activated with the help of cytokines, such as IL-12, and perhaps in some cases, in conjunction with the presentation of self-antigens rather than microbial antigens (57, 58). This has been demonstrated in the case of *Streptococcus pneumoniae* infection, where type I NKT cell activation is strongly dependent on IL-12, while CD1d deficiency greatly reduced but did not fully abrogate NKT cell activation (59).

Glycolipid Activation of Type I NKT

The first antigen shown to activate type I NKT cells was α -galactosyl ceramide (αGalCer), which was isolated from a marine sponge in a screen for compounds that prevented tumor metastases in mice and changed by medicinal chemistry from the parental compound, Agelasphin-9b (Figure 1). αGalCer is now widely considered the prototypical antigen for human and mouse type I NKT cells. αGalCer is a glycosphingolipid, in which an α -anomeric galactose is connected to a ceramide backbone. The ceramide consists of a sphingoid base, which carries an N-amide-linked saturated C26 acyl chain. Interestingly, a new



study identified α -glycosyl ceramides in immune cells in mice, where they could play an important role in the development of iNKT cells (60, 61).

α GalCer binds to CD1d with the C26 acyl chain in the A' pocket and the sphingoid base in the F' pocket (Figure 1). This binding orientation exposes the galactose moiety above the CD1d-binding groove for interaction with the TCR and subsequent NKT cell activation.

Glycosphingolipids from *Sphingomonas* spp

The first identified and characterized microbial antigen for type I NKT cells was a glycosphingolipid from *Sphingomonas* bacteria. *Sphingomonas* are Gram-negative bacteria that lack lipopolysaccharide (LPS) and are highly abundant in the environment, including sea water (62, 63). Although *Sphingomonas* is not highly pathogenic, mice lacking type I NKT cells are defective for clearance of *Sphingomonas yanoikuyae* at early times after infection, while at later times, the bacteria was cleared without signs of any damage (64, 65). While the original TRAJ18^{-/-} mice used in those studies had a lower TCR repertoire, which could potentially contribute to some of the observed effects, a new mouse strain lacking iNKT cells is now available to assess the contribution of iNKT cells in host defense and other disease models (66, 67).

Similar to α GalCer, the antigen GalA-GSL also carried an α -linked sugar connected to a ceramide backbone (64, 68). However, instead of a galactose, the most potent antigen contained a galacturonic acid, while the ceramide lacked a hydroxyl

group at C4 of the sphingoid base (Figure 1). In addition, instead of the C26 acyl chain found in α GalCer, GalA-GSL contains a much shorter C14 fatty acid.

Borrelia burgdorferi Galactosyl Diacylglycerol Antigens

Borrelia burgdorferi is a spirochete and the causative agent of Lyme disease. Mice lacking type I NKT cells were less capable of clearing *B. burgdorferi* and they were more subject to chronic joint inflammation (69–71). One week after bacterial infection, type I NKT cells were activated *in vivo* to produce cytokines, such as IFN γ and IL-4 (70). *B. burgdorferi* is the first example of a pathogenic microbe that contain glycolipid antigens that activates type I NKT cells, and it is also the first example showing that type I NKT cell antigens do not have to be glycosphingolipids (72). *B. burgdorferi* has abundant glycosylated diacylglycerols (73, 74) with an α -anomeric galactose sugar in the sn-3 position of the glycerol. The sn-1 and sn-2 positions carry different acyl chains, most prominently palmitate (C16:0), stearate (C18:0), oleate (C18:1), and linoleate (C18:2) (Figure 1).

Using synthetic versions of the diacylglycerol antigen from *B. burgdorferi*, carrying different acyl chains at both sn-1 and sn-2 position, revealed the impact of the lipid backbone in type I NKT cell activation. The glycolipid, BbGL-2c (sn-1, oleate, sn-2, palmitate) proved to be stimulatory for mouse type I NKT cells, while BbGL-2f (sn-1, linoleate, sn-2, oleate) was the preferred antigen for human type I NKT cells (72, 75). The data suggested that despite

having an α -anomeric galactose for TCR recognition, identical to α GalCer, the nature of the lipid backbone that anchors the glycolipid to CD1d can influence T cell activation. Although diacylglycerol lipids are less potent than sphingolipids, this finding is important since diacylglycerols are widely distributed in microbes.

Streptococcus pneumoniae Glucosyl Diacylglycerol Antigens

S.pneumoniae and Group B streptococcus are important pathogens responsible for pediatric and community-acquired pneumonia. α -glucosyl-containing diacylglycerol antigens (Glc-DAG)-s2, the main iNKT antigen found in these bacteria, was the first microbial antigen identified that did not carry a galactosyl moiety (76). Instead, it is composed of an α -linked glucosyl hexose linked to a diacylglycerol backbone. Interestingly, the *sn*-2 position of the glycerol carries an unusual vaccenic (C18:1, *cis*-11) acid, while the *sn*-1 position is occupied by palmitic acid (C16:0). The requirement for this unusual combination of sugar and fatty acid appears to be quite stringent, as the positional isomer carrying a vaccenic acid in position *sn*-1 is not antigenic and that replacement of the glucosyl moiety with galactose did not restore antigenicity (36). Notably, Glc-DAG-s2 is also antigenic in human NKT cells (76), suggesting that the importance of similar synergies between lipid and polar portion of the streptococcal iNKT antigens is maintained in humans.

Bacteroides fragilis Glycosphingolipid Antigens

Bacteroides fragilis is a commensal bacteria found in humans and mice, where it colonizes the gut, and is characterized by an unusually high percentage of sphingolipids in its membranes. iNKT antigens were reported in these bacteria, which structurally resemble the prototypical antigen α GalCer (Figure 1) (77, 78), while carrying features also found in the original antigenic sphingolipid isolated from marine sponges, such as Agelasphin-9b. These antigens were found to have either stimulatory or inhibitory effects on iNKT cells, playing a critical role in iNKT homestasis during development. While no structural information is currently available on how these antigens are presented by CD1d, their structural similarity to well-characterized sphingolipids suggests a conserved CD1d-binding mode, with the sphingosine chain bound in the F' pocket and the fatty acid chain in the A' pocket.

Helicobacter pylori Glucosyl Cholesterol Antigens

Glycolipid antigens derived from *Helicobacter pylori*, the bacterium associated with the etiology of gastritis and peptic ulcers, have been recently described as cholesteryl α -glucoside antigens (79). In particular, the lipid cholesteryl phosphatidyl α -glucoside (α CPG) was shown to bind to CD1d and be able to stimulate iNKT cells. Clinical severity of gastric atrophy upon infection of *H. pylori* was correlated with the expression of the microbial enzyme cholesteryl α -glucosyltransferase (α CgT), which generates the iNKT cell antigen cholesteryl α -glucoside (79). $J\alpha 18^{-/-}$ mice lacking iNKT cells have an elevated burden of *H. pylori*, suggesting a protective role of iNKT cells against certain

clinical isolates of *H. pylori*. While this study did not address whether the phosphatidyl moiety or the cholesterol moiety binds inside the CD1d-binding groove, unpublished data from our own lab indicated that the mouse CD1d can in fact bind cholesterol derivatives. How such a bulky and rigid lipid moiety is bound within the CD1d-binding groove is currently unclear and will require further structural characterization.

Entamoeba histolytica Acylated Lysophosphatidyl Inositol Antigen

Another microbe that contains unusual iNKT cell antigens is *Entamoeba histolytica*, an intestinal protozoan parasite that causes amebiasis, resulting in significant morbidity and mortality worldwide (80). Clinical manifestations include liver abscesses, and animal models of experimental amebic liver abscess (ALA) demonstrated a role for IFN- γ in the control of *E. histolytica* invasion. As iNKT cells are predominantly located in the liver of mice and potently produce copious amounts of IFN- γ , it is not surprising that $J\alpha 18^{-/-}$ mice have considerably larger liver abscesses compared to wildtype mice, indicating a protective role of iNKT cells in parasite control. The iNKT cell antigen was generated upon lysosomal processing of a lipopeptidophosphoglycan to the active form EhPIb, 1-O-[(28:0)-lyso-glycero-3-phosphatidyl]-2-O-(16:0)-inositol (81). This lipid correlates with a model in which the C28:0 chain binds in the A' pocket of CD1d, while the inositol headgroup is exposed above the CD1d-binding groove and the inositol-linked C16 fatty acid inserts into the F' pocket of CD1d. However, no published data using a synthetic version of this antigen are available to date, which would be important to address the question of how such a structure binds to CD1d to activate iNKT cells.

Antigen Loading and Processing

Lipid antigens are generally water-insoluble and if not bound to proteins themselves, will be embedded into cellular membranes. As a consequence, there has to be an active process in which lipids are extracted from the membranes of APCs and/or transferred from another protein into the binding groove of CD1d. While some antigens, such as α GalCer, can be loaded directly into CD1d molecules on the cell surface, antigen presentation generally is enhanced by the internalization of glycolipids into acidic endosomal compartments (82). As cell-surface expressed CD1 recycles back through cellular compartments, CD1 encounters lysosomal lipid transfer proteins, such as saposins A–D (83–87), which facilitate the transfer of glycolipids into CD1. Saposins can extract lipids from membranes, directly bind them in hydrophobic pockets, and transfer them onto CD1d. However, the mechanism of lipid transfer and whether saposins directly bind CD1d has not been well studied. In addition to saposins, a variety of cells, including DCs, secrete apolipoprotein E, which enhances the presentation of glycolipid antigens to iNKT cells because it can interact with glycosphingolipid antigens and enhance their uptake (88). Furthermore, microsomal triglyceride transfer protein (MTTP), an ER chaperone protein, has been reported to assist in lipid loading into nascent CD1d, as it passes through the secretory pathway on the way to the cell surface (89). As a

result, endogenous lipids are found associated with cell-surface expressed CD1d.

In addition to lipid loading, lipid processing can also occur in acidic compartments. Using the synthetic glycosphingolipid antigens Gal(α1,2)αGalCer, carbohydrate antigen processing was demonstrated in APCs (90). Gal(α1,2)αGalCer itself is unable to directly activate iNKT cells but once the terminal galactose is removed by the lysosomal enzyme α-galactosidase A, thereby generating the highly antigenic monosaccharide, αGalCer, iNKT cell activation could be observed (90). Processing of microbial antigens has also been observed for the *Entamoeba histolytica* antigen, EhPIb. However, in contrast to the removal of a carbohydrate, removal of a fatty acid through a phospholipase (PL) is necessary to generate the antigen. Another example of microbial antigen processing is provided by hexamannosylated phosphatidyl-myo-inositols (PIM₆), from *Mycobacterium tuberculosis*, which is presented by CD1b. PIM₆ is processed to the dimannoside form PIM₂ by α-mannosidase, a process that is greatly enhanced by CD1e (13). Also, recognition of iGb3 is believed to require processing from iGb4 by an enzyme or enzymes having the β-subunit found in lysosomal hexosaminidases A and B (91).

In summary, processing of antigens can occur both in the carbohydrate moiety, as well as the lipid backbone and appears to constitute a common feature of both endogenous and microbial antigens.

Structural Basis of Glycolipid Presentation by CD1d

While the list of NKT cell antigens is rapidly expanding, most structural work on the presentation of glycolipids by CD1d has focused on dual chain lipids, such as diacylglycerol lipids or sphingolipids. Only limited structural data are available on the presentation of lysolipids, which are characterized by possessing only one alkyl chain, or other types of lipids, such as cholesterol derivatives. The few current examples of lysolipids include the presentation of lyso-PC by human CD1d, which is an antigen for iNKT cells and lyso-sulfatide by mouse CD1d, which is an antigen for a subset of type II NKT cells (92, 93). Both single chain lipids appear to bind in the F' pocket of CD1d but activate distinct NKT cell populations. Lysophospholipids are derived from diacylglycerol lipids upon PL A1, A2, or B digestion, generating lysolipids with a single fatty acid located either at the sn-1 (PLA2, B) or sn-2 (PLA1, B) position. However, the current list of microbial lysolipids able to activate iNKT cells is limited. While *Entamoeba histolytica* contains a lysolipid with a fatty acid at the sn-1 (EhPIa), only the lipid that contains an additional fatty acid at the inositol moiety (EhPIb) appears to be antigenic for iNKT cells (Figure 1) (81).

Microbial dual alkyl chain lipids binding to CD1d have been well characterized, both for ceramide-based glycosphingolipids and for diacylglycerol-containing glycolipids (33, 36, 64, 68, 72, 75, 76, 94, 95). We now know that sphingolipids bind in a conserved orientation to CD1d, with the longer acyl chain (typically up to C26) filling the larger A' pocket, and the shorter sphingoid base (~C18) filling the smaller F' pocket (96, 97). This binding

orientation is maintained even when the fatty acid or the sphingosine moiety is truncated to eight or nine carbons, respectively, which would allow for a reversed binding orientation (26, 98). The binding orientation is likely orchestrated by a network of conserved H-bond interactions between the core mouse CD1d residues, Asp80, Asp153, and Thr156 (Asp151 and Thr154 are the equivalent residues in human CD1d) and the polar moieties of the rigid ceramide backbone (Figure 1C). If the fatty acid chain is too short to fill the A' pocket completely, spacer lipids, such as a C16:0 fatty acids (palmitic acid), are recruited to occupy the remainder of the pocket, at least for proteins that were recombinantly expressed in insect cells (26, 95, 99). Spacer lipids have also been observed in the F' pocket for glycosphingolipids, such as OCH, where the sphingoid base had been shortened (35, 98). The conserved binding orientation places the carbohydrate moiety in a rather similar position for TCR engagement. The first identified microbial antigens for iNKT cells were from *Sphingomonas* spp., which contained the glycosphingolipids α-glycuronosylceramide (containing either a glucuronic or galacturonic moiety). Structural data revealed that the overall binding to CD1d was similar compared to αGalCer, with slight different interactions based on the lack of the 4-OH group of the sphingoid base. This resulted in a slightly deeper binding of the sphingoid base inside the F' pocket and a concomitant loss of well conserved electron density for the galacturonic acid headgroup at the CD1d surface (95). This less well-defined presentation of the headgroup was likely the major determinant for the reduced antigenicity compared to αGalCer and a reduced TCR-binding affinity (33). Interestingly, a subset of non-canonical Vα10 NKT cells had been described earlier that can also recognize αGalCer but have a preference for glucose-containing antigens, such as αGlcCer, and the microbial antigen, α-glucuronosylceramide. Despite sharing only 40% sequence conservation with the Vα14 chain, the Vα10 NKT cells have a CD1d-docking mode similar to type I NKT cells (100). iNKT cell antigens that contain ceramide backbones are generally more potent than antigens that are based on a diacylglycerol backbone. In fact, the fine structure of the diacylglycerol backbone greatly influences the potency of iNKT cell agonists. While the carbohydrate moiety is connected to the sn-3 position of the glycerol, the two fatty acids occupy the sn-1 and sn-2 position. This leads to a greater diversity in the lipid backbone of diacylglycerol lipids, as the fatty acids can vary in length and saturation. During the structural characterization of *B. burgdorferi* α-galactosyl diacylglycerolipid binding to mouse CD1d, we observed two binding orientations of the diacylglycerolipid backbone. The two antigens, BbGL-2c and BbGL-2f, bind with the oleic acid (C18:1) in the A' pocket and the palmitic acid (C16:0, BbGL-2c) or linoleic acid (C18:2, BbGL-2f) in the F' pocket. However, as the oleic acid is at the sn-1 position in BbGL-2c but in the sn-2 position on BbGL-2f, this results in the reversed binding orientation (Figure 3B). It has been demonstrated that only BbGL-2c can potently activate mouse iNKT cells, suggesting that the reversed binding orientation observed for BbGL-2f renders the antigen non-antigenic, even though the same galactose epitope is presented (75). However, presentation of this galactose moiety above the CD1d-binding groove is altered, suggesting that the fine positioning of the carbohydrate

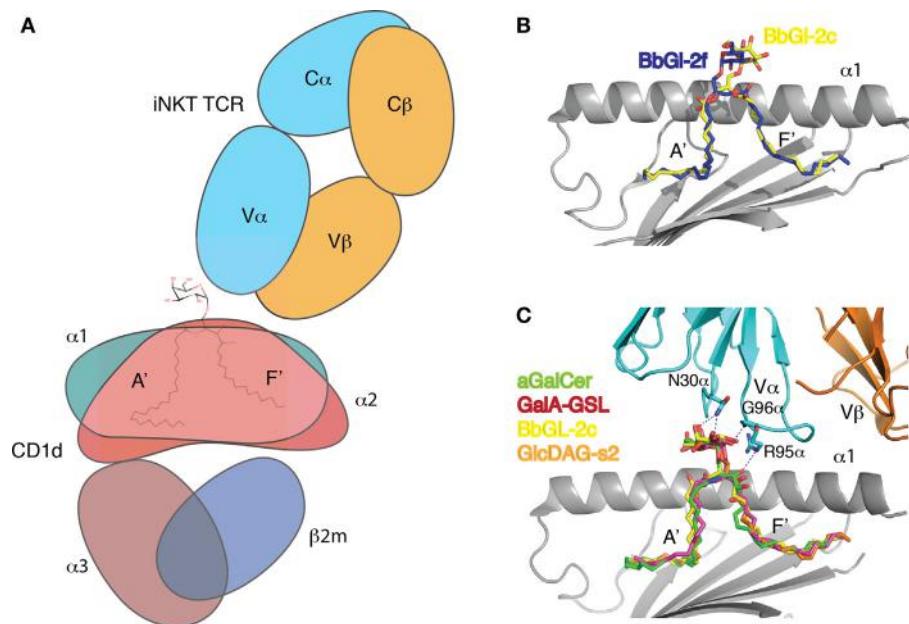


FIGURE 3 | TCR recognition of CD1d-presented glycolipids. (A) Cartoon representation of the CD1d- α GalCer-V α 14V β 8.2 TCR ternary complex. **(B)** Individual fatty acids can affect the binding orientation of diacylglycerolipid antigens. BbGL-2c binds with the *sn*-1-linked oleic acid in the A' pocket, while BbGL-2f inserts its *sn*-2 linked oleic acid. This affects presentation of the

galactose moiety. **(C)** The glycolipids are exclusively contacted by CRD1 α and CRD3 α residues of the V α 14 chain of the TCR α , while the V β 8.2 chain is offset to the C-terminal end of the α 1-helix, and only contacts CD1d directly. Note that all glycolipids superimpose well as they are molded into the same position by the TCR.

moiety at the TCR interface by the precise structure of the lipid backbone greatly affects antigenicity. It is intriguing to speculate that microbes can synthesize specific glycolipids in an attempt to invade immune recognition by iNKT cells; however, both BbGL-2c and BbGL-2f are abundantly expressed by *B. burgdorferi* (73).

Streptococcus pneumoniae contains α -Glc-DAG, in contrast to the galactose or galacturonic acid moieties found in *B. burgdorferi* and *Sphingomonas* spp. Glucose-containing glycolipids are generally considered as weaker antigens based on early studies using α GalCer analogs (101). The precise lipid structure contained an *sn*-3-linked α -glucose, a palmitic acid at *sn*-1, and a cis-vaccenic acid (C18:1, *cis*-11) at *sn*-2 (Glc-DAG-s2) (76). Replacing the glucose with a galactose surprisingly resulted in a glycolipid that was not able to activate murine iNKT cells (Gal-DAG-s2) (36). Similar observations were made when the *cis*-vaccenic fatty acid was replaced against oleic acid (C18:1, *cis*-9), which effectively only shifts the unsaturation two carbons toward the end of the chain, or when the *cis*-vaccenic acid was linked to the *sn*-1, rather than *sn*-2 position of the glycerol (36, 76). As such, the streptococcal antigen exhibited a unique interplay between the lipid backbone, the precise location of the unsaturation at the *sn*-2 linked fatty acid, and the glucose moiety. Presentation of this glycolipid by CD1d shared similarities with the presentation of the borrelial antigen BbGL-2c, which had the oleic acid at the *sn*-1 position. While both lipids bound in opposite orientations inside the CD1d-binding pocket, both the galactose and the glucose moiety were presented in a more upright tilted orientation to the TCR. This was a direct consequence of loss of intimate H-bond

interactions with the core residue Asp153, which binds the 2"- and 3"-OH of α GalCer (Figure 1C).

T Cell Recognition of CD1d–Glycolipid Complexes

In recent years, structural studies shed light on the recognition of the different microbial glycolipids by the V α 14V β 8.2 TCR of murine iNKT cells (33, 36). Similar to the structure of the V α 14V β 8.2 TCR bound to mCD1d- α GalCer (38), the microbial antigens are contacted by the TCR using a highly conserved binding chemistry. The TCR binds with the invariant V α 14 α 18 chain directly above the carbohydrate headgroup with a footprint centered above the F' pocket. In stark contrast to TCR recognition of pMHC, the TCR beta chain does not participate in direct antigen recognition. The highly variable complementarity-determining region (CDR)3 β loop exclusively contacts CD1d and is involved in controlling autoreactivity (102). The antigen is recognized by the TCR alpha chain, specifically CDR1 α and CDR3 α . In addition, certain antigens, such as isoglobotrihexosyl ceramide (iGb3) or phosphatidyl inositol (PI), are also contacted by the framework residue, Lys68, while α GalCer analogs with aromatic groups attached to the 6"-OH of the galactose can be in contact with CDR2 α residue Gln52 (103–105). CDR1 α exclusively contacts the headgroup, while CDR3 α contacts both headgroup, the hydroxyls of the lipid backbone, and the F' roof, indicating that the overall recognition and binding orientation is dominated by CDR3 α (Figure 3). For α GalCer, the 3" and 4"-OH groups of the galactose epitope make a hydrogen bond to Asn30 of CDR1 α ,

while the 2"-OH interacts with the backbone nitrogen of Gly96 of CDR3 α . Arg95 contacts the 4'-OH of the sphingoid base. All or most of those interactions are also observed in the microbial glycolipid structures but depending on the type of carbohydrate, the interaction with the axial 4"-OH can be lacking (e.g., in glucose, the 4"-OH is in equatorial configuration, pointing away from the TCR). Also, depending on the nature of the lipid backbone, the interaction between Arg95 of CRD3 α and 4'-OH of the sphingoid base is not always formed. Surprisingly, while the carbohydrate moieties of three two microbial lipids, GalA-GSL, BbGL-2c, and Glc-DAG-s2, are presented by CD1d in different orientations before TCR engagement, all the lipid headgroups superimpose well with that of α GalCer after TCR binding. The combined biophysical and structural data revealed the basis of why α GalCer is such a potent antigen and high affinity ligand for the TCR. The TCR binds α GalCer using a lock and key mechanism, while microbial antigens, especially the carbohydrate headgroups have to be re-oriented by the TCR to allow for the conserved binding footprint on CD1d (33, 37, 106, 107). One could argue that the lipid structures of the analyzed microbial antigens are similar to α GalCer, despite the obvious differences in the lipid backbones. As such, the TCR not surprisingly binds to those antigens using a conserved binding chemistry. However, the conserved binding chemistry of the TCR has also been observed in the more complex β -anomeric glycolipid, iGb3, where the TCR completely flattens the trihexosyl group to form similar interactions with the β -anomeric glucose. In fact, the β -anomeric glucose is molded upon TCR binding into a position where it mimics the observed flat binding of α -anomeric carbohydrates (104, 108). TCR-binding kinetics also correlates well with the structural change that the TCR induces in the ligands upon binding to the CD1d-glycolipid complexes. All the microbial antigens that are not based on a ceramide backbone and as such differently presented by CD1d have a 10 \times or more reduced TCR association rate. Surprisingly, however, the TCR dissociates 70 \times faster from GalA-GSL compared to α GalCer. While both antigens are presented in a similar orientation, and also form the same number of H-bond interactions, we wondered whether there are any structural changes in CD1d. Indeed, as previously reported, the short chain α GalCer analog PBS-25 induces the closure of the F' roof upon binding to CD1d, while the F' roof is not closed by any of the microbial antigens (26, 33, 36). The F' roof is the major binding site for the

TCR α chain and upon binding of the TCR, the F' roof is formed regardless of the bound glycolipid. That correlates with a model in which the TCR induces a structural change in CD1d for the TCR α chain to bind to, and the necessary binding energy to keep the roof closed is taken out of the TCR binding energy. As a result, the TCR dissociates faster from CD1d-glycolipid complexes that do not have a preformed F' roof (33, 36). Further mutational studies targeting individual residues within the F' roof highlighted the importance of this roof in the overall stability of the ternary complexes, without affecting TCR association rates, suggesting a two step-binding mechanism in which the antigen is bound first and then the CD1d molecule (36).

Conclusion

The TCR of iNKT cells can recognize a vast range of antigens. While novel microbial antigens for iNKT cells continue to be identified, a clear structural pattern has emerged that the TCR recognizes with preferred specificity. The pattern consists of an α -anomeric monohexosyl sugar, linked to a lipid backbone that can either be based on a ceramide, a diacylglycerol, or potentially a cholesterol moiety. The type of sugar that is preferentially recognized would depend on the precise structure of the lipid backbone but generally contains a galactose, glucose, or derivatives thereof. While it is still difficult to predict which DAG backbone would give rise to iNKT cell antigens, the binding of glycosphingolipids is more conserved and many interactions with the TCR can now be modeled with confidence. Deviation from this pattern can be compensated by the unique binding properties of the TCR, with regards to the structural changes that it can induce in both lipid headgroup orientation and in CD1d. Those changes would be reflected in a lower TCR binding affinity and potency. As such, the most potent glycolipid antigens for iNKT cells, regardless of source, are glycosphingolipids followed by α -glycosyl diacylglycerolipids. Binding of other types of lipid antigens has not been structurally assessed but will be the focus of continuing studies, since many microbes also produce glycolipids that deviate from the known structures.

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Dynamics of NKT-cell responses to chlamydial infection

Sudhanshu Shekhar¹, Antony George Joyee² and Xi Yang^{1,2*}

¹ Department of Medical Microbiology, Faculty of Medicine, University of Manitoba, Winnipeg, MB, Canada, ² Department of Immunology, Faculty of Medicine, University of Manitoba, Winnipeg, MB, Canada

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S. M. Mansour Haeryfar,
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*Correspondence:

Xi Yang,

Department of Immunology, Faculty of Medicine, University of Manitoba,
471 Apotex Centre, 750 McDermot Avenue, Winnipeg, MB R3E 0T5,
Canada
yangxi@cc.umanitoba.ca

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Natural killer T (NKT) cells have gained great attention owing to their critical functional roles in immunity to various pathogens. In this review, we provide an overview of the current knowledge on the role of NKT cells in host defense against and pathogenesis due to *Chlamydia*, which is an intracellular bacterial pathogen that poses a threat to the public health worldwide. Accumulating evidence has demonstrated that NKT cells, particularly invariant NKT (iNKT) cells, play a crucial role in host defense against chlamydial infections, especially in *C. pneumoniae* infection. iNKT cells can promote type-1 protective responses to *C. pneumoniae* by inducing enhanced production of IL-12 by dendritic cells (DCs), in particular CD8α+ DCs, which promote the differentiation of naive T cells into protective IFN-γ-producing Th1/Tc1 type CD4+/CD8+ T cells. This iNKT-cell-mediated modulation of DC function is largely dependent upon CD40–CD40L interaction, IFN-γ production, and cell-to-cell contact. In addition, iNKT cells modulate the function of natural killer cells. NKT cells may be also involved in the pathogenesis of some chlamydial diseases by inducing different patterns of cytokine production. A better understanding of NKT-cell biology will enable us to rationally design prophylactic and therapeutic tools to combat infectious diseases.

Keywords: CD1d restriction, NKT cells, dendritic cells, *Chlamydia*

Introduction

Natural killer T (NKT) cells represent a unique population of innate lymphocytes that express the natural killer (NK)- and T-cell markers, such as NK1.1 and αβ T-cell receptor (TCR) (1). NKT cells are divided into two subsets, type I or invariant NKT (iNKT) and type II NKT cells, depending upon their TCR forms and cognate ligands. iNKT cells possess the invariant αβ TCR (iTCR) that recognizes glycolipid and lipid antigens presented to them by CD1d molecules. iNKT cells are the most widely studied subset of NKT cells. Activation of iNKT cells leads to the rapid production of Th1, Th2, and Th17 cytokines and chemokines (2). On the other hand, type II NKT cells do not express the iTCTR and are reactive to sulfatides (1). Because of their distinct immunologic characteristics and crucial functions in host immune responses to different pathogens, NKT cells have gained much attention in recent years (3). In this review, we focus on the functional role of NKT cells, especially iNKT cells, in conferring T-cell immunity against chlamydiae, which are obligate intracellular bacteria that cause a range of human diseases worldwide (4). In particular, we describe how iNKT cells bridge innate and adaptive immunity by modulating the function of dendritic cells (DCs) during chlamydial infection. These findings

Abbreviations: Ag, antigen; C, *Chlamydia*; CD40L, CD40 ligand; DC, dendritic cell; IC, infected cell; IL-12, interleukin-12; iNKT, invariant natural killer T cell; iTCTR, invariant T cell receptor; Tc, CD8+ T cytotoxic cell; Th1, T helper cell.

present a rational basis for developing effective prophylactics and therapeutics against infectious diseases.

Chlamydial Infections and Their Pathophysiology

Chlamydia has a biphasic life cycle, consisting of two distinct forms, elementary body (EB) and reticulate body (RB). EB is an extracellular and metabolically inactive, but stable form, which is responsible for dissemination of infection from one person to another. On the contrary, RB is an intracellular and metabolically active form (5). EBs attach and enter the epithelial cells through endocytosis. Following their entry into the cell, the EBs undergo germination to give rise to RBs. The RBs so formed multiply by binary fission in enlarging vacuoles called inclusion bodies. As the inclusion body expands following maturation, the RBs re-differentiate into EBs, which are released by the cells to infect more cells (6).

Chlamydial species belong to the taxonomic family Chlamydiaceae. Using 16s and 23s rRNA sequencing, the family Chlamydiaceae has divided into two genera and nine species (7). Out of these nine chlamydial species, *C. trachomatis* and *C. pneumoniae* (also called as *Chlamydophila pneumoniae*) are clinically significant species that cause a variety of human diseases. *C. trachomatis* has three human serovars, including serovars A–C, D–K, and L1–L2. Serovars D–K of *C. trachomatis* are the most common cause of bacterial sexually transmitted diseases (STDs), but can also cause neonatal pneumonia and conjunctivitis (4, 8–11). They cause 90 million cases of STDs each year across the globe, with approximately 3 million cases alone in the United States (11). The clinical manifestations of chlamydial genital infection in women include urethritis, cervicitis, upper genital tract infection, and perihepatitis. If untreated, infected women can develop pelvic inflammatory disease, which have serious consequences, such as infertility, ectopic pregnancy, and abortion. In men, *C. trachomatis* infection can cause urethritis, epididymitis, seminal vesiculitis, and prostatitis (8, 9). It is notable here that majority of infected people are asymptomatic and only about 20% of infected women and 30% of men show clinical signs of chlamydial infection and so are subjected to antibiotic treatment. A range of broad spectrum antibiotics such as erythromycin and tetracycline are effective against *Chlamydia*, although an accurate and timely diagnosis of chlamydial infections presents a challenge to the clinician due to their diverse clinical manifestations (4, 12). Serovars L1–L2 of *C. trachomatis* cause lymphogranuloma venereum, which is a venereal disease with lesions in genital tissues, particularly the tissue-draining lymphatics and lymph nodes. These genital tract chlamydial infection increases the chance of women to be infected with human immunodeficiency virus and human papilloma virus-induced cervical neoplasia (13, 14). Apart from genital tract infection, serovars A–C of *C. trachomatis* cause trachoma, which is the leading cause of infectious blindness worldwide that affects about 84 million people with active disease. Pathologic lesions in trachoma include the development of follicles and inflamed conjunctivae that lead to cloudy and vascularized cornea, trichiasis, corneal ulcer, and blindness. Transmission of *C. trachomatis* for trachoma takes place by contaminated fingers or fomites or

through placenta in infected mothers (15, 16). On the other hand, *C. pneumoniae* causes a variety of respiratory diseases, including sinusitis, pharyngitis, bronchitis, and community-acquired pneumonia that are common throughout the world (17). A higher prevalence of chlamydial infection is however noted in third world countries compared to the developed ones. Humans are the only known reservoir for *C. pneumoniae*. In recent years, there are various reports based on epidemiological, immunological, and pharmacological studies that indicate an association of *C. pneumoniae* infection with cardiovascular and neurodegenerative diseases, such as atherosclerosis, Alzheimer's disease, and multiple sclerosis (18–20). Despite considerable efforts, it still remains a challenge to develop a safe and effective chlamydial vaccine due to inadequate knowledge of protective immunity and immunopathology of chlamydial infections. This is important because of the fact that the immune responses also contribute to the pathogenesis of chlamydial diseases (21). An effective vaccine strategy therefore requires the identification of antigens/adjuvants, which evoke protective but not pathologic immune responses.

Coexistence of *Chlamydia* and its host imposes an evolutionary pressure on both of them. The host's immune system has developed to defend the body from chlamydial infections, whereas *chlamydiae* are equipped with various evasion mechanisms to escape the host's immune system. Pathogenesis of chlamydial diseases is the result of this host-pathogen interaction. Chlamydial infection leads to the activation of mucosal epithelial cells. Activation of epithelial cells induces secretion of multiple cytokines and chemokines, such as IL-1, TNF- α , IL-8, GM-CSF, and IL-6, which cause infiltration of immune cells at the primary site of infection. These immune cells include, but not limited to, neutrophils, monocytes, NK cells, and T cells. Infected epithelial cells and neutrophils secrete potent proteolytic enzymes like elastase and MMPs to cause tissue damage (22–24). Persistence of chlamydial infection can lead to the continuous release of proinflammatory cytokines from the epithelial cells which results in tissue damage. On the other hand, immune responses have also been held responsible for the tissue damage. Although IFN- γ + CD4 T cells induce immunity to chlamydial infection, they might have detrimental effects on the primary site of infection resulting in collateral damage (25). CD4 T cells producing IL-4 can elicit immunopathology via suppression of protective responses (26). Autoreactive T cells specific for *Chlamydia* and host proteins such as heat-shock protein 60 have also been described, although the mechanism of their development can be assigned to the phenomenon of molecular mimicry (27). A reduced pathology in IL-knockout (KO) mice, compared to the wild-type (WT), during *Chlamydia*-infected mice suggests a detrimental role for IL-10 in this infection model (28, 29). Therefore, overt responses by immune cells can culminate into pathology during chlamydial infection.

Anti-Chlamydial Host Immunity

Many studies in animal models and clinical settings have demonstrated that T cells play a crucial role in control of chlamydial infections. In genital infection of *C. trachomatis*, CD4+, but not CD8+, T cells are indispensable for resolution of primary as well as secondary infections (30–33). Similar function for CD4+ T

cells has been described in *C. muridarum*, a mouse biovar of *C. trachomatis*, and lung and genital tract infection (34). Although both CD4+ and CD8+ T cells contribute to immunity to *C. pneumoniae* lung infection, the predominant role is played by CD8+ T cells (35). The type of T-cell immunity has a profound effect on whether the infection is contained or culminates into pathology. Th1/Tc1 responses characterized by IFN- γ production by CD4+/CD8+ T cells are the major form of protective immunity (34). It is also shown that IL-17/Th17, in cooperation with Th1, responses exert anti-chlamydial adaptive immunity, especially in lung infections (36–38). In contrast, Th2 immunity characterized by secretion of cytokines such as IL-4, IL-5, and IL-13 are more associated with inflammatory and pathologic changes (34). IL-10, a Th2 and immunoregulatory cytokine, has also been annexed with pathologic responses (28). Therefore, promotion of Th1/Tc1 and, in certain conditions, Th17 responses are more likely beneficial for resolution of chlamydial infection, whilst the increase of Th2 responses culminates into pathology.

Activation of iNKT Cells

Activation of iNKT cells is achieved through two mechanisms; CD1d-dependent and CD1d-independent. In CD1d-independent mechanism, iNKT cells are activated without involvement of CD1d molecules. This type of activation is mediated through innate or inflammatory stimuli irrespective of the presence of foreign microbial antigens, possibly in conjunction with self-glycolipid antigen recognition (39). Recent data further illustrate that the innate stimuli such as cytokines appear to be the predominant means of iNKT-cell activation, even with bacteria that carry iNKT cell agonists (40). On the other hand, in CD1d-dependent mechanism, antigens are presented by CD1d molecules expressed on antigen-presenting cells (APCs) such as DCs for interaction with iTCR that possesses a conformation that is able to recognize glycolipid and lipid antigens processed and presented to them by CD1d molecules (41, 42). Interaction between iTCR and its cognate ligand leads to the activation of iNKT cells, as evidenced by massive production of a variety of cytokines, such as Th1 (IFN- γ), Th2 (IL-4), and Th17 (IL-17) cytokines and chemokines (43). The biochemical and physiological nature of iNKT-cell-specific ligands has been deciphered by many recent studies. Kawano et al. for the first time identified a lipid antigen specific for iNKT TCR, α -galactosylceramide (α -GalCer), which was originally isolated from a marine sponge, *Agelasmauritanicus* (44). Since α -GalCer is a potent ligand for iNKT-cell activation and has been instrumental in understanding the biological properties of iNKT cells, it is referred to as a prototypic antigen for these cells. The ability to activate iNKT cells is however not limited to α -GalCer. A variety of microbial antigens have also been shown to activate iNKT cells, such as α -glucuronosylceramide from *Sphingomonas* species (45–47), α -galactosyldiacylglycerol from *Borrelia burgdorferi* (48), and phosphatidylinositol-mannosidase from *Mycobacterium bovis* BCG (49, 50). In an attempt to identify chlamydial lipid antigens which active iNKT cells, we tested a previously reported glycolipid exoantigen from *C. muridarum* (GLXA) for activation of iNKT cells (51, 52). We found that intravenous injection of GLXA into WT mice led to an enhanced

production of IFN- γ and IL-4 in mouse sera, which was not seen in $\text{J}\alpha 18$ -KO mice that lack iNKT cells only. Following GLXA treatment, iNKT cells underwent activation and produced IFN- γ and IL-4 (52). These findings suggest that chlamydial GLXA acts as a specific ligand for iNKT-cell activation. In line with these findings, Jiang et al., using APC-free culture system, have shown that both iNKT as well as type II NKT-cell hybridomas were activated when cultured with ultraviolet-killed *C. muridarum* (53). It is known that iNKT and type II NKT cells are activated by different ligands. For example, lipid and glycolipids are ligands for iNKT-cell activation, whereas hydrophobic antigens such as sulfatides induce specific activation of type II NKT but not iNKT cells. Since *C. muridarum* activated iNKT and type II NKT cell hybridomas, it is likely that there are different chlamydial antigens for activating these cell types (53). Therefore, further studies on identification and purification of different chlamydial antigens for iNKT- and type II NKT-cell activation may be crucial for anti-chlamydial vaccine development. Based on the current data, it appears reasonable to conclude that iTCR is involved in activation of iNKT cells through interaction with chlamydial antigens.

iNKT Cells in Protective Immunity Against Chlamydial Infection

Recent studies in mice have provided significant evidence on the role of NKT cells in protective immunity to various infections, including chlamydial infections (54, 55). Activation of iNKT cells by injection of α -GalCer in mice mounted a strong protective immunity to intranasal *C. pneumoniae*, intra-articular *C. trachomatis*, and intravaginal *C. muridarum* infection (54, 56, 57). In these studies, BALB/c mice were used for intra-articular *C. trachomatis* and vaginal *C. muridarum* infection, and C57BL/6 mice for intranasal *C. pneumoniae* infection; however, the outcomes of the infections were similar following α -GalCer treatment (54, 56, 57). To better understand the protective function of NKT cells *in vivo* during chlamydial infections, we and other groups used various experimental approaches, including transgenic/KO mice. During *C. trachomatis* intra-articular infection, CD1d-KO mice, which lack both iNKT and type II NKT cells, experienced enhanced pathology and higher bacterial burden compared to the WT mice, indicating a protective role for NKT cells in this infection model (57). To directly examine the contribution of iNKT cells in host defense against *C. pneumoniae* infection, we infected $\text{J}\alpha 18$ -KO mice through intranasal route (54). Upon infection challenge, more severe body weight loss, pathological changes, and higher organism growth were observed in $\text{J}\alpha 18$ -KO mice than in the WT mice (54), which suggested a protective effect of iNKT-cell activation on *C. pneumoniae* infection. We further assessed the impact of iNKT cells on T cells in the context of cytokine response to *C. pneumoniae* infection (54). Intracellular cytokine analysis demonstrated that the WT mice, compared to the KO, displayed a robust type-1 CD4 and CD8 T cell response, characterized by IFN- γ production in *C. pneumoniae* infection. Furthermore, CD4 T cells of $\text{J}\alpha 18$ -KO mice reflected an enhanced Th2 (IL-4) response than those from the WT mice (54). Thus, iNKT cells contribute to the

development of protective Th1/Tc1 responses against *C. pneumoniae* infection.

Bridging Innate and Adaptive Immunity

It is becoming clearer that innate and adaptive immune systems do not work in isolation, but rather interact with each other to give rise to an optimal immune response against infections. A significant example in this context is the case of innate lymphocytes that have been shown to bridge innate and adaptive immunity by modulating DCs (58). To study the impact of iNKT cells on DC function, α -GalCer as a model antigen has been widely used. Whether this is true in case of real infections has been addressed by some recent studies (59–63). Our recent studies using a mouse model of *C. pneumoniae* infection have done an in-depth analysis of the impact of iNKT cells on DC function for the elicitation of T-cell immunity in a real infection setting (62, 63) (Figure 1). Adoptive transfer of DCs isolated from the spleens of *C. pneumoniae*-infected $\text{J}\alpha 18\text{-KO}$, in contrast to the WT, mice promoted infection and pathology in naïve recipient mice upon challenge with chlamydial infection (62), suggesting that iNKT cells are crucial for DCs to confer protective Th1/Tc1 immunity. Overall, these data provided direct evidence on the role of iNKT cells in modulating DC function thereby enhancing protective immunity in an *in vivo* model of infection. Since DCs demonstrate a high degree of heterogeneity consisting of various subsets, we further investigated whether this modulating effect of iNKT cells was biased to a DC subset. CD8 $\alpha+$ and CD8 $\alpha-$ DCs are important DC subsets residing in the lymphoid tissues such as the spleen. While CD8 $\alpha+$ DCs induce Th1 responses, CD8 $\alpha-$ DCs skew Th2 responses (64). These DC subsets were purified from $\text{J}\alpha 18\text{-KO}$ and WT mice following chlamydial infection and then adoptively transferred to naïve recipient mice that subsequently received chlamydial infection. While both the groups of mice receiving CD8 $\alpha+$ and CD8 $\alpha-$ DCs from WT mice showed significant resistance to infection compared to those from $\text{J}\alpha 18\text{-KO}$ mice, the WT CD8 $\alpha+$ DC recipients had superior protection (63). Collectively, these data provided the first direct evidence that iNKT cells preferentially promote the functional development of a subset of DC to generate protective immunity against infections. Since the local pulmonary immune responses may not be similar to that in the splenic environment, we also

examined the iNKT cell–DC interaction in the lung, which is the primary site of infection where predominant inflammatory and immunologic changes occur (65). These findings, however, were not different from what we observed in splenic DC studies, which suggested that DCs residing in different anatomical compartments induce similar immune responses during chlamydial lung infection. Apart from DCs, alveolar macrophages (AMs) are also a critical immune cell population in the lung that regulates immune responses against pulmonary pathogens. In *C. muridarum* infection, iNKT cells were found to affect both the phenotype as well as function of AMs (unpublished observation). Altogether, these findings suggest that iNKT cells exert protective T-cell immunity to *C. pneumoniae* through modulating the function of APCs. How do iNKT cells modulate the DC function? In *C. pneumoniae* infection, the expression of CD40L and IFN- γ by iNKT cells was found to be upregulated (62). To directly examine the contribution of CD40L and IFN- γ in the modulating effect of iNKT cells on DC, we cocultured iNKT cells with DCs and then used blocking antibodies against these molecules. Blockade of either CD40L or IFN- γ significantly reduced the enhancing effect of iNKT cells on IL-12 production by DCs. However, the enhanced effect on IL-12 production was completely abrogated when physical contact between these cells was prevented (62). These data conclude that CD40–CD40L interaction, IFN- γ production, and cell-to-cell contact are critical for iNKT cells to modulate DC function during chlamydial infection.

Recent reports indicate a modulating effect of NKT cells on the function of NK cells. *In vivo* administration of α -GalCer in mice induced NK cells to produce IFN- γ as well as cause cytotoxicity (66, 67). Since NKT and NK cells have been shown to contribute to immunity against chlamydial infections, we focused on whether NKT cells influence the functional role of NK cells during infection (68). We found a reduced expansion of NK cells in $\text{J}\alpha 18\text{-KO}$ mice following *C. muridarum* infection. A lower percentage of IFN- γ -producing, but higher CD107a+ degranulating, NK cells were observed in $\text{J}\alpha 18\text{-KO}$ than in WT mice. These data suggest that iNKT cells have a differential effect on NK cell functions. They enhance IFN- γ production by NK cells but inhibit their cytotoxic activities during chlamydial infection (68). Whether the interaction between iNKT and NK cells shape the adaptive immunity merits further exploration.

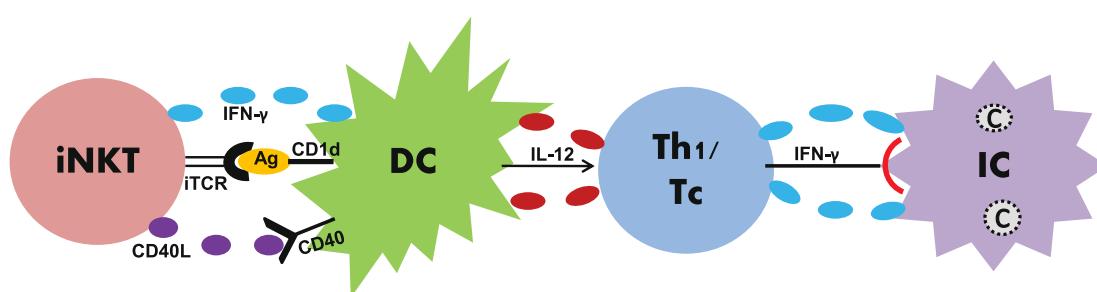


FIGURE 1 | Induction of anti-chlamydial T-cell responses by iNKT cells through DC modulation. During chlamydial infections, iNKT cells induce DC maturation through IFN- γ , CD40–CD40L binding, iTCR–Ag interaction, and

cell-to-cell contact. Once matured, DCs induce enhanced production of IL-12 that skews Th1/Tc responses. Th1/Tc responses characterized by IFN- γ production lead to the clearance of intracellular Chlamydiae.

iNKT Cells in Chlamydial Pathology

Although a growing wealth of evidence indicates a protective role for iNKT cells in chlamydial infections, some studies have implicated them in eliciting pathologic responses (53, 54, 69). *In vivo* stimulation of iNKT cells by α -GalCer increased *C. muridarum* burden in the lungs of BALB/c mice (69). However, following *C. muridarum* lung infection, CD1d-KO mice (BALB/c background) displayed reduced body weight loss, lung pathology, and chlamydial growth compared to WT mice, which suggested that NKT cells induce immunopathology to *C. muridarum* (69). Similar pathogenic effects of NKT-cell activation were recorded in response to murine genital tract infection with *C. muridarum* (53). When challenged with genital *C. muridarum* infection, WT mice exhibited severe pathologic changes such as oviduct dilation and fibrosis compared to the CD1d-KO mice (53). Altogether these findings suggested a promoting effect of NKT, including iNKT cell, activation on *C. muridarum* infection, which is in contrast to their protective role in *C. pneumoniae* infection (54). The reason behind why iNKT cells act differently in the outcome of infection with these two chlamydial species is still unclear and so warrants further investigation. It appeared that while both pathogens share many biological features, the cellular response and immunological course during infection are different in addition to the differences in growth characteristics and host specificity of these two pathogens. In broad sense, these results suggest that NKT-cell activation effect or the activation itself is pathogen or even species specific. Since *Chlamydiae* may have various antigens for iNKT cells, it is possible that the antigenic variability among the antigens from different chlamydial strains might explain the differential iNKT-cell responses. It is also possible that NKT cells including iNKT cells can play variable roles in different conditions or stages of infection, same as other cell types like CD4 and CD8 T cells. On the other hand, the detrimental role of NKT cells in chlamydial infection has been mostly reported in the studies using CD1d-KO mice that are in BALB/c background and have deficiency in both iNKT and type II NKT cells. Therefore, the observed distinction between chlamydial strains should be more carefully studied. In addition, in-depth studies on the characterization of glycolipid antigens derived from different chlamydial species and analyses of iNKT-cell activation effects in different routes and stages/conditions of infection would provide more insight into the iNKT-cell-mediated pathologic mechanisms in chlamydial infection.

NKT-Cell Subsets in Chlamydial Infections

Invariant NKT and type II NKT cells have distinct characteristics for their phenotype and function. Whether these cell subsets induce differential immune responses to chlamydial infections is not fully understood. To elucidate the specific roles of iNKT and type II cells in anti-chlamydial immunity, we used CD1d-KO and $\text{J}\alpha 18$ -KO mice because the former lack both iNKT and type II NKT cells but the latter are deficient in only iNKT cells. We found that CD1d-KO, in contrast to WT, mice showed increased resistance to *C. muridarum* lung infection. Similar outcome of *C. muridarum* infection was found in case of $\text{J}\alpha 18$ -KO. These findings indicate a detrimental role for both iNKT and type II

NKT cells in *C. muridarum* infection (54, 69). On the contrary, CD1d-KO and $\text{J}\alpha 18$ -KO mice displayed increased susceptibility to *C. pneumoniae* lung infection compared to their respective WT control mice (54), which advocates that NKT-cell subsets induce protection. Taking account of these data, it appears that the protective or pathogenic roles of NKT-cell subsets are mainly driven by the type of bacterial species used to infect mice. Of note, CD1d-KO and $\text{J}\alpha 18$ -KO mice used in these studies were having BALB/c and C57BL/6 genetic backgrounds, respectively. Since the outcome of chlamydial infection might be impacted by genetic differences, it would be prudent to use different KO mice with similar genetic background to arrive at a definitive conclusion.

In contrast to the immune function of NKT-cell subsets in chlamydial infections, iNKT and type II NKT cells have been shown to have differential impact on the outcome in certain other models, especially for anti-tumor immunity. *In vitro* stimulation of murine and human iNKT cells with α -GalCer led to an enhanced lysis of tumor cells in a perforin- and granzyme B-dependent fashion, which suggested a direct protective role of these cells in tumor lysis (70, 71). Using a methylcholanthrene (MCA)-induced fibrosarcoma mouse model, Smyth et al. showed that $\text{J}\alpha 18$ -KO mice treated with different doses of MCA developed fibrosarcoma, while the control B6 mice did not develop tumors (72). Upon adoptive transfer of the liver lymphocytes from WT mice, $\text{J}\alpha 18$ -KO mice, when injected with MCA, exhibited enhanced protection against tumor growth compared to the $\text{J}\alpha 18$ -KO mice that received either the liver lymphocytes from $\text{J}\alpha 18$ -KO mice or PBS (73). These findings indicate a clear role for iNKT cells in protective immunity to tumor development. In contrast to the anti-tumor activities of iNKT cells, type II NKT cells are reported to suppress the tumor immuno surveillance (74, 75). CD1d-KO, in contrast to $\text{J}\alpha 18$ -KO and WT, mice promoted the growth of subcutaneous 15-12RM fibrosarcoma and CT26-L5 colon carcinoma, which indicates that type II NKT cells inhibit the tumor immuno surveillance (74). Similarly, type II NKT cells were suppressive in the immune responses to B-cell lymphomas (75). Overall, these data point out that iNKT cells confer protective immunity to tumors, whereas type II NKT cells promote pathology. In a broader perspective, the data from chlamydial infection and tumor models shed significant light on different roles played by iNKT and type II NKT cells in diverse disease settings. This emphasizes the careful analysis of the impact of NKT-cell subsets on the outcome of diseases because findings from one experimental model cannot be extrapolated to another.

Conclusion and Future Directions

Invariant NKT cells play an important role in immunity to chlamydial infections. These cells not only induce innate responses but also shape adaptive responses, bridging innate and adaptive immunity. In doing so, iNKT cells modulate the function of DCs through enhanced cytokine production, CD40-CD40L binding, iTCR-antigen interaction, and cell-to-cell contact. In addition, iNKT cells can modulate the function of NK cells that can also modulate DC function. While significant insights

have been provided toward understanding the iNKT-cell biology in chlamydial infections, the following questions need to be addressed in the times to come.

- (1) The involvement of iNKT cells in both protective immunity and pathology in chlamydial infections has been reported. Notably, the protective and detrimental role of NKT cells in chlamydial infection was mostly shown in $\text{J}\alpha 18\text{-KO}$ and $\text{CD}1\text{d-KO}$ mice, respectively. Considering the differences of the mice in genetic background (C57BL/6 vs. BALB/c) and NKT cells (iNKT only vs. iNKT and type II NKT cells), a more detailed study to exclude the influences of these variations need to be performed.
- (2) The molecular basis for the influence of iNKT cells on spleen and lung DCs need to be studied in-depth. In addition, it would be interesting to see if there is any impact of iNKT cells on the migration pattern of DC/DC subsets, which is crucial for priming T cells in the lymphoid tissues.

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- (3) The impact of iNKT cells on other immune cells, apart from NK cells and DCs, merits further investigation. For example, AMs are considered to be a critical immune cell population in pulmonary pathogen defense. How do iNKT cells modulate AMs to influence the outcome of chlamydial infection?
- (4) How to prophylactically and therapeutically target iNKT cells for inducing protection without having any significant side effects? Can chlamydial lipid antigens be used in vaccine design strategies for promoting DC function?

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iNKT cells and their potential lipid ligands during viral infection

Anunya Opasawatchai^{1,2} and Ponpan Matangkasombut^{1,3*}

¹ Department of Microbiology, Faculty of Science, Mahidol University, Bangkok, Thailand, ² Faculty of Dentistry, Mahidol University, Bangkok, Thailand, ³ Systems Biology of Diseases Research Unit, Faculty of Science, Mahidol University, Bangkok, Thailand

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Luc Teyton,
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Qatar

*Correspondence:

Ponpan Matangkasombut,
Department of Microbiology,
Faculty of Science, Mahidol
University, 272 Rama VI Road,
Bangkok 10400, Thailand
ponpan@post.harvard.edu,
ponpan.mat@mahidol.edu

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Invariant natural killer T (iNKT) cells are a unique population of lipid-reactive CD1d-restricted innate-like T lymphocytes. Despite being a minor population, they serve as an early source of cytokines and promote immunological crosstalk thus bridging innate and adaptive immunity. Diseases ranging from allergy, autoimmunity, and cancer, as well as infectious diseases, including viral infection, have been reported to be influenced by iNKT cells. However, it remains unclear how iNKT cells are activated during viral infection, as virus-derived lipid antigens have not been reported. Cytokines may activate iNKT cells during infections from influenza and murine cytomegalovirus, although CD1d-dependent activation is evident in other viral infections. Several viruses, such as dengue virus, induce CD1d upregulation, which correlates with iNKT cell activation. In contrast, herpes simplex virus type 1 (HSV-1), human immunodeficiency virus (HIV), Epstein–Barr virus, and human papilloma virus promote CD1d downregulation as a strategy to evade iNKT cell recognition. These observations suggest the participation of a CD1d-dependent process in the activation of iNKT cells in response to viral infection. Endogenous lipid ligands, including phospholipids as well as glycosphingolipids, such as glucosylceramide, have been proposed to mediate iNKT cell activation. Pro-inflammatory signals produced during viral infection may stimulate iNKT cells through enhanced CD1d-dependent endogenous lipid presentation. Furthermore, viral infection may alter lipid composition and inhibit endogenous lipid degradation. Recent advances in this field are reviewed.

Keywords: iNKT, CD1d, virus, lipid

Introduction

Since they were first described in the late 1980s, invariant natural killer T (iNKT) cells have been recognized as a minor, but unique lipid-reactive population of T cells with diverse functions in the immune system. The number of iNKT cells in human peripheral blood is highly variable and ranges from 0.03 to 0.78% of lymphocytes (1). They function similarly to the cells of the innate immune system as they display less specificity and more rapid activation compared to adaptive immune cells (2). The term “invariant” comes from the expression of almost invariant T cell receptors (TCR), V α 14 J α 18 in mice and V α 24 J α 18 in human, paired with limited V β chain (3). Unlike conventional T cells, which recognize peptide antigens presented on MHC molecules, iNKT cells recognize lipid antigens presented on CD1d. A member of the CD1 family, CD1d is a non-polymorphic MHC class I-like molecule, expressed on antigen-presenting cells (APCs). CD1d is present on dendritic cells (DC), B cells, monocytes, and macrophages and also on cells of non-hematopoietic origin, such as lung, gastrointestinal and cervical epithelial cells, and hepatocytes (4, 5).

Although not the main focus of this review, it should be noted that in addition to iNKT or type 1 NKT cells, there is another NKT cell population called diverse NKT (dNKT) or type 2 NKT cells. The dNKT cells express TCRs that are more diverse and recognize different sets of lipid antigens compared to iNKT cells (6). Furthermore, other CD1 family members, such as CD1a, b, c, present other types of lipid structures and are able to activate CD1-reactive, non-NKT T cells, such as $\gamma\delta$ T cells (7).

Activated iNKT cells can rapidly produce various T helper cell cytokines and crosstalk with other populations of cells in the immune system. Thus, they are an important factor in determining the outcome of the overall immune responses in various disease models, such as asthma (8), autoimmune diseases (9), cancer (10), and infectious diseases (11). The diverse roles of iNKT cells from anti-microbial immunity to regulatory functions in autoimmune diseases are partly due to the bidirectional activation between iNKT cells and DCs. In the presence of infection or pattern recognition receptor stimulation, iNKT-DC interactions, through CD40-CD40L, induce NF- κ B activation, enhancing pro-inflammatory DC maturation and IL-12 production (12). Simultaneously, DCs present lipid on CD1d and produce IL-12 activating iNKT cells. The activation of these pathways results in the induction of innate and adaptive immune responses, including transactivation of NK cells (13) and enhanced response of CD4 $^{+}$ and CD8 $^{+}$ classical T cells to peptide antigens (2, 14, 15). In contrast, interactions between iNKT cells and immature DC, without other stimuli, trigger tolerogenic DC maturation. Tolerogenic DCs in turn induce regulatory T cells preventing autoimmunity (12).

Invariant natural killer T cells can be activated directly by the cognate interactions between their invariant TCRs and CD1d-loaded with exogenous or endogenous lipid antigen, and indirectly by the combination of pro-inflammatory cytokines (11). The first identified exogenous lipid antigen for iNKT cells was α -galactosylceramide (α -GalCer), a glycosphingolipid. Subsequently, pathogenic bacteria-derived glycolipids from *Borrelia burgdorferi* (16) and *Streptococcus pneumoniae* (17) were found to bind CD1d and be presented to iNKT cells. In the absence of microbial-derived or exogenous lipid antigens, such as in the case of Gram-negative *Salmonella* infection (18, 19), iNKT cell activation can also be mediated by presentation of endogenous lipid antigens via cognate interaction between CD1d and iNKT cell TCR, as well as cytokine-mediated activation (11).

Viruses are another example of microbes that lack lipid antigens, yet there is growing evidence for the involvement of iNKT cells in several viral infections (20). The mechanisms underlying iNKT cell activation during viral infection remain ambiguous. While some studies suggest cytokine-mediated activation, others indicate possible lipid-loaded CD1d-dependent activation. Several lines of study have clearly demonstrated that some viruses downregulate surface CD1d expression, attenuating the iNKT cell response as an evasion strategy, supporting a role for CD1d-dependent iNKT cell activation in viral clearance (21–25).

In this review, we summarize the current information on the role of iNKT cells, CD1d, and lipid antigens during viral infection. Importantly, potential CD1d-loaded lipid antigens as iNKT cell ligands in viral infection will be discussed and proposed.

iNKT Cells in Viral Infection

Both protective and pathogenic roles of iNKT cells in various viral infections have been demonstrated in mice and human. Mice lacking iNKT cells displayed worsened disease outcomes for several viral infections including herpes simplex virus type 1 and 2 (HSV-1, 2) (24, 26, 27), murine cytomegalovirus (MCMV) (28), respiratory syncytial virus (RSV) (29), and influenza virus (30–32). In human, human immunodeficiency virus (HIV) is known to infect CD1d-restricted T cells (33), resulting in reduced iNKT cell numbers in HIV-infected patients after seroconversion (34). Moreover, X-linked lymphoproliferative syndrome patients, who have mutations in SLAM-associated protein, an adaptor protein important for iNKT cell development, are more susceptible to severe Epstein–Barr virus (EBV) infection suggesting a protective role for iNKT cells against EBV infection (35–37).

Beneficial roles of iNKT cells are also demonstrated by the enhanced anti-viral immunity and improved clinical outcomes following treatment with α -GalCer, a potent iNKT cell stimulant, in HIV (38), MCMV (39), RSV (29), hepatitis B virus (HBV) (40), and influenza virus infections (41). Co-administration of α -GalCer with inactivated influenza virus resulted in boosted antibody production and enhanced cellular responses to subsequent infections in immunized mice (42). In contrast, iNKT cells are also known to have pathogenic roles following hepatitis C virus (HCV) infection (43), and promote chronic lung disease in Sendai virus-infected mice (44). Recently, iNKT cells have been shown to play a deleterious role in dengue virus (DENV) infection in mice (45), and iNKT cell activation was found to be correlated with poor clinical outcomes in dengue infected patients (46).

Modes of iNKT Cell Activation During Viral Infection

As viruses contain no known exogenous lipid antigens, it is possible that they may activate iNKT cells using cytokine signals alone or through CD1d-bound endogenous lipid antigens. For some viruses, such as influenza (31) and MCMV (47), cytokines secreted during infection alone could potentially activate iNKT cells. While the significance of CD1d-dependent iNKT cell activation in viral infection remains controversial, APC stimulation by viral toll-like receptor (TLR) agonists has been shown to lead to a shift in cellular lipid metabolism toward antigenic lipids as well as CD1d-dependent iNKT cell activation (48, 49). Moreover, some viruses downregulate CD1d expression, presumably to evade iNKT cell recognition, suggesting that CD1d-bound endogenous lipid antigens might be involved in iNKT cell response during viral infection. Because dNKT cells are also reactive to CD1d-loaded lipids, the up- or downregulation of CD1d in viral infection could also affect dNKT cells. Likewise, the expression of different CD1 isoforms could also affect the functions of other CD1-reactive T cells such as $\gamma\delta$ T cells.

Regulation of CD1d in Viral Infection

CD1d Upregulation

CD1d expression is upregulated in response to viral danger signals, and the increase in expression could lead to higher iNKT

cell response (23, 50). DC maturation in response to viral TLR agonists leads to higher cell surface CD1d expression (49). The increase in cell surface CD1d in response to viral TLR agonists was shown to be mediated both at the transcriptional level and through enhanced cellular distribution of CD1d toward the surface (50). Apart from viral TLR stimulation, type I interferons, known for their anti-viral function, can also induce higher levels of CD1d mRNA transcripts (50). In actual viral infections, CD1d was upregulated in cardiac endothelial cells in mice infected with coxsackievirus B3 virus (51), hepatocytes from HCV infected patients (52), and in monocytes from DENV-infected patients (46).

The upregulation of CD1d in response to viral danger signals could therefore be a possible mechanism for initiating the iNKT cell response to the viral infection. This notion has been supported by experiments demonstrating that iNKT cell cytokine production in response to attenuated HSV was reduced upon blocking CD1d with a monoclonal antibody (50). In addition, induction of CD1d expression in EBV-transformed B cells has also been shown to rescue IFN- γ production in iNKT cells (23). However, blocking of CD1d through the use of an antibody in attenuated HSV-infected DC could not completely abrogate the iNKT cell response (50). Moreover, induction of CD1d expression in healthy B cells did not result in an enhanced iNKT cell response (23). These findings suggest that additional soluble factors, such as cytokines produced during viral infection, might act in concert with CD1d antigen presentation to optimize the iNKT cell response.

CD1d Downregulation: A Strategy to Subvert iNKT Cell Recognition?

Another piece of evidence supporting a CD1d-dependent response during viral infection is the finding that some viruses downregulate surface CD1d. This ability has been hypothesized to be a strategy to subvert iNKT cell recognition. The earliest reports of viral-infection-induced CD1d downregulation were from lymphocytic choriomeningitis virus (LCMV), vaccinia virus (VV), and vesicular stomatitis virus (VSV) (53) infections. Mice with acute LCMV, VV, and VSV infections showed reduced surface CD1d expression on DCs and macrophages (53). A subsequent analysis demonstrated that the VSV protein could affect cellular CD1d distribution resulting in inhibition of CD1d-mediated antigen presentation (54). HSV-1 (21, 55–57) and Kaposi sarcoma-associated herpes virus (KSHV) (25) also utilize their viral proteins to disturb CD1d trafficking, in these cases, through interaction with the CD1d cytoplasmic tail, a site important for CD1d sorting. While HSV viral proteins modify and signal CD1d for lysosomal degradation (57), interaction with KSHV proteins increases CD1d internalization from the cell surface (25). EBV, another member of herpes viruses, has recently been shown to downregulate CD1d expression on EBV-transformed B cells abrogating the recognition by iNKT cells (23). In contrast to HSV and KSHV, the decrease in CD1d expression during EBV infection is a result of altered CD1d transcription (23). Human papillomavirus (HPV) employs yet another strategy to suppress surface CD1d expression, utilizing a viral protein E5 to trap CD1d molecules inside the ER-promoting proteasomal degradation (58). Three different HIV proteins VpU (59), Nef (22, 60), and gp120 (61) participate in CD1d downregulation, but

whether CD1d downregulation results in the loss of iNKT cell recognition in HIV-infected patients is still unknown.

These examples highlight several strategies employed by viruses to achieve one goal, to prevent CD1d from reaching or accumulating at the cell surface (Figure 1). The downregulation of CD1d can diminish the iNKT cell response and worsen the outcome of several viral infections, suggesting that iNKT cells might be a significant player in combating against certain viral infections. Together, these findings demonstrate the importance of CD1d-dependent iNKT cell activation in the cellular response to viral infection even though viruses contain no known exogenous lipid antigens. The next challenge in understanding how iNKT cells are modulated by viral infections is the identification of potential endogenous lipid antigens that could serve as iNKT cell ligands.

Possible Self-Lipid Ligands for iNKT Cells

Due to the lack of virus-derived lipid antigens, host cellular lipids are the most likely source of CD1d ligands that are presented to activate iNKT cells during viral infection. Endogenous lipid antigens are required for iNKT cell selection in the thymus and possibly play a role in activating iNKT cells in the periphery (3). The advancement in the search for endogenous lipid ligands has begun to provide insights into the biology of CD1d-bound mammalian lipids that could induce the iNKT cell response. However, the role of endogenous lipids as well as their regulation during viral infection remains largely unknown.

Cellular Lipid Antigens

Glycosphingolipids

Several lines of evidence have suggested a role for mammalian glycosphingolipids (GSLs) in the development and peripheral activation of iNKT cells. Among these GSLs, isoglobotrihexosylceramide (iGb3) was proposed to be involved in thymic iNKT cell selection and peripheral iNKT cell activation (18, 62). However, its importance in these processes remains to be clarified (63, 64).

Glucosylceramide (GlcCer) derivatives can initiate an iNKT cell response in CD1d-dependent manner (48, 49, 65). β -anomeric GSLs were previously considered as the candidate endogenous iNKT ligands as these lipids are the most abundant form of GSLs in mammalian tissues. In addition, only β -transferases for GlcCer and galactosylceramide are present in the mammalian genome (66) and α -anomeric GSLs were not thought to be present in mammals (67). However, recent findings, using more sensitive lipid detection methods, suggest that α -anomeric GSLs might be sparingly present in the mammalian cells (66, 68). Interestingly, the activity of GlcCer from mammalian tissue, formerly ascribed as β -GlcCer (48), is now found to account for a low level of α -anomeric GSL that appears to impact iNKT activity. Removal of β -GlcCer from the lipid fraction using glucocerebrosidase treatment did not alter iNKT cell activity while inhibition of α -anomeric GSL with a monoclonal antibody diminished the effect (66, 68). In addition, only α -GalCer but not β -GluCer-loaded CD1d tetramer could stain splenocytes and DN32 NKT hybridoma (66). Consistent with these observations, mice treated with an antibody against α -linked monoglycosylceramide exhibited impaired iNKT cell development (66). This suggests

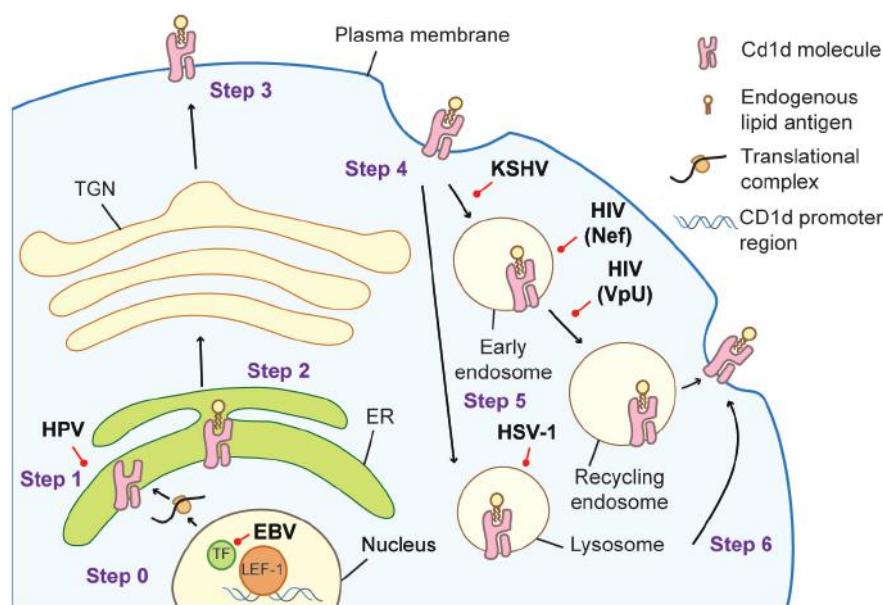


FIGURE 1 | Cellular trafficking of CD1d molecules in an antigen-presenting cell (APC) and the strategies viruses employed to interfere with successful antigen presentation to iNKT cells. Step 0: CD1d gene is transcribed. EBV infection results in the association of LEF-1 at the CD1d promoter region interfering with its transcription (23). Step 1: newly synthesized CD1d molecules assemble with the β_2 -microglobulin subunit in the endoplasmic reticulum (ER). HPV utilizes its E5 protein to retain CD1d in the ER (58). Step 2: endogenous lipid antigen is loaded on CD1d. Step 3: loaded CD1d

traffics to the plasma membrane. Step 4: CD1d is internalized into the endocytic compartments. The MIR protein of KSHV can promote endocytosis (25). The HIV protein Nef accelerates CD1d internalization (22, 60), while VpU retains it in early endosome (59). HSV-1 infection results in CD1d retention in the lysosomal limiting membrane (21), and two HSV-1 proteins gB and US3 direct CD1d to lysosomal degradation (55). Step 5: the exchange for the antigenic lipid occurs in the lysosome. Step 6: CD1d returns to the plasma membrane to present lipid antigen to iNKT cell membrane to present lipid antigen to iNKT cells.

that α -linked monoglycosylceramides, such as α -GalCer and α -GlcCer, might be the iNKT cell selecting self-antigen in the thymus (66). The availability of α -GlcCer is tightly regulated by degradation with catabolic enzymes of the ceramide and glycolipid pathway (66). However, the detailed mechanisms underlying the synthesis of α -anomeric GSLs in mammals remain largely unknown.

Non-Glycosphingolipids

Apart from GSLs, other lipid species have also been suggested as possible iNKT cell stimuli (69). Phosphatidylinositol (PI) (70) and phosphatidylcholine (PC) (71) were among the first endogenous CD1d-bound lipids reported. Mammalian lysophospholipids and lysosphingomyelin stimulate iNKT cell hybridomas with varying strength among the clones examined (72). A recent study also demonstrated that ether-bonded phospholipids generated in the peroxisomes of mouse thymus could serve as iNKT cell selecting ligands, as mice lacking the enzyme required for their generation displayed a marked decreased in iNKT cell number (73).

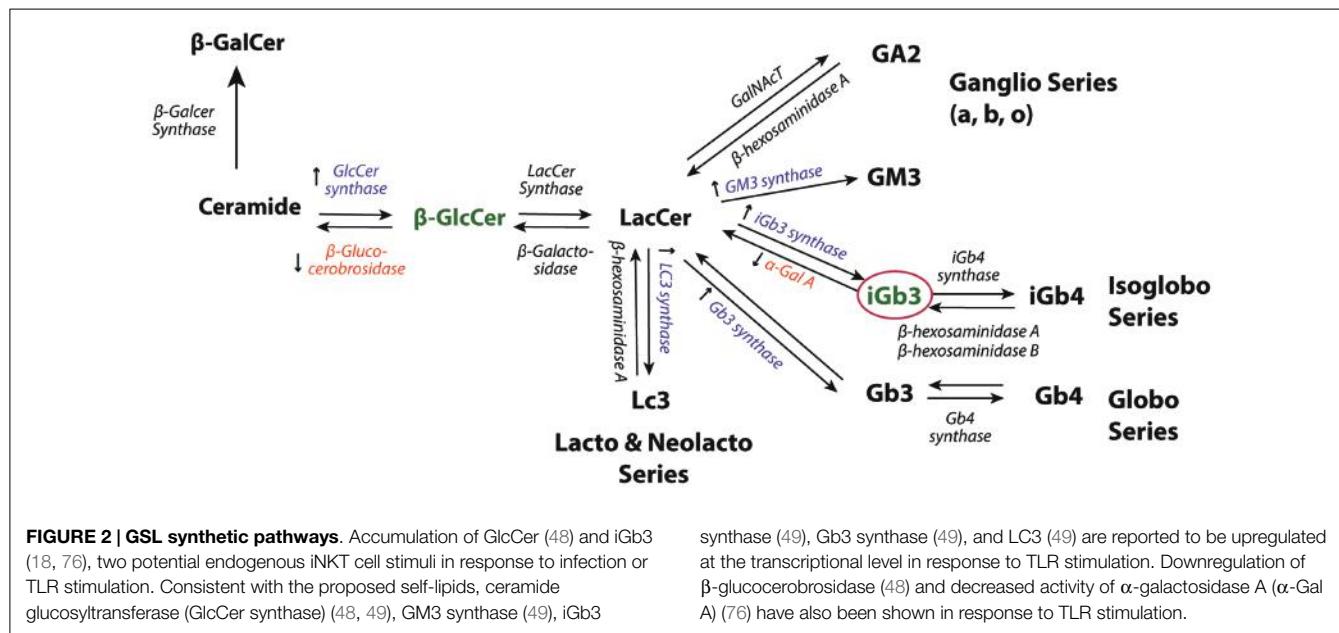
Regulation of Endogenous Lipid Antigens Presented in Response to TLR Stimulation and Infection

Limited evidence is available regarding CD1d-dependent endogenous lipid presentation to iNKT cells during viral infection. By presenting self-lipid antigens, the host is at risk for undesirable auto-reactivity, necessitating tight control of this process. Unresolved questions regarding endogenous lipid antigens utilized in

the response to viral infection include what are the correct form of lipid antigen, the appropriate magnitude of release, as well as location, and temporal control that would provide a beneficial effect but limit adverse consequences to the host. Several studies suggested the possible involvement of the pro-inflammatory signals that enhance CD1d-dependent self-lipid presentation (48, 49, 74), self-lipids “alteration” during viral infection (75), or the inhibition of enzymes that degrade endogenous lipid antigens (76).

Several innate pro-inflammatory signals may induce iNKT cell activation through CD1d presentation of endogenous lipid antigens. Once DCs are stimulated with agonists for endosomal TLRs known to recognize viral genomes, such as TLR3 (77), TLR7 (74), and TLR9 (49, 78), they may mount an iNKT cell response by the presentation of endogenous lipid antigen in concert with the production of cytokines such as IL-12 (74, 77, 78) and type I interferon (49). Although several enzymes involved in the biosynthesis of GSLs including GlcCer synthase and sialyltransferases were found upregulated in response to TLR stimulation (Figure 2), the lipid antigen being presented remains elusive (49, 74). β -GlcCer was proposed to be the endogenous ligand involved in the iNKT cell response to TLR stimulation (48) but whether low levels of α -GlcCer contamination could be the active ligand in this situation was not examined (66, 68). Although α -linked monoglycosylceramide has been suggested as the selecting ligand for iNKT cell development (66), it is not known whether it plays a role in viral infection.

The alteration of self-lipid antigens to “antigenic” lipids that could activate CD1d-restricted NKT cells was reported in the



mouse model of HBV infection (75). Hepatocytes infected with HBV could induce NKT cell activation in a process that required CD1d, a microsomal triglyceride transfer protein (MTP) and secretory phospholipases. The antigenic lipids were found to be lysophospholipids, specifically lysophosphatidylethanolamine (PE) (75). Surprisingly, *in vitro* analysis indicated that iNKT cells were not activated by CD1d-presented lysophospholipids, instead a dNKT cells appeared to be the target. Moreover, the activation of iNKT cells was shown to be cytokine mediated during *in vivo* murine HBV infection (75). Therefore, the nature of lipid antigens, differences in TCR structure, and the mode of docking of different lipid antigens might contribute to their activation efficacy on different NKT cell subsets.

Decreased degradation of endogenous lipid antigens has been suggested to mediate iNKT cell auto-reactivity and activation in response to TLR stimulation (76). The activation of MyD88-dependent-TLR 4 and 9 could lead to a decrease in the enzymatic activity of α-galactosidase A, an enzyme that acts as a rate limiting step in endogenous lipid degradation and results in the accumulation of lysosomal lipid and iNKT cells activation (76) (Figure 2). α-Gal-A is proposed to play a role in regulating the cellular levels of GSLs during physiologic conditions, but decreases in its activity may allow the level of self-lipid to reach the threshold of iNKT cell stimulation during infection (76).

Alteration of Lipid Metabolisms During Viral Infection

Recent findings indicate that viruses can modulate host lipids to accommodate their life cycle. Several cellular lipids have been identified to be crucial for their entry, replication, and budding (79). Altering the host metabolism as a strategy to facilitate their replication has been reported for human cytomegalovirus (80), DENV (81), and HCV (82) infection. Two HCV proteins, NS5A and NS5B, appear to induce expression of the GlcCer synthase gene, an enzyme essential for the synthesis of GlcCer, a species of

self-lipid antigen (83) (Figure 2). Whether such alterations by the virus to accommodate itself could serve as a signal for the immune response to counteract the infection is not clear. Likewise, whether changes in lipid metabolism mediated by virus infection can be employed as an immune evasion strategy by diverting the lipid profile “away” from the composition that could activate iNKT cells has not yet been extensively studied.

Concluding Remarks

Despite the rapidly expanding knowledge regarding the roles of iNKT cells in viral infection, an important question remains: what are their natural ligands during viral infection? Self-lipid antigens loaded on CD1d have been proposed in the absence of microbial-derived lipid antigens. Recent advances using viral TLR agonists have identified potential species of cellular lipids as iNKT cell stimuli. However, iNKT cell lipid ligands important in actual viral infections have not been established. As several viruses are known to interfere with host cellular lipid metabolism, alteration of cellular lipid regulation may also affect self-lipid antigen presentation to iNKT cells. A better understanding of self-lipid antigens in viral infection would not only provide us with a more complete picture of the complex host–virus interaction but would also reveal potential strategies to manipulate iNKT cells for desirable effects to combat against viral infections.

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CD1d expression and invariant NKT cell responses in herpesvirus infections

Brian K. Chung^{1,2*}, John J. Priatel³ and Rusung Tan^{4*}

¹ NIHR Birmingham Liver Biomedical Research Unit, Centre for Liver Research, University of Birmingham, Birmingham, UK

² Institute of Clinical Medicine, Faculty of Medicine, University of Oslo, Oslo, Norway, ³ Department of Pathology and

Laboratory Medicine, University of British Columbia, Vancouver, BC, Canada, ⁴ Department of Pathology, Sidra Medical and Research Center, Doha, Qatar

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USA

*Correspondence:

Brian K. Chung,
NIHR Biomedical Research Unit,
Centre for Liver Research, Institute of
Biomedical Research, University of
Birmingham, 5th Floor, Room 512,
Edgbaston B15 2TT, UK
b.chung@bham.ac.uk;

Rusung Tan,
Department of Pathology, Sidra
Medical and Research Centre,
Doha, Qatar
rtan@sidra.org

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Invariant natural killer T (iNKT) cells are a highly conserved subset of unconventional T lymphocytes that express a canonical, semi-invariant T cell receptor and surface markers shared with the natural killer cell lineage. iNKT cells recognize exogenous and endogenous glycolipid antigens restricted by non-polymorphic CD1d molecules, and are highly responsive to the prototypical agonist, α -galactosylceramide. Upon activation, iNKT cells rapidly coordinate signaling between innate and adaptive immune cells through the secretion of proinflammatory cytokines, leading to the maturation of antigen-presenting cells, and expansion of antigen-specific CD4+ and CD8+ T cells. Because of their potent immuno-regulatory properties, iNKT cells have been extensively studied and are known to play a pivotal role in mediating immune responses against microbial pathogens including viruses. Here, we review evidence that herpesviruses manipulate CD1d expression to escape iNKT cell surveillance and establish lifelong latency in humans. Collectively, published findings suggest that iNKT cells play critical roles in anti-herpesvirus immune responses and could be harnessed therapeutically to limit viral infection and viral-associated disease.

Keywords: iNKT cells, CD1d, herpesvirus, viral immunity, immunotherapy

Introduction

Herpesviridae is a family of large DNA viruses that contain between 100 and 200 genes within an icosahedral capsid composed of viral proteins, mRNAs, and a lipid bilayer envelope (1). In humans, herpesviruses frequently infect both immunocompetent and immunocompromised hosts, with high-prevalence rates ranging from 60 to 90% in the adult population (2, 3). Common human herpesviruses include herpes simplex type 1 (HSV-1) and type 2 (HSV-2), varicella zoster virus (VZV), human cytomegalovirus (HCMV), Epstein–Barr virus (EBV), human herpesvirus 6 (HHV-6), and Kaposi's sarcoma-associated herpesvirus (KSHV). Primary infections with herpesviruses are frequently mild or asymptomatic and lead to lifelong viral latency within the host. However, reactivation of viral replication in immunocompromised individuals often leads to life-threatening infections and malignancies (4).

Host immune responses are critical for restraining and abrogating viral replication, controlling viral load, and limiting disease severity (5–10). For example, HSV and HCMV infections in immunocompetent individuals trigger a rapid expansion of natural killer (NK) cells and virus-specific cytotoxic T lymphocytes (CTL) that are important for eliminating infected cells (3, 11, 12). In response, herpesviruses have evolved sophisticated strategies to evade NK cell and CTL recognition that allow herpesviruses to achieve lifelong survival. In the case of CTL, whose T cell receptor (TCR) bind virus-peptide–MHC

class I complexes on the infected cell surface, herpesviruses have been shown to disrupt many steps of MHC class I presentation, including the transfer of cytosolic peptides into the ER, the loading of peptides onto newly synthesized MHC complexes, and the trafficking of MHC-peptide molecules from the cytosol to the plasma membrane (13, 14). In contrast to CTL, NK cells lack TCR and respond to reduced MHC class I expression induced by herpesvirus infection (13, 15). Inhibitory NK surface markers, such as killer cell immunoglobulin-like receptors (KIR), leukocyte immunoglobulin-like receptors (LIR), and CD94/NKG2 (15, 16), monitor the expression of self-MHC class I and prevent the activation of NK cells. Herpesvirus infections that downregulate MHC class I surface expression in order to evade CTL are more susceptible to NK cells and hence, some herpesviruses also express viral homologs of MHC class I to escape NK cell detection (14, 17). The existence of these back and forth CTL and NK cell evasion strategies underscores their presumed importance in controlling herpesvirus infection and provides a rationale for why multiple immune subsets are necessary to effectively combat herpesviruses.

Natural killer T (NKT) cells are a unique group of CD1d-restricted innate-like lymphocytes and patients deficient in NKT cells develop severe and fatal herpesvirus infections (18–24). These findings, in concert with observations showing that herpesviruses downregulate surface expression of CD1d (25, 26), suggest an important role for NKT cells in the immune response to herpesviruses. NKT cells are distinct from NK cells as they express TCR, but unlike CTL, NKT cells emigrate from the thymus primed to respond and aid in early anti-viral defenses. In this review, we focus on invariant natural killer T (iNKT) cells, a population of NKT cells, which recognize the exogenous lipid antigen, α -GalCer (27). We highlight the role of iNKT cells in herpesvirus infections and the significance of CD1d expression in controlling herpesvirus replication.

iNKT Cells – Unconventional T Lymphocytes

Invariant natural killer T cells are a subset of T lymphocytes that express a canonical, semi-invariant TCR and surface

markers typically found on NK cells and activated CTL (28–30). iNKT cells are positively selected in the thymus by the non-polymorphic glycoprotein, CD1d, and recognize CD1d-restricted glycolipid antigens presented by antigen-presenting cells (APC) in the periphery. Human iNKT cells are CD4+, CD8+ or CD4–CD8– and bear a V α 24–J α 18 TCR rearrangement that preferentially associates with V β 11 (31, 32). In mice, CD8+ iNKT cells are rare and the majority express a V α 14–J α 18 TCR α -chain paired with V β 8, V β 7, or V β 2 (33, 34). Human and mouse iNKT cells both display an effector memory phenotype (CXCR3+, CXCR4+, CD44+, CD69+, CD161+ in humans, NK1.1+ in mice) (35, 36) and are strongly activated by α -GalCer, a non-mammalian glycosphingolipid originally isolated from a marine sea sponge (27). In contrast to iNKT cells (type I), variant NKT cells (diverse or type II) are unresponsive to α -GalCer, and react to sulfatide and phospholipid antigens (37). Type II NKT cells are largely excluded from this review as much less is known about their role in viral infection.

iNKT Cell Activation in Viral Infection

In recent years, evidence from multiple clinical and animal studies suggest that iNKT cells enhance the control of herpesvirus replication (18–24, 35, 38–41) (Table 1). However, the identity of the lipid antigen(s) that presumably drive iNKT cell activation remains elusive. By contrast, several bacteria-derived lipid antigens containing α -linked glycans similar to α -GalCer have been reported [α -glucuronosylceramide and α -galacturonosylceramide (42–44), α -galacosydiacylglycerol (45), and α -glucosyldiacylglycerol (46) from *Streptococcus pneumoniae*, *Sphingomonas paucimobilis*, and *Borrelia burgdorferi*, respectively]. Unlike bacteria, herpesviruses do not express virus-specific lipids; therefore, in the absence of pathogen-derived antigens, iNKT cells likely recognize endogenous self-lipids presented by CD1d (47). Supporting this assumption are several lines of evidence showing that CD1d is required to activate iNKT cells following human herpesvirus infection (25, 48–53). Moreover, hepatitis B infection has been

TABLE 1 | Effect of human herpesvirus infection on CD1d expression and iNKT cells.

Virus	Human CD1d expression	Mechanisms	iNKT cell deficiency			
			Mouse	Human	α -GalCer	References
HSV-1	↑ (Low-viral dose) ↓ (High-viral dose)	Glycoprotein B (gB); serine–threonine kinase, US3	↑ Viral titer	–	–	(48, 51, 55–59)
HSV-2	–	–	↑ Viral titer ↑ Mortality	–	↑ Protection	(49, 60–65)
VZV	–	–	–	↑ Disease	–	(23, 40)
EBV	↓	↓ CD1d transcription	–	↑ Viral titer ↑ Disease	–	(18–21, 24, 53, 66–71)
HHV-6A/B	–	–	–	–	–	–
HHV-7	–	–	–	–	–	–
CMV	↓	glycoprotein US2	↑ Viral titer ↑ Mortality	–	↓ Viral titer	(52, 72–77)
KSHV	↓	Modulator of immune recognition-1 and -2 (K3 and K5)	–	–	–	(25, 78, 79)

HSV-1, herpes simplex virus-1; HSV-2, herpes simplex virus-2; VZV, varicella zoster virus; EBV, Epstein–Barr virus; HHV6A/B, human herpesvirus 6A/B; HHV-7, human herpesvirus-7; CMV, cytomegalovirus; KSHV, Kaposi's sarcoma-associated herpesvirus; –, unknown.

shown to induce the expression of endogenous lipid antigens (lysophospholipids) in human and mouse hepatocytes (54), suggesting that herpesvirus infection may trigger the presentation of analogous self-lipids on CD1d.

Lysophospholipids were identified as endogenous iNKT cell antigens by screening the responsiveness of human iNKT cell clones to synthetic preparations of CD1d-bound ligands (80). Similar filtering procedures were used to identify the glycosphingolipid, β -D-glucopyranosylceramide, as a physiologically relevant self-antigen for iNKT cells (81). Whether these self-antigens are presented by APC during herpesvirus infections is not yet known but the recognition of viral nucleic acids by Toll-like receptors (TLR)-3, -7, and -9 has been shown to induce the synthesis of β -D-glucopyranosylceramide (21), implying that glycosphingolipid antigens may be expressed in herpesvirus infections (81). APC treated with TLR-3, -7, -8, and -9 agonists also enhance transcription of enzymes involved in glycosphingolipid synthesis and the inhibition of these pathways abolishes the reactivity of iNKT cells to TLR-stimulated APC (82, 83). Together, these findings suggest that herpesvirus may activate early iNKT cell responses during infection by inducing the presentation of endogenous lipids antigens on CD1d.

In addition to antigen activation, iNKT cells can react to herpesvirus replication in a CD1d/TCR-independent manner through the actions of proinflammatory cytokines and costimulatory molecules on APC (47). iNKT cells express high levels of IL-12R and are sensitive to IL-12, as well as IL-2, IL-18, and type I IFN released following bacterial (84, 85) and murine cytomegalovirus (MCMV) infection (72, 86). iNKT cells also respond to IL-23 and IL-25 (87, 88), and stimulation by these cytokines induces IL-17 production and amplify inflammatory anti-viral responses (89, 90). Thus, the activation of iNKT cells during herpesvirus infections may involve two pathways; TCR signaling provided by the recognition of lipid antigen(s) and antigen-independent stimuli supplied via cytokines and co-stimulation molecules.

T cell receptor-dependent and -independent activation of iNKT cells can both elicit the substantial release of cytokines and chemokines, including IFN- γ , TNF- α , TNF- β , GM-CSF, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IL-21, CCL3/MIP-1 α , CCL4/MIP-1 β , CCL5/RANTES, and eotaxins (91, 92). IFN- γ , TNF- α , and TNF- β are known to have direct inhibitory effects on viral replication and GM-CSF, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IL-21, CCL3/MIP-1 α , CCL4/MIP-1 β , CCL5/RANTES activate APC, NK cells, CD4+, and CD8+ T lymphocytes, and promote iNKT cells to migrate to sites of inflammation (36, 92). The early production of cytokines and chemokines by iNKT cells may boost the regulation of anti-herpesvirus defenses by triggering the activation of innate and adaptive immune responses. Further work is necessary to substantiate the production and effect of these cytokines and chemokines *in vivo* as the majority of these molecules are secreted by iNKT cells only after powerful TCR stimulation by α -GalCer and have not been directly assessed during herpesvirus infections.

Along with their potent cytokine abilities, activated iNKT cells can also kill target cells through their expression of perforin/granzyme, TRAIL, and FasL (91). Additional assessment is also required to fully delineate the importance of iNKT cell cytotoxicity in herpesvirus infections but B cells transformed by EBV are susceptible to iNKT-mediated cytolysis *in vitro* (53), suggesting

that iNKT cells may directly prevent the proliferation of virus-transformed cells.

Herpes Simplex Virus-1

Herpes simplex virus-1 is an α -herpesvirus that infects mucocutaneous epithelium and establishes latency in sensory ganglia (2). HSV-1 is commonly associated with oral and ocular lesions. However, genital HSV-1 infections now account for over half of genital herpes episodes in North American and European countries (93–96). Studies in HSV-1 murine models support a role for iNKT cells in controlling herpesvirus infection: CD1d- and α 18-deficient mice infected with HSV-1 experience higher viral loads and morbidity compared to wild-type littermates (48). iNKT cells may be dispensable in some strains of HSV-1 infection (55) but help control HSV-1 strains that persist in sensory neurons indicating that iNKT cells may be important for restricting the reactivation of HSV-1 (56).

A role for iNKT cells in HSV-1 infection is also supported by observations that HSV-1 alters CD1d presentation, which implies that HSV-1 may modulate CD1d expression to evade iNKT cell recognition. While low-dose HSV-1 infection in human myeloid dendritic cells (DC) increases surface CD1d expression (51, 57), infection with high-viral titers triggers the rapid re-distribution of surface CD1d molecules to the limiting membrane of lysosomes and the *trans*-Golgi network (Figure 1), an action mediated by HSV-1 glycoprotein B (gB) and the viral serine-threonine kinase, US3, which inhibits the activation of iNKT cells (26, 58). HSV-1 may also suppress the

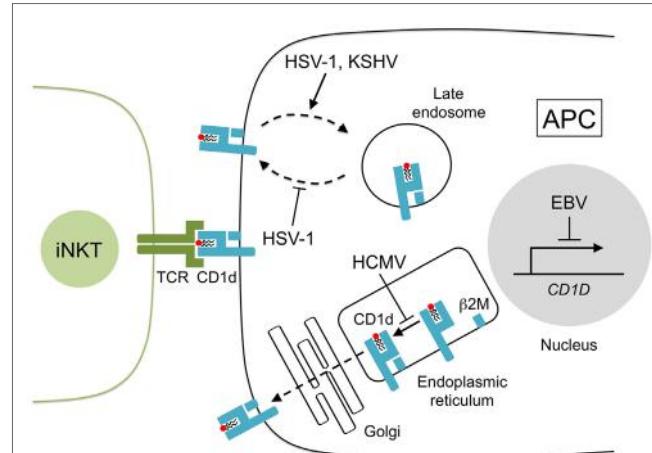


FIGURE 1 | CD1d presentation is disrupted by human herpesviruses.

Lipids are loaded onto newly synthesized CD1d heavy chains and arranged with β 2-microglobulin (β 2M) in the endoplasmic reticulum. CD1d-lipid complexes are transported to the cell surface through the Golgi network by exocytosis. CD1d-lipid molecules are recycled from the cell surface by endocytosis and CD1d ligands are exchanged in the late endosome. Herpesviruses inhibit CD1d presentation to iNKT cells in several ways: HCMV glycoprotein US2 interacts with CD1d in the endoplasmic reticulum reducing iNKT cell activity, HSV-1 glycoprotein B, and serine-threonine kinase US3 remove CD1d from the cell surface and prevent its return to the surface, KSHV modulator of immune recognition-1 and -2 (also known as K3 and K5) downregulate surface CD1d by sequestering its expression to the late endosomes, and EBV transformation of B cells suppresses CD1d transcription.

stimulation of iNKT cells in a CD1d-independent manner as HSV-1 infection in keratinocytes has no effect on CD1d but still impairs iNKT cell activation through an undetermined contact-dependent mechanism (59). These findings support the participation of iNKT cells in anti-HSV-1 responses as HSV-1 appears to have evolved specific mechanisms that suppress iNKT cell function.

Herpes Simplex Virus-2

Herpes simplex virus-2, also an α -herpesvirus, shares significant DNA sequence homology with HSV-1 (97) but is more often linked with genital mucocutaneous infections and persistence in innervating sensory neurons than mucocutaneous epithelium (98). Vertical transmission of HSV-2 by infected mothers to newborns results in neonatal herpes, a serious disease with high rates of neurological complications and mortality (99, 100).

In mice, iNKT cells appear to strongly influence HSV-2 replication as CD1d-deficient animals are 10-fold more susceptible to severe infection compared to wild-type controls (49). iNKT cells are early producers of IFN- γ in HSV-2 infection (49) and can also secrete large quantities of IL-21 that can trigger NK cell and CTL function (60), reduce infection severity and improve host survival (61). IL-21 production by iNKT cells may be particularly critical in limiting HSV-2 replication at the site of infection as the IL-21R expression is upregulated on vaginal epithelia 1–3 days post-infection, and similar to CD1d-deficient mice, IL-21R-deficient animals have increased viral loads and higher mortality to HSV-2 (61).

Severely reduced iNKT cell numbers and a complete lack of NK cells are also observed in IL-15-deficient mice (62) providing further evidence that iNKT cells play a role in controlling HSV-2 as IL-15-deficient mice display a heightened sensitivity to HSV-2 infection (100-fold) compared to CD1d-deficient mice (49). This finding supports the notion that iNKT cells are important in anti-HSV-2 defenses and that they may act synergistically with NK cells to augment host responses to HSV-2. It must be noted, however, that IL-15 can mediate innate immunity against HSV-2 independently of iNKT cells and NK cells (63), and that IL-15 is released by human peripheral blood mononuclear cells (PBMC) upon HSV-2 infection (64).

iNKT cells could be an effective immunotherapy against HSV-2 as intranasal and intravaginal immunization with α -GalCer and HSV-2 glycoprotein (gD) elicits robust innate immunity, the development of systemic gD-specific antibodies and strong secondary responses to HSV-2 proteins in mice (65). Intravaginal immunization provides complete protection against lethal vaginal HSV-2 infection, which supports further evaluation of α -GalCer as an adjuvant for HSV-2 vaccines.

Varicella Zoster Virus

Varicella zoster virus is a neurotropic α -herpesvirus that commonly causes varicella (chicken pox) and subsequently herpes zoster in humans (101). Since its introduction in 1974 (102), live attenuated varicella vaccine has been routinely used worldwide with a wide-safety profile in healthy children although a small number of apparently normal children have been described to develop severe complications such as pneumonitis (23, 40, 103, 104). Immune phenotyping in two of these patients revealed a profound reduction of peripheral blood iNKT cells (23, 40). In the first case, an 11-year-old girl developed a papulovesicular rash and adverse respiratory

illness several weeks after receiving varicella vaccine (23). Analysis of her peripheral lymphocytes at 2 and 4 months after her recovery showed a striking lack of iNKT cells and a complete absence of IFN- γ production by her PBMC following α -GalCer stimulation. The second report describes a 6-year-old boy that presented with vesicular rash and life-threatening pneumonitis 3 weeks after varicella vaccination (40). He too had a reduced number of peripheral iNKT cells, but unlike the first patient, α -GalCer elicited an IFN- γ response from his PBMC, albeit approximately two-fold less than controls. IFN- γ production by his conventional T cells was also decreased upon stimulation with a low concentration of the poly-colonial T cell mitogen, PHA, suggesting that the patient may have had a global IFN- γ defect in addition to low iNKT cell numbers. CD1d expression on the surface of his APC was undetectable and CD1d RNA levels were approximately two-fold lower compared to controls. This observation raises the interesting possibility that circulating iNKT cell numbers in this patient may have been affected by the absence of CD1d on his APC. These case reports suggest that iNKT cells may be activated during VZV infection and future studies quantifying their activation and expansion following VZV vaccination would help delineate the contribution of iNKT cells to anti-VZV defenses.

Epstein-Barr Virus

Epstein-Barr virus is a γ -herpesvirus and primary infection in childhood is generally asymptomatic whereas exposure in adolescence or young adulthood often presents as infectious mononucleosis (IM) (66). EBV is strongly associated with several cancers including nasopharyngeal carcinoma in immunocompetent adults, and a variety of B cell and other malignancies in immunocompromised individuals with AIDS or following transplant immunosuppression (105).

There is extensive evidence that iNKT cells are a critical component of immune responses to EBV, but much of the data are inconclusive or circumstantial because it originates from humans with rare monogenic disorders and clinical case reports. Boys with mutations in the *SH2D1A* gene, which encodes SLAM-associated protein (SAP), have a complete absence of iNKT cells (18–20) and develop X-linked lymphoproliferative disease (XLP) (67, 68), a form of severe and often fatal IM typically triggered by EBV infection (66). It is difficult to ascribe the symptoms of XLP to iNKT cell defects alone because SAP mutations impair iNKT cell development and also disrupt the function of NK cells, CD4+, and CD8+ T cells (69).

Patients with defects in X-linked inhibitor of apoptosis (XIAP) also present with an XLP-like syndrome and have reduced iNKT cell numbers (21). However, the link between iNKT cells and XIAP is unclear given that XIAP-deficient mice have normal numbers of iNKT cells, whereas SAP-deficient mice closely mimic the phenotype of XLP patients and share an impaired development of iNKT cells (70). These findings suggest that patients lacking SAP or XIAP may be susceptible to EBV because of different signaling defects despite exhibiting a similar absence of iNKT cells.

A case report on two sisters who died from an EBV-associated lymphoproliferative disorder resembling XLP strengthens the argument that iNKT cells are involved in the normal control of EBV replication (24). Genetic studies on the two siblings revealed that both

sisters had inherited a homozygous mutation in IL-2-inducible T cell kinase (*ITK*) and immune phenotyping revealed a total absence of iNKT cells, a finding that is recapitulated in *ITK*-deficient mice (71). This study, along with the previous reports in XLP patients, implies genetic mutations that impair iNKT cell development (*SH2D1A*, *XIAP*, *ITK*) may be critical risk factors in determining susceptible to EBV-associated diseases. Additional studies are warranted to clearly elucidate the contribution of iNKT cells in anti-EBV responses and determine if iNKT cells can be targeted for use in EBV vaccines.

iNKT cells may also be involved in the control of EBV-associated cancers. We have shown that the transformation of human B cells into lymphoblastoid cell lines (LCL) rapidly triggers the loss of CD1d transcription and surface expression due to the increased binding of lymphoid enhancer-binding factor 1 (LEF-1) to the CD1d promoter region (53) (Figure 1). LEF-1 is a nuclear protein and dimerizes with β-catenin to suppress CD1d promoter activity (73, 106). Treatment of LCL with the retinoic acid receptor agonist, AM580, prevents the accumulation of LEF-1 at the CD1d promoter, restores the transcription and surface expression of CD1d, and activates human iNKT cell lines to recognize LCL even in the absence of α-GalCer. These findings suggest that EBV transformation may induce the expression of endogenous lipid antigens and that the modulation of the retinoic acid pathway could improve iNKT cell regulation of EBV malignancies.

Human Cytomegalovirus

Human cytomegalovirus is a polytropic β-herpesvirus and the largest member of the herpesvirus family (100). Infection by HCMV is usually asymptomatic but primary and reactivated disease in immunocompromised individuals is associated with significant morbidity and mortality (7, 74). HCMV appears to evade iNKT cell surveillance by expressing the HCMV glycoprotein, US2, which interacts with CD1d (75) and facilitates its proteasomal degradation *in vitro* (76) (Figure 1). The precise contribution of iNKT cells during HCMV infection *in vivo* is less conclusive but murine cytomegalovirus (MCMV) has been widely used as an experimental model for HCMV and in this model, iNKT cells appear to assist early immune responses against MCMV (52, 72, 77) despite an earlier report to the contrary (107). As expected, iNKT cells produce substantial levels of IFN-γ and perforin shortly after MCMV challenge but the addition of TCR blockers or CD1d antibody prior to infection had minimal effect on iNKT cell function (72) indicating that iNKT cell activation by MCMV may be CD1d-independent and could be a consequence of IL-12 production by TLR-9-stimulated APC (86, 108). The relevance of iNKT cells in anti-HCMV defenses requires future clarification as $\text{J}\alpha 18$ -deficient mice (specifically lack iNKT cells) show similar mortality rates as wild-type controls after high dose MCMV infection (72, 107). By contrast, CD1d-deficient mice (lack both iNKT cells and type II NKT cells) show an increased MCMV susceptibility (72) suggesting that type II NKT cells may play a larger role than iNKT cells in the regulation of HCMV.

Kaposi's Sarcoma-Associated Herpesvirus

KSHV is a γ-herpesvirus that can cause malignancies including Kaposi's sarcoma, primary effusion lymphoma, and multicentric

Castleman's disease (1, 78, 79). A putative role for iNKT cells in anti-KSV responses was inferred by the finding that KSHV infection of B cells leads to the sequestering of CD1d to the endocytic pathway and a subsequent loss of iNKT recognition (25). CD1d is directed away from the cell surface by the KSHV-encoded ubiquitin ligases, modulator of immune recognition (MIR)-1, and MIR-2 (also known as K3 and K5), which ubiquitinate the cytosolic lysine residues of CD1d and prevent CD1d from recycling to the plasma membrane (25) (Figure 1). MIR-2 also downregulates the expression of the NKG2D ligands, MHC class I-related chain A (MICA), and MICB (109). NKG2D signaling is known to activate iNKT cell function in the absence of TCR stimulation (110); therefore, the loss of NKG2D signaling may represent another mechanism by which KSHV can control iNKT cell activation during infection.

Conclusion

Mounting evidence supports a significant role for iNKT cells in bridging innate and adaptive immune defenses during herpesvirus infection. Clinical case reports and animal studies demonstrate that iNKT cells may prevent severe and fatal herpesvirus infections (Table 1). Given that herpesviruses interfere with CD1d-iNKT recognition empirically suggests that virus survival and persistence may benefit from the evasion of iNKT cell surveillance.

Significant progress over the last decade has greatly improved our understanding of iNKT cell biology but the precise nature of the CD1d-restricted antigens that activate iNKT cells in herpesvirus infections is still unknown. Discovering the identity of these virus-induced lipid antigens is a priority that will greatly improve the understanding of anti-viral iNKT cell responses *in vivo* and would provide stronger evidence that iNKT cells contribute to anti-herpesvirus defenses. These findings could also assist the development of iNKT cell-based therapies that specifically target pathways that induce the expression of lipid antigens.

Published studies have shown that herpesviruses target the transcription (53) and surface expression of CD1d (26, 58) as a general mechanism for impeding iNKT cell recognition. Thus, future work focused on accurately quantifying the expression of CD1d during herpesvirus infection may yield important insights into the kinetics of iNKT cell recognition and lead to the identification of the lipid antigen(s) that are possibly triggered by herpesvirus infections. Such findings would support the involvement of iNKT cells in the control of herpesvirus infections and the hypothesis that herpesviruses downregulate the surface expression of CD1d to evade recognition by iNKT cells.

Lastly, we have shown EBV transformation suppresses the expression of CD1d and that the activation of the retinoic acid receptor pathway using AM580 re-establishes CD1d surface expression on LCL (53). This finding suggests that maintaining or restoring CD1d expression could improve anti-herpesvirus defenses and this approach could boost anti-viral defenses when combined with the concurrent administration of α-GalCer, or other iNKT cell agonists. Such strategies may enhance the priming of innate and adaptive immune responses to herpesviruses and promote the overall development of iNKT cell immunotherapies (111).

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Regulation of NKT cell localization in homeostasis and infection

Drew Slauenwhite¹ and Brent Johnston^{1,2,3,4*}

¹ Department of Microbiology and Immunology, Dalhousie University, Halifax, NS, Canada, ² Department of Pediatrics, Dalhousie University, Halifax, NS, Canada, ³ Department of Pathology, Dalhousie University, Halifax, NS, Canada, ⁴ Beatrice Hunter Cancer Research Institute, Halifax, NS, Canada

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***Correspondence:**

Brent Johnston,
Department of Microbiology and
Immunology, Dalhousie University,
5850 College Street, Halifax, NS B3H
4R2, Canada
brent.johnston@dal.ca

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Natural killer T (NKT) cells are a specialized subset of T lymphocytes that regulate immune responses in the context of autoimmunity, cancer, and microbial infection. Lipid antigens derived from bacteria, parasites, and fungi can be presented by CD1d molecules and recognized by the canonical T cell receptors on NKT cells. Alternatively, NKT cells can be activated through recognition of self-lipids and/or pro-inflammatory cytokines generated during infection. Unlike conventional T cells, only a small subset of NKT cells traffic through the lymph nodes under homeostatic conditions, with the largest NKT cell populations localizing to the liver, lungs, spleen, and bone marrow. This is thought to be mediated by differences in chemokine receptor expression profiles. However, the impact of infection on the tissue localization and function of NKT remains largely unstudied. This review focuses on the mechanisms mediating the establishment of peripheral NKT cell populations during homeostasis and how tissue localization of NKT cells is affected during infection.

Keywords: natural killer T cells, chemokines, cytokines, homeostasis, leukocyte homing

Introduction

The clearance of bacterial, viral, fungal, and protozoan infections depends upon the coordinated activation of both the innate and adaptive arms of the immune system (1). Innate immune cells, such as macrophages, dendritic cells (DCs), neutrophils, and natural killer (NK) cells, are critically involved in the initial control and clearance of infectious organisms. However, adaptive immunity mediated by T cells and B cells is also required to generate specific sterilizing responses and provide long-lasting immunological memory. Natural killer T (NKT) cells are a subset of T cells that serve as a bridge between innate and adaptive immunity. Upon activation, NKT cells rapidly generate and secrete a diverse array of cytokines and chemokines (2–4), allowing them to shape the magnitude and polarization of host immune responses in infection (5, 6), autoimmune disease (7, 8), allergy (9, 10), and cancer (11). Compared to conventional T cells, NKT cells exhibit altered patterns of tissue localization, suggesting differences in the signals regulating homing and homeostasis. This review examines pathways important in the trafficking and maintenance of NKT cell populations under homeostatic conditions and during microbial infections. The impact of these mechanisms on NKT cell-derived anti-microbial effector functions will be discussed in terms of their ability to orchestrate both innate and adaptive immune responses.

Natural Killer T Cells

Natural killer T cells develop in the thymus from uncommitted thymic progenitors that undergo T cell receptor (TCR) rearrangement and selection. However, unlike the diverse TCR repertoire of conventional T cells that are selected via type I or type II major histocompatibility complex

(MHC), NKT cells express a restricted repertoire of TCR rearrangements that are selected via the MHC-like molecule CD1d (12, 13). While the specific selecting antigen(s) in the thymus remains unclear, several endogenous NKT cell ligands have been proposed based on NKT cell activating capacity. These include isoglobotrihexosylceramide (iGb3) (14), lysophosphatidylcholine (LPC) and lysosphingomyelin (LSM) (15), the peroxisome-derived ether-bonded compounds lysophosphatidylethanolamine (pLPE) and lysophosphatidic acid (eLPA) (16), β -glucosylceramide (β -GluCer) (17), and α -glycosylceramides (18). However, the relative roles of these candidate ligands during intrathymic NKT cell development in mouse versus human as well as their capacities to influence NKT cell functional regulation and/or tissue localization in the periphery remain undefined.

Isoglobotrihexosylceramide, which appeared to be a promising candidate for an endogenous NKT cell selecting antigen in mice, is unlikely to be an endogenous ligand for human NKT cells due to the lack of the relevant iGb3 synthase enzymes in humans (19). Furthermore, the contamination of commercial β -GluCer with α -linked species has brought into question the role of this compound. Indeed, two groups have demonstrated that highly purified preparations of β -GluCer lack NKT cell stimulatory activity (18, 20). However, Kain and colleagues (18) identified the presence of small quantities of endogenous α -linked glycosylceramides [α -GluCer and α -galactosylceramide (α -GalCer)], a class of glycolipids that were thought to be absent in mammalian cells, and identified them as possible endogenous ligands for NKT cell selection and activation. Our understanding of NKT cell development and function will continue to improve as ongoing efforts further characterize the self-lipid antigens that select NKT cells in the thymus.

Two major subsets of NKT cells can be distinguished based on their TCR repertoire and lipid reactivity. Type I or invariant NKT (iNKT) cells express an invariant TCR α chain composed of V α 14–J α 18 rearrangements in mice and V α 24–J α 18 in humans, paired with a restricted repertoire of V β chains (V β 8.2, V β 7, or V β 2 in mice, and V β 11 in humans) (21, 22). Specific detection of iNKT cells is possible through the use of CD1d tetramers loaded with α -GalCer (23, 24). Analogs of α -GalCer are potent activators of iNKT cells and can influence immune responses in many pathological states, including microbial infection (25, 26), autoimmune disease (7), allergy (27), and cancer (28). Type II NKT cells are CD1d-restricted but do not recognize α -GalCer (29). This is a more heterogeneous population of cells, expressing oligoclonal TCRs that utilize a limited collection of V α (V α 1, V α 3, V α 8) and V β -rearrangements (29–31). Comparisons of J α 18 $^{+/-}$ mice lacking type I NKT cells with CD1d $^{-/-}$ mice lacking type I and type II NKT cells suggest that the type II NKT cells are regulatory cells that can suppress anti-tumor immunity (32–34). The best characterized subset of type II NKT cells expresses a TCR that recognizes sulfatide (3-sulfated galactosylceramide) (29, 31, 35). These cells serve as an important regulatory population during inflammatory responses and can be activated by sulfatide to suppress autoimmunity (36–40). Type II NKT cells can also regulate iNKT cell responses. For example, activation of type II NKT cells by sulfatide suppresses the proliferative and cytokine responses of

iNKT cells activated with α -GalCer (33). Furthermore, in a ConA-induced hepatic injury model, sulfatide-activated type II NKT cells induced iNKT cell anergy and prevented inflammatory liver disease (37). However, dysregulated responses of type II NKT cells have also been shown to play a role in the pathogenesis of inflammatory bowel disease in both mice and humans (41, 42). While iNKT cells are more prevalent than type II NKT cells in mice, type II NKT cells appear to be the predominant subset in humans (43). This review focuses on responses of iNKT cells, and the term NKT cell will be used throughout to refer to this population.

In addition to TCR-CD1d interactions, NKT cells are also stimulated by inflammatory cytokines (44–46), neurotransmitters (47), and toll-like receptor (TLR) ligands (48–50). Following activation, NKT cells are able to produce a wide range of cytokines including interferon- γ (IFN- γ), tumor necrosis factor (TNF), interleukin-2 (IL-2), IL-4, IL-10, IL-13, IL-17, IL-21, IL-22, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (2–4, 51). However, the cytokine profile is influenced by the nature of the stimulation and the subset of NKT cells that are activated. Indeed, recent studies have identified a number of distinct lineages of NKT cells that emerge during development, each with a unique profile of transcription factors and cytokine production (52–59). Based on these profiles, NKT cells can be subdivided into NKT-1, NKT-2, NKT-17, and NKT-10 subsets, analogous to the T helper type 1 (Th1), Th2, Th17, and IL-10 producing subsets of conventional T cells. Through their secretion of various cytokines, NKT cells are able to activate other immune cells, contributing to NK cell transactivation (60), DC maturation (61, 62), T cell polarization (63, 64), and B cell antibody responses (65).

NKT Cell Homeostasis

Natural killer T cells require a number of growth factors and survival signals for their maintenance in the periphery. In contrast to the requirement for CD1d during thymic NKT cell selection and initial maturation, mature NKT cells do not require continual CD1d interactions in the periphery to support homeostatic proliferation, long-term survival, or to maintain tissue distribution (66). Instead, NKT cells rely more on signaling elicited by cytokines such as IL-15, and to a lesser extent IL-7 (66–69). However, while thymic NKT cell development and homeostatic NKT cell proliferation are impaired in IL-15-deficient mice, these populations are not abolished (67, 70). It is possible that this is due to differences in the requirement for IL-15 during development of distinct NKT cell lineages. For example, NKT-1 cells express CD122 (the IL-2/IL-15 receptor β -chain) and require IL-15 for development and homeostasis, while NKT-2/NKT-17 cells (marked by the expression of IL-17RB, a receptor for IL-25) develop normally in the absence of IL-15 (58). Accordingly, CD122 is moderately to highly expressed on NKT cells in the mouse liver and spleen, where the NKT-1 lineage constitutes the majority of the NKT population, but is not expressed on lymph node NKT cells, where NKT-17 cells are enriched (52, 54). ICOS/ICOSL interactions are also required for NKT cell homeostasis and function as survival of wild-type NKT cells transferred into ICOSL $^{-/-}$ mice was reduced, and ICOS $^{-/-}$ NKT cells were impaired in their ability to produce IL-4 and IL-13 (71). Many studies have highlighted roles for a

variety of signaling molecules and transcription factors in NKT cell development and homeostasis, including NF- κ B (72), T-bet (73, 74), c-Myc (75, 76), mTORC2 (77), calcineurin (78), Egr-2 (78), Id2 (79), Bcl-2 (80), Bcl-X_L (81), in addition to cytokine receptor subunits IL-2R β (82), IL-7R α (83), IL-15R α (84, 85), and the common gamma chain (83).

In addition to these factors, chemokine receptor signaling has also been implicated in regulating NKT cell homeostasis in the periphery. NKT cells express high levels of CXC chemokine receptor 6 (CXCR6) (86–89), and NKT cells in the liver and lungs are depleted in mice lacking CXCR6 or its ligand CXCL16 (90–92). Geissmann et al. (90) reported that NKT cells from CXCR6^{-/-} mice underwent apoptosis more rapidly in culture than NKT cells from CXCR6^{+/+} mice. *In vivo* however, CXCR6^{-/-} and CXCR6^{+/+} mice exhibited a similar frequency of apoptotic CD1d-reactive cells in liver sections and freshly isolated liver lymphocytes (90). We found no difference in the apoptosis rates of cultured NKT cells purified from the livers of CXCR6^{+/+} and CXCR6^{-/-} mice (91), but observed an accumulation of NKT cells in the bone marrow, suggesting an alteration in homing. Interestingly, mice deficient in Id2 exhibit impaired survival of liver NKT cells, which is associated with reduced expression of CXCR6 and the survival factors Bcl-2 and Bcl-X_L (79). Similarly, hepatic NKT cells from CXCR6-deficient mice expressed lower levels of Bcl-2, suggesting a role in survival (79). Despite the conflicting reports, it seems likely that CXCR6 plays a role in regulating survival of NKT cells within certain tissue environments [since NKT cell numbers are normal in most tissues (90–92)], or under specific culture conditions.

A separate study found that NKT cells in CC chemokine receptor 5 (CCR5)-deficient mice were resistant to activation-induced apoptosis, and produced more IL-4, resulting in enhanced liver injury in a model of ConA-induced hepatitis (93). Interestingly, despite an impairment of activation-induced cell death, there were no defects in Fas-mediated apoptosis in these NKT cells. In human T cells, CCR5-dependent apoptosis has been reported in response to high concentrations of the chemokine ligand CCL5 (94), or ligation of CCR5 by the human immunodeficiency virus (HIV) envelope protein gp160 (95). In these cases however, there was enhanced susceptibility to caspase-8-dependent cell death through induction of FasL (95). These studies point to a role for chemokine receptors in influencing lymphocyte survival and add to a growing body of literature demonstrating the ability of chemokine receptors to regulate a number of cellular functions in addition to their traditional roles in regulating leukocyte recruitment and positioning.

Natural killer T cell homeostasis is also regulated by the microbiome. Germ-free Swiss-Webster and C57BL/6 mice exhibit variable alterations in thymic, spleen, and liver NKT cell populations compared to conventionally housed animals (96–98). This variability may reflect differences in the conventional microbiota in control mice housed in different facilities (98). However, germ-free mice consistently exhibited increased numbers of NKT cells in the intestinal lamina propria and lungs (96, 98). NKT cell accumulation appears to result from dysregulated CXCL16 expression, and could be reversed by CXCL16 blockade or neonatal exposure to conventional microbiota (96). Bacteria of the genera *Bacteroides* comprise >50% of the bacteria in the human gut (99),

and *B. fragilis* has been shown to generate α -GalCer derivatives capable of regulating NKT cells (100, 101). One such compound, α -GalCer_{Bf}, binds to CD1d and activates NKT cells *in vitro* and *in vivo*, albeit to a lesser degree than synthetic α -GalCer (100). However, colonization of germ-free mice with *B. fragilis* led to variable expansion of NKT cells (100). *B. fragilis* also generates GSL-Bf717, an α -GalCer analog that inhibits NKT cell activity and restored NKT cell homeostasis in germ-free mice (101). Therefore, it appears that the composition of the intestinal microbiota influences the homeostasis of NKT cells within the colon and lungs, and may also exert influences on NKT cells within other tissues. Adding further complexity, NKT cells also influence bacterial colonization in the intestine (102), and engagement of epithelial CD1d contributes to intestinal epithelial cell-dependent regulation of mucosal homeostasis via IL-10 production (103), highlighting the intricate interactions which take place between host cells and the microbiota.

NKT Cell Tissue Localization Patterns

In mice, NKT cells are first detected in the thymus at day 5–6 after birth, and in the periphery after day 8 (12, 104). They populate multiple tissues and reach steady state levels by 5–6 weeks of age. In the adult mouse, NKT cell frequency is highest in the liver (12–30% of liver lymphocytes), with lower frequencies in the spleen (1–3%), lungs (5–10%), thymus (0.5–1%), bone marrow (0.4–8%), lymph nodes (0.2–1%), intestines (0.05–0.6%), and blood (0.2%) (23, 24, 98, 105–110).

In contrast to the post-natal NKT cell ontogeny in mice, NKT cells are detected in the human fetal thymus at the start of the second trimester, but the frequency declines with gestational age to reach low levels in the post-natal thymus (111, 112). Human NKT cells also distribute to the periphery during the second trimester, with a prominent distribution to the small intestine which may act as a maturation site (113). Overall, the tissue distribution of NKT cells in the periphery appears to be similar between adult humans and mice. However, the frequency of NKT cells in humans is significantly lower and is subject to considerable variability among individuals. For example, frequencies of NKT cells range from 0.05 to 1% of liver lymphocytes in humans (114, 115), and generally account for 0.01–0.1% of human peripheral blood mononuclear cells, but have been observed to constitute upwards of 3% of peripheral blood mononuclear cells in some individuals (112, 116–118). The variability in NKT cell frequency between individuals appears to be influenced by genetic factors as evidenced by identical twin studies (118). Despite these differences, NKT cells play important roles in human health and disease. Indeed, dysfunctional NKT cell responses and reduced circulating numbers of NKT cells have been reported in patients with autoimmune disorders (8) and malignancies (119–122), suggesting a role for NKT cells in maintaining immune homeostasis.

Phenotypic and Functional Differences in NKT Cell Subsets

Even though NKT cells have a restricted TCR profile, they contain phenotypically and functionally diverse subpopulations characterized by differences in surface marker expression, tissue

TABLE 1 | Chemokine receptor expression and ligand responses on circulating human NKT cells.

Receptor	Expression (%) ^a			Chemotactic response ^c	Reference
	CD4 ⁺	DN ^b	CD8 ⁺		
CCR1 ^d	2–25	55–85	30–80	CD4 ⁺ ; DN ⁺⁺ ; CD8 ⁺⁺	(87, 89)
CCR2 ^d	60–80	95–99	65–99	+++	(87, 89)
CCR3	0–4	0–4	0–4	n.d.	(87, 89)
CCR4	12–40	4–18	2–10	CD4 ⁺⁺ ; DN ⁺ ; CD8 ⁻	(87, 89)
CCR5	45–80	90–99	70–99	+	(4, 87, 89, 116)
CCR6	10–68	72–95	50–85	CD4 ⁺ ; DN ⁺⁺⁺ ; CD8 ⁺⁺⁺	(87, 89, 116)
CCR7	11–28	7–32	2–25	++	(4, 87, 89)
CCR8	11–55% total NKT cells			—	(89, 140)
CCR9	0–4	0–4	0–4	—	(87, 89)
CCR10 ^e	n.d.	n.d.	n.d.	—	(89)
CXCR1	5–10	3–8	n.d.	—	(89)
CXCR2	0–2	0–1	n.d.	—	(89)
CXCR3	75–90	95–99	80–90	+++	(4, 87, 89)
CXCR4	90–99	98–99	95–99	+++	(4, 87, 89)
CXCR5	0–4	0–4	0–4	—	(87, 89)
CXCR6	22–45	85–99	60–98	++	(87, 89, 116)
CX ₃ CR1	4–12	4–12	n.d.	—	(89)

^aRange in reported frequency of receptor positive NKT cells.^bDN = CD4⁻CD8⁻ double negative NKT cells.^cNet chemotactic migration: — (did not respond), + (2–10%), ++ (11–30%), and +++ (>30%).^dOthers report CCR1 and CCR2 on <2% of NKT cells (89, 116).^eCCR8 and CCR10 mRNA detected in NKT cell subsets (89).

n.d. – not determined.

localization, and effector functions. NKT cells in humans can be divided into CD4⁺ (12–36%), CD4⁻CD8⁻ (DN; 60–85%), or CD4⁻CD8α⁺ (1–5%) subsets (123). While the DN and CD8α⁺ subsets in human blood are phenotypically and functionally similar, the CD4⁺ subset represents a functionally distinct lineage with marked differences in cytokine profile and homing receptor expression (see **Table 1**) (4, 87, 116). For example, CD4⁻ NKT cells produce primarily Th1 cytokines such as IFN-γ and TNF, while CD4⁺ NKT cells generate both Th1 and Th2 cytokines (IFN-γ, TNF, IL-4, IL-5, IL-10, and IL-13) (4, 87, 116). However, tissue-resident NKT cells may have differences in surface marker expression and cytokine profiles (115, 124).

Natural killer T cells in mice are comprised of CD4⁺ (60–80%, depending on the tissue) and DN subsets, while CD8⁺ CD1d-restricted NKT cells are absent (24, 105, 125). A Th2-like subset of CD4⁺ NKT cells localizes to the lungs and contributes to airway hyperreactivity and asthma (126, 127), while a subset of DN IL-17-producing NKT cells localize preferentially to the lymph nodes and skin (54). Although there is little evidence for differences in cytokine profiles of CD4⁺ and DN NKT cell subsets in the liver and spleen, functional differences have been reported. For example, DN NKT cells from the liver are able to control tumors better than CD4⁺ NKT cells from the liver or NKT cells from other tissues (128). NKT-1, NKT-2, NKT-10, NKT-17, and follicular

helper-type NKT (NKT_{FH}) subsets of NKT cells have recently been identified on the basis of transcription factor profiles and select surface marker expression (52–59, 129). However, more work is needed to determine the maintenance and plasticity of these profiles since the population ratios in mice seem to change significantly over time (55). Although NKT-1, NKT-2, and NKT-17 subsets emerge in the thymus, it is likely that tissue-specific factors and microenvironmental influences act to shape the phenotype and function of NKT cells after recruitment to specific tissue sites.

Expression of Homing Receptors on NKT Cells

The trafficking behaviors of naïve and effector/memory lymphocyte subsets are a function of the specific combinations of adhesion molecules and chemokine receptors that they express. For example, naïve T lymphocytes use L-selectin (CD62L), the αLβ2-integrin (CD11a/CD18; LFA-1), and CCR7 to enter peripheral lymph nodes at high endothelial venules (130–132), while gut homing memory lymphocytes express the α4β7-integrin and CCR9 (133, 134), and memory lymphocytes targeted to the skin express the cutaneous lymphocyte antigen (CLA) and CCR4 (135). Cells capable of migrating to sites of inflammation display varying levels of CCR1, CCR2, CCR5, CXCR3, or other chemokine receptors on their surface (130, 136, 137).

Human NKT Cells

Generally, human NKT cells express homing receptors for extra-lymphoid tissues (**Table 1**), with only 10–20% of circulating NKT cells expressing the lymph node homing receptor CCR7 (87, 89). Few blood NKT cells express the chemokine receptors CCR3, CCR9, CXCR1, CXCR2, CXCR5, or CX₃CR1 (87, 89). In contrast, a majority (>60%) of NKT cells express CCR2, CCR5, CXCR3, and CXCR4, with differential expression of CCR1, CCR4, CCR6, and CXCR6 depending on the specific NKT cell subset or their tissue distribution (87, 88, 138). Multiple studies examining chemokine receptor expression on human NKT cells have observed greater frequencies of DN and CD8⁺ NKT cells expressing CCR1, CCR2, CCR5, CCR6, CXCR6, and the integrin CD49a, while CCR4 was expressed by a greater proportion of CD4⁺ NKT cells (4, 87, 89, 116, 138). The frequency of circulating NKT cells that express CCR8, a skin homing receptor expressed on the majority of human T cells in healthy skin (139), ranges from 11 to 55% (140). Adhesion molecules such as CLA, CD62L, and α4β7-integrin are present on blood NKT cells to varying levels, with few CLA⁺ NKT cells (6–19%), or CD62L⁺ NKT cells (11–24%), and a larger proportion expressing α4β7 (30–75%) (4, 87). However, the co-expression of specific adhesion molecules and chemokine receptors on NKT cells is required for homing into certain tissues. The frequency of NKT cells expressing both CD62L and CCR7 is much lower than the fraction of cells expressing either receptor alone (87). This is likely to explain the low frequency of NKT cells in peripheral lymph nodes. The identification of distinct NKT cell subsets that exhibit differential cytokine production and unique patterns of homing receptors suggests that different NKT cell subsets can be targeted to different tissues or sites of inflammation.

Mouse NKT Cells

Mouse NKT cells display significant differences in their chemokine receptor profiles and responsiveness to chemotactic ligands compared to human blood NKT cells. A majority of splenic mouse NKT cells express the receptors CCR9, CXCR3, CXCR4, and CXCR6 (88, 98), and TCR β^+ NK1.1 $^+$ cells in V α 14 transgenic mice have elevated surface expression of CCR2, CCR5, and CCR9 (141). In contrast to human blood NKT cells, mouse blood, liver, spleen, and bone marrow NKT cells lack chemotactic responsiveness to ligands for CCR1, CCR2, CCR5, and CCR6 (88). Mouse NKT cells exhibited robust migration to the CXCR3 ligand monokine induced by gamma interferon (MIG; CXCL9) and the CXCR4 ligand stromal cell-derived factor 1 (SDF-1; CXCL12), while the CXCR6 ligand, CXCL16, only induced modest migration of NKT cells despite a large proportion of these cells expressing CXCR6 (Table 2) (88). This suggests that NKT cell responsiveness to CXCL16 and other chemokines is regulated. Consistent with this, responsiveness of CD8 $^+$ T cells to CXCL16 is dependent on activation (86), and increased chemotactic responsiveness to CXCL16 was observed in thymic NKT cells (91). Other tissue-specific differences among NKT cell subsets in terms of their chemokine receptor expression patterns or their chemotactic activity include the findings that a subset of NKT cells in the spleen, bone marrow, and blood, but not the liver, were responsive to the CCR7 ligand secondary lymphoid-tissue chemokine (SLC; CCL21), while a subset of CXCR5 $^+$ NKT cells were only present in the spleen and migrated in response to the CXCR5 ligand B cell-attracting chemokine 1 (BCA-1; CXCL13) (88). Ligands for CCR4 could mobilize lung NKT cells into the airways (108), but did not elicit chemotaxis of NKT cells derived from the spleen, liver, bone marrow, or blood (88), suggesting differences in CCR4 expression or regulation. Similarly, NKT cells in skin and peripheral lymph nodes express CCR6 and migrate in response to the ligand macrophage inflammatory

protein 3 α (MIP-3 α ; CCL20) (54), while spleen, liver, bone marrow, and blood NKT cells do not (88). As CCR6 expression on peripheral lymph node NKT cells correlates with a NKT-17 transcription profile, it will be interesting to correlate NKT cell localization and homing receptor expression in other tissues with the transcription factor expression patterns that have recently been used to classify NKT cell subsets.

Differential chemokine receptor expression on distinct NKT cell subsets suggests the potential to regulate homing to different tissue sites. However, the lack of functional chemotactic responses to many chemokine ligands suggests that chemokine receptor signaling is altered or regulated. Rather than contributing to tissue localization, some chemokines may play important roles in regulating NKT cell survival or effector functions. There is currently little known about the chemokine receptor profiles on activated NKT cells in comparison to resting NKT cells. *In vitro* activation of human NKT cells with α -GalCer upregulated CCR6 protein expression on DN NKT cells relative to CD4 $^+$ NKT cells, while the CD8 $^+$ NKT cell subset displayed increased mRNA for CX $_3$ CR1 (142). Whether these changes mediate alterations in NKT cell localization and/or activity remain to be seen.

NKT Cell Homing and Maturation

During the developmental progression of thymocytes from immature DN precursors through the CD4 $^+$ CD8 $^+$ double positive (DP) stage to mature conventional single positive cells, a subset of chemokine receptors regulate cellular trafficking and positioning through the cortex (CXCR4), subcapsular zone (CCR9), and medulla (CCR4, CCR7) (143–146). It is unclear whether positioning is similar during NKT cell development, as these cells are selected via DP thymocytes rather than thymic stromal cells (68, 147). However, while CCR4 is not expressed on thymic NKT cells, CCR7 controls NKT cell development by enabling access to IL-15

TABLE 2 | Chemokine receptor expression and ligand responses on mouse NKT cells.

Receptor	Expression (%) ^a	Chemotactic response ^b					Reference
		Spleen	Liver	Bone Marrow	Blood	Other	
CCR1	n.d.	—	—	—	—	—	(88)
CCR2	23	—	—	—	—	—	(88, 141)
CCR3	n.d.	—	—	—	—	—	(88)
CCR4	n.d.	—	—	—	—	Lung: ++	(88, 108)
CCR5	20–60	—	—	—	—	—	(88, 93, 141)
CCR6	PLN: 70–80	—	—	—	—	PLN: ++	(54, 88)
CCR7	Thymus: 15–60	+	—	+	+	+	(88)
CCR8	n.d.	—	—	—	—	—	(88)
CCR9	18–80	—	—	—	—	—	(88, 98, 141)
CCR10	n.d.	—	—	—	—	—	(88)
CXCR2	n.d.	—	—	—	—	—	(88)
CXCR3	80–96	++	++	++	++	++	(88)
CXCR4	55–58	+	+	++	++	++	(88)
CXCR5	Spleen: 35–38	+	—	—	—	—	(88)
CXCR6	92–94	+	n.d.	n.d.	n.d.	Thymus: +	(88, 91)
CX $_3$ CR1	n.d.	—	—	—	—	—	(88)
XCR1	n.d.	—	—	—	—	—	(88)

^aRange in reported frequency of receptor positive NKT cells.

^bNet chemotactic migration: — (did not respond), + (<25%), and ++ (>25%).

n.d., not determined; PLN, peripheral lymph nodes.

trans-presentation in the thymic medulla (148, 149). NKT cells upregulate the chemokine receptor CXCR6 during/after positive selection (91), which could also facilitate positioning within the medulla where the ligand CXCL16 is expressed (86).

Interestingly, the NKT cell pool in the thymus contains both “immature” NK1.1⁻ and mature NK1.1⁺ subsets (150). The expression of T-bet during maturation of NK1.1⁻ NKT cells induces the expression of CCR5 and CXCR3 (74). The interaction of CXCR3 with interferon- γ -induced protein 10 (IP-10; CXCL10) expressed by medullary thymic epithelial cells retains mature NK1.1⁺ NKT cells in the thymus as a long-lived resident population (151) (**Figure 1**). However, it remains unclear what role these retained mature NKT cells might play within the thymus as they appear to be absent in humans (112), and conventional T cell development is unaffected in NKT-cell deficient (CD1d^{-/-}) mice (152).

We have shown that NKT cells begin expressing high levels of CXCR6 in the thymus during the transition from CD4⁺CD8⁺ NKT cells to CD4⁺ and DN NKT cells following positive selection (91). CXCR6-deficiency does not affect thymic NKT cell development, but CXCR6^{-/-} mice exhibited a defect in the accumulation of mature CD1d-restricted NK1.1⁺ NKT cells in the periphery. Similarly, treatment of mice with a blocking antibody against CXCL16 did not inhibit accumulation of NK1.1⁻ recent thymic emigrants in the liver, but led to a defect in the accumulation of mature NK1.1⁺ NKT cells (91). These data point to a potential role for CXCR6 and CXCL16 in mediating maturation of NK1.1⁻ recent emigrant NKT cells, and retention and/or survival of mature NKT cells in the liver. A role for CXCR6 in retention is supported by the redistribution of NKT cells to the bone marrow in CXCR6^{-/-} mice (91), while others have also implicated CXCR6 in NKT cell survival (79, 90).

CXCR6^{-/-} mice also exhibited impaired cytokine production by liver and spleen NKT cells following activation with α -GalCer.

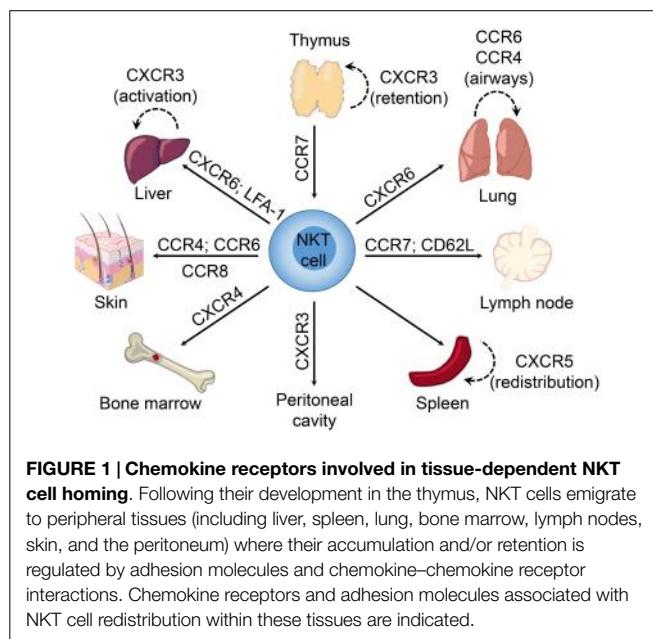


FIGURE 1 | Chemokine receptors involved in tissue-dependent NKT cell homing. Following their development in the thymus, NKT cells emigrate to peripheral tissues (including liver, spleen, lung, bone marrow, lymph nodes, skin, and the peritoneum) where their accumulation and/or retention is regulated by adhesion molecules and chemokine-chemokine receptor interactions. Chemokine receptors and adhesion molecules associated with NKT cell redistribution within these tissues are indicated.

(2, 91). It is likely that CXCR6 delivers co-stimulatory signals to NKT cells as CXCL16 is expressed as a transmembrane protein on antigen-presenting cells (86, 153), and DCs from CXCL16^{-/-} mice are impaired in their ability to stimulate IFN- γ production from wild-type NKT cells (92). A reduction in IL-4 production by CXCR6^{-/-} NKT cells results from decreases in preformed IL-4 mRNA transcripts (91). Therefore, CXCR6 is critical for normal NKT cell development and function in addition to NKT cell homing and homeostasis. In addition to NKT cells, CXCR6 plays a role in regulating cytokine polarization in conventional T cell subsets. CXCR6 expression defines polarized subsets of Th1 and Th17 effector T cells *in vivo* (154, 155), and T cells from CXCR6^{-/-} mice exhibit impaired IFN- γ and IL-17 production in response to antigen restimulation *in vitro* (156).

Reporter mice in which the *Cxcr6* coding region was replaced with green fluorescent protein (*Cxcr6*^{gfp/+}) have been used to show that liver NKT cells are localized within the vasculature, crawling along the luminal surface of liver sinusoids. Interestingly, although CXCR6-deficiency resulted in a significant reduction in NKT cell accumulation within the liver, it did not alter the crawling behavior of hepatic NKT cells (90), suggesting that other signals contribute to this behavior. The α L β 2-integrin (LFA-1) also appears to be important for the accumulation or retention of NKT cells within the liver, as mice deficient in LFA-1 have significantly reduced numbers of liver NKT cells (157, 158). Moreover, blockade of LFA-1 and intercellular adhesion molecule 1 (ICAM-1) interactions resulted in a marked reduction in hepatic NKT cell numbers with a concomitant increase in NKT cell frequency within the peripheral blood (110).

Similar to the liver, NKT cells accumulate in the lung via CXCR6 (91), and reside as an intravascular population (108, 110). This strategic positioning may facilitate sensing of airborne antigens or infection as airway exposure to glycolipids or microbial cell wall components induced accumulation of NKT cells in the lung interstitium and bronchoalveolar space (108). This rapid redistribution of NKT cells preceded local expansion of the intravascular cells and did not appear to involve recruitment of NKT cells from the periphery. Multiple chemokines were induced in the lung after exposure to airborne NKT cell ligands (108), including known NKT cell attractants such as thymus and activation regulated chemokine (TARC; CCL17), MIG/CXCL9, and BCA-1/CXCL13 (88, 89, 159). It was suggested that CCR4 may be important in regulating NKT cell redistribution in the lung, since aerosolized delivery of exogenous TARC/CCL17 was sufficient to drive extravasation of NKT cells into the lung parenchyma (108). These findings are consistent with previous work demonstrating that CCR4 mediates localization of NKT cells to the airways following aerosolized antigen challenge or delivery of α -GalCer to the lungs (160).

In contrast to circulating naïve conventional T lymphocytes, few NKT cells in mouse or human blood express both CD62L and CCR7 (87, 88), which is consistent with the relative scarcity of NKT cells within the lymph nodes. However, a subset of “immature” NK1.1⁻ NKT cells exhibited chemotaxis in response to CCR7 ligands *in vitro* (88). This was initially interpreted as a role for CCR7 in mediating the exit of immature NKT cells from the thymus, since chemotactic responsiveness to CCR7 ligands was

not detected within mature NK1.1⁺ NKT cell subsets. However, this is also consistent with observations that a small subset of IL-17-generating NK1.1⁻ NKT cells accumulates preferentially within lymph nodes (3, 54). Within the lymph nodes, NKT cells are highly motile and are located mainly in the interfollicular region and in the medulla, but are absent in the paracortex where most naïve conventional T cells reside (161). In contrast to this *in situ* distribution under resting conditions, adoptively transferred NKT cells derived from the liver, spleen, or lymph nodes of TCR transgenic mice localized primarily to the lymph node paracortex (162). While the basis for these distinct distribution patterns was not investigated, it is possible that alterations in homing properties were induced by the manipulations involved in isolation, purification, and transfer of NKT cells.

Natural killer T cell populations resident in peripheral lymph nodes and skin exhibit an NK1.1⁻CD4⁻ phenotype, associated with expression of the retinoic acid receptor-related orphan receptor γ T (ROR γ T) transcription factor, and generate IL-17 following activation (3, 54, 163, 164). Similar to Th17 cells, IL-17-producing NKT cells within the peripheral lymph nodes and skin in mice express CCR6 and migrate in response to the ligand MIP-3 α /CCL20 (54), which has been shown to be involved in the recruitment of pathogenic Th17 cells to inflammatory sites in models of autoimmunity (165, 166). While it is unclear whether NKT-17 cells in the skin are distinct from those in the peripheral lymph nodes, it is thought that these cells are recruited via CCR6 and retained at epithelial sites by interactions between the α E-integrin (CD103) and E-cadherin (54). An NK1.1⁻ NKT cell population that produces IL-17 was also identified within the lung and shown to contribute to airway neutrophilia upon activation (56). While it is suggested that the NKT-17 lineage develops in the thymus of mice (55, 164), human and murine NKT cells can be differentiated into IL-17-producing cells in the presence of proinflammatory cytokines, such as IL-1 β and IL-23, along with transforming growth factor- β (TGF- β) (167, 168). Furthermore, both CCR6⁺ and CCR6⁻ NKT cells from human blood contained cells that could produce IL-17 (167). This suggests plasticity in NKT cell populations, with the ability to be reprogramed in response to factors in the local tissue environment.

A subset of NKT cells in the spleen, but not in other tissues, expresses CXCR5 and actively migrates in response to BCA-1/CXCL13 (88), a chemokine that mediates homing to B cell zones in lymphoid tissues (169–172). Subsequent studies demonstrated that NKT_{FH} cells (CXCR5⁺PD-1^{hi}) could provide cognate help to B cells, leading to the formation of antibody-producing plasma cells (65, 129, 173, 174). In contrast to the intravascular localization of NKT cells in the liver and lungs (90, 110), NKT cells in the spleen are widely distributed under basal conditions, dispersed throughout the red and white pulp (175), the periarteriolar lymphoid sheath (110), the marginal zone (176), and occasionally in close proximity to the vasculature (177). Exogenous glycolipid antigens or infection with *Streptococcus pneumoniae* induced rapid accumulation and immobilization of splenic NKT cells in close proximity to marginal zone DCs and macrophages (175, 176). Importantly, the number of splenic CD1d-tetramer⁺ NKT cells were not significantly altered, suggesting that the accumulation in these areas of the spleen is due to redistribution

and not enhanced recruitment of NKT cells from the peripheral blood (175).

Role of NKT Cells in Microbial Host Defense

Many studies have implicated roles for NKT cells in the immune responses elicited by microbial pathogens (6, 178–180). NKT cells respond to a range of infectious organisms through the recognition of microbial lipids presented via CD1d⁺ antigen presenting cells (181–187). For example, α -galactosyldiacylglycerol from *Borrelia burgdorferi* (the causative agent of Lyme disease) (185), lipophosphoglycan from *Leishmania donovani* (188), α -glucosyldiacylglycerol from *S. pneumoniae* (189), α -glucuronosylceramide and α -galacturonosylceramide from *Sphingomonas* species (182–185), and a cholesteroyl α -glucoside from *Helicobacter pylori* (186) are all recognized by the invariant TCR of NKT cells within the context of CD1d.

However, NKT cell activation is not restricted to microbes that contain lipid antigens recognized directly by the V α 14-J α 18 TCR on iNKT cells. Other microbial products stimulate antigen presenting cells via pattern recognition receptors (TLRs, NOD-like receptors, etc.), causing enhanced accumulation of weak self-lipid antigens and the production of NKT cell-stimulating cytokines (IL-12, IL-18, and type I IFNs) (46, 184, 190–193). Endogenous lipid ligands induce weak signaling through the NKT cell TCR that is not sufficient for full NKT cell activation, but primes NKT cells to produce IFN- γ upon exposure to the cytokines IL-12 and IL-18 (194). Moreover, there is evidence that CD1d-presented antigens may not be required and IL-12, IL-18, or type I IFNs alone or in combination may be sufficient to drive NKT cell activation and IFN- γ production (193, 195).

The mode of activation may have implications for NKT cell localization and effector functions. *In vitro*, NKT cells form stable conjugates with α -GalCer-pulsed DCs and subsequently lose motility, whereas NKT cells incubated with DCs in the presence of exogenous IL-12 and IL-18, or LPS-treated DCs, exhibit unaltered migration patterns (194). Consistent with this, NKT cell activation through intravenous delivery of exogenous glycolipid caused CD1d-dependent NKT cell arrest within liver sinusoids (90, 196) and induced rapid accumulation of NKT cells in the marginal zone of the spleen (175, 176). In contrast, while IL-12 and IL-18 treatment induced CD1d-independent arrest in liver sinusoids (196), these cytokines did not induce NKT cell redistribution to the marginal zone within the spleen (175). This suggests a role for CD1d engagement and cell-cell interactions in regulating the specific localization and redistribution of NKT cells, while cytokine stimulated NKT cells likely adhere to local integrin ligands in response to inside out signaling. There could also be differences in the chemotactic signals and localization gradients elicited by antigenic versus cytokine stimuli.

NKT Cells in Bacterial Infections

Borrelia burgdorferi

Lyme disease is caused by *B. burgdorferi*, a bacterial spirochete that generates the NKT cell-stimulating glycolipid, α -galactosyldiacylglycerol (185). NKT cell-deficient mice

(CD1d^{-/-} and Jα18^{-/-}) exhibit increased bacterial burden when infected with *B. burgdorferi* (197, 198). Under homeostatic conditions, NKT cells actively crawl within hepatic sinusoids (90). However, in mice systemically infected with *B. burgdorferi*, the majority (~80%) of sinusoidal NKT cells arrested and formed clusters in stable contact with *B. burgdorferi*-containing Kupffer cells (197). Interestingly, antibody blockade of either CXCR3 or CD1d inhibited NKT cell arrest and cluster formation (197). Kupffer cells release substantial amounts of MIG/CXCL9 early following infection with *B. burgdorferi* (197), suggesting that a chemotactic gradient facilitates recruitment and interaction of CXCR3⁺ NKT cells with CD1d⁺ Kupffer cells. This response reflected a redistribution of hepatic NKT cells as there was little recruitment of additional NKT cells to the liver.

Intriguingly, the most prominent phenotype in *B. burgdorferi*-infected NKT cell-deficient mice was a greater abundance of bacteria in the joints (197, 198). This suggests that NKT cells play a role in limiting the emigration of *B. burgdorferi* out of the vasculature in Lyme disease-associated arthritis. In contrast to the intravascular distribution and patrolling behavior of NKT cells in the liver, Lee et al. (199) found that NKT cells in the joint were distributed throughout the extravascular tissue with the majority remaining stationary and in close contact with the blood vessels. Following *B. burgdorferi* infection, the spirochetes were found to adhere to the inner wall of joint blood vessels and attempt to extravasate into the tissue. Extravascular NKT cells in the vicinity of the adherent pathogen increased their crawling activity, suggesting the release and recognition of pathogen- or host-derived chemotactic factors, possibly complement-derived anaphylatoxins (199). Moreover, NKT cells played a critical role in clearance of *B. burgdorferi* from the joint tissue via direct granzyme-dependent killing (199). In contrast to the CD1d-dependent responses to *B. burgdorferi* in the liver, NKT cell-mediated pathogen recognition and killing activity in the joint was not dependent on CD1d interactions. This study highlights the importance of NKT cell positioning with respect to their antimicrobial function and identifies a functionally unique subset of bactericidal NKT cells in the joint, since liver and spleen NKT cells were unable to directly recognize and kill *B. burgdorferi*. Interestingly, NKT cells were found to be present in the normal joint under homeostatic conditions (199), and infection with *B. burgdorferi* did not result in enhanced accumulation of NKT cells within the joints in mice (198).

Extravascular NKT cells have also been detected in knee joints of *B. burgdorferi* infected patients (199, 200). Although there are fewer NKT cells in human joints compared to mice, synovial fluid from patients with osteoarthritis, rheumatoid arthritis, and Lyme arthritis contained increased numbers of activated NKT cells (199, 200). It is unclear if this represents redistribution or expansion of local NKT cells or recruitment of NKT cells from other sites. While this appears to contrast with the lack of increased NKT cell accumulation in mice with Lyme borreliosis, it could result from differences in the time course of disease or differences in the precise compartment within the joint from which samples were collected. Lee et al. (199) demonstrated that NKT cells were not uniformly distributed throughout the joint but rather were found predominantly at the joint surface, outside of the joint capsule. The

increased number of NKT cells detected in synovial fluid samples from arthritis patients could represent a redistribution of extravascular joint-resident NKT cells into the joint capsule, which may be promoted by the presence of *B. burgdorferi* within synovial fluid. Consistent with this, Katchar et al. (200) observed a significantly higher proportion of NKT cells in the synovial fluid of patients with antibiotic-responsive Lyme arthritis (*B. burgdorferi* present in joint fluids in all patient samples) compared to the nearly undetectable levels of NKT cells in those with antibiotic-refractory Lyme arthritis (10 of 15 patients lacked detectable *B. burgdorferi* in joint fluids). T cells and NK cells were detected at similar levels in the synovial fluid of both patient groups, further suggesting that NKT cells play a key role in host defense against *B. burgdorferi*, and their absence may contribute to excessive inflammation and immune dysregulation in the joints of antibiotic-refractory Lyme arthritis patients.

Chlamydia

Chlamydia species are obligate intracellular pathogens that can cause numerous disease states in humans, including lung infection (201), gastrointestinal infection (202), urogenital infection (203), and reactive arthritis (204). Using an animal model of *C. trachomatis*-induced arthritis, Bharhani et al. (205) demonstrated that NKT cells play a role in ameliorating joint inflammation. Mice deficient in NKT cells (CD1d^{-/-}) exhibited enhanced arthritis severity, while α-GalCer treatment of *C. trachomatis*-infected wild-type mice increased the accumulation of NKT cells within synovial tissues, reduced bacterial load, suppressed expression of inflammatory chemokines [macrophage inflammatory protein-2 (MIP-2) and IP-10/CXCL10], and decreased infiltration of inflammatory cells into the inflamed joint. Moreover, while Bharhani et al. (205) could not detect NKT cells in synovial tissues of control mice, synovial NKT cell populations were detected in *C. trachomatis*-infected mice, suggesting active recruitment of NKT cells to inflamed joints in these mice. However, since others have identified NKT cells in the joints of control mice (199), it is unclear whether the increased proportion of synovial NKT cells following α-GalCer treatment of *C. trachomatis*-infected mice was due to further recruitment of NKT cells, or resulted from local NKT cell expansion.

Natural killer T cells have been shown to respond rapidly to infection and regulate microbial immunity in response to *C. muridarum* infections in the lung and genital tract of mice (206, 207), where treatment with α-GalCer enhances IFN-γ production to increase host resistance (207). Jiang et al. (208) reported elevations in bacterial burden and inflammatory cell infiltrate in the genital tract of CXCR5^{-/-} mice infected with *C. muridarum*. While CXCR5-deficiency did not alter NKT cell accumulation in the genital tract, CXCR5^{-/-} mice exhibited increased NKT cell activation *in vitro* and *in vivo* in response to *C. muridarum* infection. Enhanced production of IFN-γ by NKT cells from CXCR5^{-/-} mice suggests a possible role for CXCR5 in regulating the activity of NKT cells. However, enhanced NKT cell activity in CXCR5^{-/-} mice did not provide greater protection against *C. muridarum* genital tract infection *in vivo* (208), implicating important roles for other CXCR5⁺ immune cells in mediating protective responses.

Streptococci/Cryptococci

CD1d-dependent activation of NKT cells in response to α -glucosyldiacylglycerol has been demonstrated in mice infected with *S. pneumoniae* and group B *Streptococcus* (causative agents of neonatal infections in humans) (189). This supports earlier studies implicating a critical role for NKT cells in early host defense against *S. pneumoniae* infection via their production of IFN- γ and recruitment of neutrophils to infected lungs (25, 209). Consistent with a TCR-dependent activation mode, NKT cell activation and cytokine production in response to *S. pneumoniae* was associated with increased NKT cell GFP expression in Nur77-GFP mice, a reporter strain that upregulates GFP in response to TCR-mediated stimuli, but not TCR-independent inflammatory stimuli (210, 211). The frequency of lung NKT cells increased following *S. pneumoniae* infection, and following intratracheal infection with the fungal pathogen *Cryptococcus neoformans* (25, 212). In both cases, the increased NKT cell frequency may be dependent upon CCR2 and monocyte chemotactic protein 1 (MCP-1)/CCL2 mediated recruitment since the frequency of NKT cells was significantly reduced in the lungs of infected CCL2 $^{-/-}$ mice compared to wild-type mice (25, 212). However, further studies are required to elucidate the relative contributions of NKT cell expansion and mobilization from lung parenchyma versus the recruitment of circulating NKT cells from the blood.

Natural killer T cells were found to promote antibody isotype switch, affinity maturation, and long-term memory B cell responses against pneumococcal capsular polysaccharides following delivery of a liposome nanoparticle vaccine containing *S. pneumoniae* capsular polysaccharide and a NKT cell-stimulating lipid (213). Antibody responses elicited by the vaccine were dependent upon cognate CD1d-restricted interactions between NKT cells and B cells, a process that might be predicted to require direct B cell help provided by NKT_{FH} cells. However, very little induction of CXCR5 $^+$ PD-1 hi NKT_{FH} cells was observed in immunized mice, suggesting a mostly extrafollicular response. These findings suggest that the inclusion of NKT cell ligands in microbial antigen-presenting liposomal particles may represent a simple and effective alternative to the conjugate vaccines currently used to elicit strong cognate help to B cells to promote protective and long lasting antibody responses.

Bacterial Sepsis

Chemokine receptor-mediated regulation of lymphocyte activation and homing has also been described during sepsis. In a mouse model of sepsis caused by cecal ligation and puncture (CLP), Herzig et al. (214) observed a CXCR3-dependent increase in peritoneal NK cell and T cell accumulation, likely due to increased concentrations of the chemokines MIG/CXCL9 and IP-10/CXCL10 in the peritoneal cavity. In contrast, CLP did not result in an increased accumulation of NKT cells within the peritoneal cavity, but did decrease CXCR3 expression on NKT cells in the liver. The authors suggested this could be due to NKT cells becoming activated during CLP, causing the internalization and down-regulation of CXCR3 (214). Interestingly, the peritoneal cavity of CXCR3 $^{-/-}$ mice was nearly devoid of NKT cells prior to and following induction of CLP, while CXCR3-deficiency had no impact on the numbers of NKT cells in the spleen (214). This

suggests CXCR3 is important for the accumulation of NKT cells within the peritoneum under normal physiological conditions.

The anaphylatoxins (C3a and C5a), generated during complement activation, are chemotactic molecules that may also influence NKT cell localization and activation. NKT cells express high levels of mRNA, but not protein, for C5a receptor (C5aR) under homeostatic conditions (215). However, upon *Escherichia coli*-induced sepsis in mice, C5aR protein is rapidly expressed on splenic NKT cells (215). Interestingly, NKT cells from C5aR $^{-/-}$ mice infected with *E. coli* expressed lower levels of the activation marker CD69 and had reduced secretion of IFN- γ and TNF, suggesting that C5aR signaling regulates the activation of NKT cells in this model (215). Cognate C5a/C5aR interactions on NKT cells were also identified as a critical factor for NKT cell recruitment during sepsis based on the observations that C5aR $^{-/-}$ mice had markedly reduced numbers of NKT cells in the spleen and peritoneal cavity following infection (215). Furthermore, *E. coli* infection induced greater accumulation of C5aR $^+$ versus C5aR $^-$ NKT cells in the spleen of mixed bone marrow chimeras (215). The absence of C5aR and NKT cells were both associated with increased survival following infection, suggesting NKT cells contribute to the overwhelming inflammation in sepsis.

Mycobacterium

Activation of NKT cells via α -GalCer has been shown to contribute to protection against *M. tuberculosis* in mice (216). Moreover, adoptive transfer of NKT cells decreased mycobacterial burden in the lung and spleen, and NKT cells were able to inhibit intracellular replication of *M. tuberculosis* within infected macrophages *in vitro* (217). CXCR6 on lung T cells has been proposed as a marker for protective immunity to *M. tuberculosis* after intranasal immunization in mice, with CXCR6 and CXCL16 playing a critical role in mediating the localization of T cells within the airways (218). It is possible that the CXCR6-CXCL16 axis is also important for NKT cell localization in these tissues as well, since lung NKT cells are reduced under baseline conditions in CXCR6 $^{-/-}$ mice (91).

Phosphatidylinositol-mannosides (PIMs) are phospholipid antigens located in the membranes of mycobacteria, some of which activate human and murine NKT cells via CD1d (181). Despite its inability to trigger expansion of NKT cells (181), PIM₂ causes recruitment of NKT cells to the skin upon subcutaneous injection (219). Although this recruitment is TCR-independent, the mechanism is unclear. Intratracheal infection of mice with *M. bovis* bacillus Calmette-Guérin (BCG) induced NKT cell mobilization into the airways, which was profoundly impaired in CCR6 $^{-/-}$ mice (220). In this study, lung parenchymal NKT cells in *M. bovis* BCG-infected wild-type mice were found to have high expression of CCR6, likely imbuing them with responsiveness to the high levels of MIP-3 α /CCL20 induced in the lungs by *M. bovis* BCG infection (220). The number of NKT cells in the lung parenchyma did not differ between wild-type and CCR6 $^{-/-}$ mice, suggesting that the >90% reduction in mobilization of NKT cells to the luminal airways in *M. bovis* BCG-infected CCR6 $^{-/-}$ mice was due to a requirement for CCR6 in the airway infiltration but not lung localization of NKT cells (220).

NKT Cells in Viral Infections

Hepatitis Viruses

In patients with chronic hepatitis B virus (HBV) infections, the frequency of circulating CD4⁺ NKT cells is lower than that in asymptomatic carriers or healthy controls, but infection did not impair the ability of peripheral NKT cells to produce IFN- γ or IL-4 in response to activation with either α -GalCer or the mitogen phorbol 12-myristate 13-acetate (PMA) (221). Interestingly, the frequency of NKT cells increased significantly following antiviral therapy (221). However, it is unclear whether this reflects changes in proliferation, survival, or homing of NKT cells.

Although there has not been an exhaustive examination of chemokine receptors on NKT cells in patients infected with HBV, the frequency of NKT cells expressing CCR5 and CCR6 was comparable between chronic HBV patients and healthy controls (221). The migratory responses of NKT cells from chronic HBV patients to CCR5 and CCR6 ligands were either very modest (RANTES/CCL5) or not detectable (MIP-3 α /CCL20) compared to medium alone (221). However, the responses in this study were not compared to chemotactic responses of NKT cells from healthy controls.

A wide spectrum of clinical disease can occur following HBV infection, ranging from an asymptomatic carrier state, to self-limiting acute disease, chronic hepatitis, cirrhosis, liver failure, and hepatocellular carcinoma (222). A study in India reported a significantly increased frequency of circulating NKT cells (CD3⁺CD56⁺CD16⁺) and higher levels of MIP-1 β /CCL4 among patients with acute HBV infection, but not HBV-induced liver failure, compared to healthy controls (223). However, a separate study reported a decline in circulating NKT cell (CD3⁺CD56⁺) frequencies in acute HBV patients in the first few weeks following hospital admission, which the authors suggested could be due to trafficking of NKT cells to the liver where they play a role in local HBV immunity (224). These studies need to be interpreted cautiously as CD3⁺CD56⁺/CD16⁺ populations exhibit only partial overlap with the iNKT cell population.

Interestingly, Inoue et al. (225) reported higher surface expression (mean fluorescence intensity) of CXCR3 on circulating NKT cells isolated from patients with chronic hepatitis C virus (HCV) infection, while expression of CCR4, CCR7, or CD62L did not differ compared to healthy donors. The enhanced expression of CXCR3 may facilitate the trafficking of NKT cells to or within the liver due to the increased hepatic levels of MIG/CXCL9 and IP-10/CXCL10 during HCV infection (226, 227). Whether increased numbers of hepatic NKT cells during chronic HCV infection would be beneficial is unclear since NKT cells from HCV⁺ patients produce more IL-13 and other Th2 cytokines (225), which could contribute to liver fibrosis during chronic viral hepatitis (228).

Dengue Virus

A recent study examining the role of NKT cells in the pathogenesis of dengue virus infection in humans found that peripheral NKT cell numbers were not altered over the course of dengue virus infection (229). However, NKT cells displayed an activated phenotype that correlated with increased disease severity (229).

Similarly, NKT cells exhibit an activated phenotype and appear to play a detrimental role during dengue infection in mice (230). NKT cell-deficient mice ($J\alpha 18^{-/-}$) exhibited resistance to lethal infection, which was associated with decreased systemic and local inflammatory responses, reduced production of inflammatory cytokines (IL-6, IFN- γ , and IL-12p40), and reduced levels of CXCL1, a chemokine known to rapidly mobilize and activate neutrophils (230). In wild-type mice, mast cells responding to dengue virus infection upregulated chemokine expression (RANTES/CCL5, SDF-1/CXCL12, and fractalkine/CX₃CL1), and mediated recruitment of NKT cells (CD3⁺NK1.1⁺) into the skin at sites of dengue virus infection (231). Taken together, these data suggest NKT cells play a critical role in the pathogenesis of dengue disease.

Influenza Virus

Influenza virus is a respiratory pathogen that can be the cause of serious airway disease, particularly among children and the elderly. The number of circulating NKT cells were reduced in patients with severe cases of pandemic H1N1 influenza infection (232), but it is unclear whether this impacted disease progression. In rodent models, NKT cells play protective roles in influenza infection through multiple mechanisms. They have been reported to suppress excessive monocytic infiltrate (233), influence the generation of virus-specific CD8⁺ T cell responses (234), enhance the cytolytic activities of NK cells and virus-specific CD8⁺ T cells (via IFN- γ production) (235), and selectively lyse virally infected cells through a CD1d-dependent mechanism (233). Interestingly, NKT cells were also found to reduce the expansion and immunosuppressive activity of influenza-induced myeloid-derived suppressor cells (236), an immune modulatory activity of NKT cells that has also been shown in cancer models (237).

Much of the research on NKT cells in influenza infection focuses on the potential for NKT cell-stimulating glycolipid agonists such as α -GalCer to act as vaccine adjuvants. Several studies have shown that intranasal immunization of inactivated influenza or a live attenuated influenza vaccine, together with α -GalCer or its derivatives, induced high levels of influenza-specific systemic IgG and mucosal IgA, influenza-specific CD8⁺ T cell memory responses, and complete protection against influenza viral challenge in mice (238–242). Intranasal administration of α -GalCer was shown to increase the NKT cell populations in nasopharyngeal-associated lymphoid tissue (NALT) and regional cervical lymph nodes, but not the spleen, indicating that nasal administration of α -GalCer influences the local NKT cell population size without altering the systemic NKT cell population (242). Interestingly, expression of CXCL16 was upregulated in NALT and cervical lymph nodes following vaccination. NKT cell accumulation within these tissues and influenza-specific mucosal IgA levels were reduced in CXCL16^{-/-} mice (242). Therefore CXCR6–CXCL16 interactions contribute to the increased population of NKT cells following nasal influenza vaccination either by regulating homing or expansion of these cells. It would be interesting to determine whether influenza vaccination in conjunction with intranasal α -GalCer administration also increases the NKT cell population in the lung and/or airways, and if so, whether this increase is also impaired in CXCL16^{-/-} mice.

Human Immunodeficiency Virus

Natural killer T cells are highly susceptible to infection with HIV-1 due to the expression of multiple co-receptors for viral fusion and entry, including CD4 and the chemokine receptors CCR5, CXCR4, and CXCR6 (138, 243–248). Indeed, NKT cell frequency is reduced in patients with HIV-1 infection, with a preferential depletion of the CD4⁺ NKT cell subset prior to depletion of conventional CD4⁺ T cells (243, 248–250). Interestingly, the frequency of CCR5⁺ NKT cells was higher in HIV-1⁺ patients, while CXCR6⁺, CCR2⁺, and CCR7⁺ NKT cell frequencies were reduced compared to healthy individuals (251, 252). Circulating NKT cell populations have been shown to recover early following effective antiretroviral therapy, but treatment failed to restore CXCR6 or CCR2 expression on NKT cells (252). Some have speculated that the rapid recovery of NKT cells after treatment is partly due to NKT cell redistribution from tissue sites to the circulation, a phenomenon that has been observed for conventional T cells in HIV-1⁺ patients following therapy (253, 254).

In addition to influencing circulating NKT cell numbers, HIV-1 infection also impairs the proliferative and cytokine-producing capacities of persisting NKT cells in chronic HIV-1⁺ patients (255, 256). However, the role of NKT cells in HIV-1 infection remains unclear since some studies report no correlation between NKT cell numbers and HIV disease progression (249), while others have suggested an association between higher levels of CD4⁺ NKT cells and lower plasma viremia (243). NKT cell activation with α -GalCer has shown promise as a vaccine adjuvant in animal models when combined with delivery of HIV-1 DNA and peptide antigens (257, 258), suggesting that NKT cells have the potential to play important roles during HIV-1 infection.

Natural killer T cells from HIV-1⁺ patients expand *in vitro* following treatment with IL-15 and IL-12 (251), and a combination of antiretroviral therapy with exogenous IL-2 promotes a greater increase in circulating NKT cell numbers than standard therapy alone (259). Therefore, the reduced peripheral NKT cell population in HIV-1-infected individuals is likely due to a combination of factors, which include direct HIV-1 infection of NKT cells and subsequent cell death, tissue redistribution of NKT cells, and impaired generation and/or responsiveness to cytokines that promote NKT cell survival. A better understanding of the mechanisms contributing to NKT cell depletion in HIV-1⁺ patients could lead to the development of new therapeutic strategies to restore NKT cell numbers and lead to better clinical outcomes following HIV-1 infection.

Conclusion and Outstanding Questions

Our understanding of the distinct phenotypic and functional subsets of NKT cells continues to improve, allowing for clearer interpretations of how NKT cells contribute to health and disease. Under homeostatic conditions, NKT cells can be found in many tissues throughout the body, and NKT cell accumulation within specific sites can be linked to the expression of specific chemokine receptors and adhesion molecules that mediate tissue homing, retention, and/or survival (e.g., liver accumulation via CXCR6 and LFA-1). Upon activation, local NKT cell populations can expand and use chemotactic signals to relocate within a tissue.

However, in most cases, there is little or no evidence that NKT cells are recruited to sites of inflammation from the blood or other tissues. Despite their low numbers, NKT cells influence the magnitude and polarization of immune responses in a wide array of contexts ranging from antimicrobial and antitumor responses to autoimmunity. However, many questions remain regarding the roles of NKT cells in these conditions.

Patients with chronic microbial infection, autoimmune disorders, and malignancies often have alterations in the number and functional activity of NKT cells (7, 119, 122, 260, 261). Some have speculated that reduced NKT cell numbers in the peripheral blood of these patients are linked to NKT cell trafficking to diseased tissue sites associated with these disorders (120, 224, 262–264). However, reduced NKT cell numbers or other NKT cell defects in many disease states may be associated with the standard therapies used to treat the disease rather than the disease itself. For example, reduced NKT cell frequencies were not observed in patients with myelodysplastic syndrome or multiple myeloma prior to treatment, but defects in peripheral NKT cells emerged following initiation of standard therapy (265, 266). In most diseases in which NKT cell numbers are affected, further investigation is required to track whether alterations in NKT cell numbers are due to altered trafficking or redistribution of NKT cells to various tissue sites. This will require that studies examine patients multiple times over the course of disease development and ideally include multiple tissues and treatment-naïve groups, rather than only examining patients on a single occasion after disease onset as most studies have done to date. Doing so will allow clearer correlations to be made between altered NKT cell numbers/function (i.e., altered subset frequencies and cytokine production) and disease progression, and ultimately provide evidence as to whether NKT cell defects are a cause or consequence of the disease process.

Under homeostatic conditions, NKT cells appear to be tissue-resident populations and exhibit very little exchange with NKT cells in the circulation, as evidenced by studies using parabiotic congenic mice. NKT cells in the blood in these pairs reach almost equal (50%) chimerism, while those in the lung, liver, spleen, lymph nodes, bone marrow, and other tissues did not recirculate, with nearly all NKT cells in these tissues originating from the host (59, 108, 110). In contrast, conventional CD4⁺ and CD8⁺ T cells, B cells, and NK cells rapidly recirculate and equilibrated in these tissues (110). This poses interesting questions regarding NKT cell redistribution during microbial infection. Multiple studies described above have observed greater NKT cell accumulation in affected tissues in a variety of infections. However, in many studies, the authors have not distinguished between the possibilities of NKT cell recruitment into the tissue versus expansion and relocalization of tissue resident NKT cells. Regardless, their accumulation at sites of infection ensures NKT cells are exposed to potential activating stimuli, either directly through specific recognition of microbial lipid antigens or indirectly through self-glycolipid and cytokine stimulation. Intriguingly, NKT cells may not need to be present in an affected tissue site in order to respond and subsequently influence the immune response within the host. For example, liver NKT cells responding directly to noradrenergic neurotransmitters were shown to release anti-inflammatory cytokines that induced a state of immune

suppression that rendered mice susceptible to bacterial infection following ischemic cerebral stroke (47). Similarly, NKT cells are activated in the liver during the induction of contact hypersensitivity reactions (267). Therefore, a lack of NKT cell accumulation within inflamed peripheral tissues during infection may not preclude effective (or deleterious) antimicrobial immune responses mediated by tissue-resident NKT cells at a distant site.

A number of NKT cell subsets have been described that exhibit distinct phenotypes and functions in terms of surface marker expression and cytokine profiles. For example, lymph node resident CCR6⁺ CD4⁻ NK1.1⁻ NKT cells described earlier express the transcription factor ROR γ t and produce IL-17 in response to inflammatory signals (54). Unique transcriptional programs have been identified for NKT-1, NKT-2, NKT-10, and NKT-17 subsets of NKT cells within the thymus (52–58). Recent data reveal that NKT cell lineage fate is regulated by let-7 (let-7) microRNAs (miRNAs), which target *Zbtb16* mRNA (encoding PLZF) to post-transcriptionally regulate the expression of PLZF protein (268). The expression of let-7 miRNAs was dynamically regulated during NKT cell development, with IL-15 and other stimuli present in the thymic medulla contributing to upregulated let-7 miRNAs and reduced levels of PLZF protein during NKT cell differentiation.

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NKT cells with downregulated levels of PLZF differentiated into IFN- γ -producing NKT-1 cells. Conversely, reduced expression of let-7 miRNAs resulted in greater levels of PLZF protein and a thymic bias toward NKT-2 and NKT-17 differentiation. However, this bias was less evident in the peripheral tissues (liver, spleen, and lymph nodes) of mice with reduced let-7 miRNAs (268), suggesting the relative frequencies of NKT cell effector subsets are influenced by differential migration and expansion of certain NKT cell effector lineages within specific tissue microenvironments. Nevertheless, it will be important to determine whether different NKT cell subsets *in vivo* represent committed lineages of cells with distinct homing receptors or if these subsets exhibit plasticity and are able to adopt various functional roles depending upon soluble and cell-associated signals received within a given tissue microenvironment. Furthermore, a focused research effort is needed to investigate the relative roles of distinct NKT cell subsets during microbial infection.

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Imaging of NKT cell recirculation and tissue migration during antimicrobial immunity

Terry L. Delovitch ^{*}

Laboratory of Autoimmune Diabetes, Department of Microbiology and Immunology, Robarts Research Institute, Western University, London, ON, Canada

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Thierry Mallevaey,
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Irah L. King,

McGill University, Canada

***Correspondence:**

Terry L. Delovitch
del@robarts.ca

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This Opinion article outlines the relative paucity and emphasizes the need to enhance our knowledge of how subsets of natural killer T (NKT) cells mediate immune mechanisms of elimination of microbial pathogens at sites of inflammation or infection. To date, most studies of how NKT cell subsets migrate upon antigen stimulation have focused on NKT cell activation in the spleen, lymph nodes (LN) and liver (1). Thus, there currently exists an unmet need to determine the patterns of recirculation and tissue migration of NKT cell subsets and interacting antigen-presenting cells (APCs) that occur at relevant mucosal surfaces in several other organs, including the lung, intestine, and colon. This article proposes and highlights the benefit of *intravital cellular imaging* *in vivo* of type I and type II NKT cell subsets as an important methodology that may enable the visualization of NKT-APC cellular interactions at mucosal surfaces and enhance the application of this methodology to clinical therapy of antimicrobial immunity.

T Cell Recirculation and Migration into Tissues

During an immune response, T cells and B cells traffic to and recirculate between blood and peripheral lymphoid tissues prior to activation by antigen (1). Chemokines attract T cells to various sites of interaction with antigen-presenting dendritic cells (DCs) in the spleen and LN. After further encounter with antigen, T cells divide and differentiate into effector T cells (Teff) that migrate to different sites of infection to combat and destroy microbial pathogens (2). Cytokines secreted by Teff also help to clear infectious pathogens from these sites. Interactions between T cells and DCs at various sites of inflammation in LN are crucial for promoting subsequent immunity to microbes (2). These observations underscore the importance of understanding how T cell recirculation, localization, and interaction *in vivo* in target tissues mediate effective immune responses that either trigger or prevent inflammation and antimicrobial immunity.

Type I and Type II NKT Cell Subsets

Little is known about the various factors that mediate the recirculation, localization, and interactions of subsets of NKT cells *in vivo* in target tissues and lead to antimicrobial immunity. NKT cells display surface T-cell antigen receptors (TCR) expressed by both conventional T cells and NK cells, such as CD56/161 (humans) and NK1.1 (mice) (3–5). NKT cells recognize lipid antigens presented by CD1d MHC class I like molecules (2–15) on various APCs, including DCs, macrophages (Mφ), B cells, thymocytes, adipocytes, and hepatocytes. While the CD1a, CD1b, CD1c, CD1e, and MR1 MHC class I like molecules are also expressed on APCs and can activate various T cell subsets, only analyses of CD1d-mediated responses of type I and type II NKT cell subsets will be presented here. The development of type I NKT cells occurs in the thymus and depends on the activity of several transcription factors including promyelocytic leukemia zinc finger (PLZF), T box transcription

factor (T-bet), retinoic acid receptor-related orphan receptor- γ T (ROR- γ T), and GATA-binding protein 3 (GATA-3) (2, 5).

Type I NKT cells respond to α - and β -linked glycolipids. For example, stimulation of type I NKT cells by the α -galactosylceramide (α GalCer) glycolipid agonist induces the secretion of many cytokines that elicit both Th1 [interferon- γ (IFN- γ) and Th2 [interleukin-4 (IL-4) and IL-13] responses (2, 7–17). Type I NKT cells are more prevalent than type II NKT cells in mice than in humans (18–20), and comprise about 50% of murine intra-hepatic lymphocytes (21–23). The type I NKT cell invariant TCR is encoded mainly by a germline V α gene (V α 14/J α 18 in mice and V α 24/J α Q in humans), and more diverse non-germline V β chain genes (V β 8.2/7/2 in mice and V β 11 in humans) (1–20, 24–26). The semi-invariant TCR on type I NKT cells preferentially binds to CD1d via its α -chain (3, 6, 15, 25).

Type II NKT cells constitute a minor subset in mice, but are more predominant in humans (18, 27). Most type II NKT cells do not recognize α -linked glycolipids, but rather respond to sulphatide, a self-antigen that occurs naturally on cell membranes in the central nervous system (myelin sheath), pancreas, kidney, and liver. Sulphatide-reactive type II NKT cells may protect from autoimmune diseases by down-regulation of inflammatory responses elicited by type I NKT cells (28, 29). In contrast, non-sulphatide-reactive type II NKT cells may be pathogenic in other diseases, such as ulcerative colitis (UC) (30). Sulphatide-reactive type II NKT cells express oligoclonal TCRs and express a limited number of V α and V β chains. The antigen specificity of type II NKT cells appears to be conferred by their surface TCR V β -chain (31).

CD1d and NKT Cell-Mediated Antimicrobial Immunity

Antimicrobial defense may be mediated by extensive cross-regulation between CD1d, NKT cells, and microbes that function predominantly at mucosal surfaces (32–34). The display of microbes at mucosal surfaces, mainly during early postnatal development, controls NKT cell trafficking and function in the intestine, lung, and intestine. Microbial recognition at these sites determines the susceptibility to NKT cell-mediated inflammatory disorders. Conversely, CD1d expression controls the composition of the intestinal microbiota. Whereas microbiota reduce the number and activity of type I NKT cells at mucosal sites, an elevated number and function of type I NKT cells may be stimulated by microbiota in peripheral tissues (32). Thus, crosstalk between microbiota and type I NKT cells influences mucosal homeostasis and its dysregulation in a bidirectional manner in inflammatory disorders.

In human inflammatory bowel disease (IBD) and infectious hepatitis, type II NKT cells are causal to inflammation (10). In contrast, intestinal inflammation in oxazolone-induced colitis, a mouse model of human UC, is dependent on CD1d and type I NKT cells that express IL-17 and secrete IL-13 (10, 35). Thus, intestinal microbiota influence pathogenic responses in NKT cell-mediated intestinal inflammation. The outcome of these responses depends on the time of microbial exposure, NKT cell subset(s) involved, nature of microbial lipid antigens

recognized, and type of APC that presents CD1d-restricted antigens to NKT cells. CD1d-restricted interactions of type I NKT cells with intestinal epithelial cells (IECs) promote IL-10 secretion and mucosal homeostasis, while CD1d-dependent interactions with bone marrow-derived APCs contribute to intestinal inflammation (36). Further experimentation may reveal whether these various responses result from the expression of different costimulatory molecules by IECs and professional APCs or whether cell-type-specific differences in CD1d trafficking and lipid acquisition contribute to this outcome. The central questions that need to be addressed are: (1) how do specific microbes control mucosal NKT cell abundance and function and determine health vs. disease, (2) what are the pathways of antigen-dependent and cytokine-dependent activation in NKT cells, and (3) do specific alterations in intestinal microbiota (e.g., in patients with IBD) (37) contribute to intestinal inflammation by the differential homing, proliferation, and activation of NKT cell subsets.

Like the intestine, the lung is a site of interaction between commensal microbiota and mucosal NKT cells. Insufficient microbial colonization during neonatal life leads to increased quantities and environmental sensitivity of type I NKT cells in lungs leading to susceptibility to asthma. This notion is supported by the result that exposure to antibiotics during early life but not late life enhances susceptibility to asthma in mice (38). In addition, elevated numbers of type I NKT cells are found in the lungs of germ-free mice. The latter finding requires the hypermethylation of the Cxcl16 chemokine gene and increased expression of the CXCL16 chemokine protein, which binds to the CXCR6 cognate chemokine receptor found on NKT cells (39). These alterations are associated with increased airway resistance, eosinophil infiltration, and proinflammatory cytokine production during ovalbumin (OVA)-induced asthma in mice (39). Thus, the development, migration, and function of type I NKT cells at mucosal surfaces may be influenced by commensal microbiota (6).

Tracking of T Cells *In vivo* by Intravital Cellular Imaging

Studies of NKT cell-mediated inflammation at different mucosal surfaces (e.g., intestine, lung, colon) illustrate that increased understanding of the mechanisms of differential recirculation, migration, proliferation, and activation of NKT cells during pathological responses requires the use of a technology that enables the visualization of these NKT cell events in real-time *in vivo*. The technique of two-photon (2P) microscopy coupled with *intravital* imaging enables one to track the location, movement, and interactions of cells (40–44). As such, 2P microscopy has improved our knowledge of T cell-DC and T cell-B cell interactions by recording how such cells function in resting tissue and undergo interaction, information exchange, and response to pathogens (40–43, 45). This methodology has also provided much new information about cellular pathways that arise during disease progression by illustrating the outcome of specific events in real-time (40–44). *Intravital* imaging and quantification of cell dynamics *in vivo* requires the use of fluorescently tagged proteins that are expressed transgenically in a cell-type-specific fashion to

monitor the migration of single cells from blood vessels to tissues at a maximum tissue depth of 300–400 μm.

Initial studies on T cell–APC interactions during the establishment of peripheral tolerance were conducted with conventional CD4⁺ T cells and APCs in the LN and spleen, and showed that the time of contact between CD4⁺ T cells and APCs may vary from long-lived (days) to short-lived (a few hours) (40, 43). This difference in time of T cell–APC contact may influence the relative capacity of an agent administered *in vivo* to treat a given disease and induce (pre-disease) or restore (post-disease) immune tolerance. For example, CTLA-4 and PD-1 inhibitory receptors on Teff or regulatory T (Treg) cells can suppress immune responses by limiting the times of effective interactions of T cells with DCs (44, 46, 47). During chronic inflammation, cytokine delivery requires long-term T cell–APC contacts. However, only a relatively small number of cytokine molecules may be secreted at a low antigen concentration (43, 44, 46, 47). At a high concentration of antigen, the duration of T cell–APC contacts may be sufficiently long to elicit a chronic inflammatory response. Protection against inflammation is more likely to occur at a significantly lower antigen concentration (43). Further experimentation is required to analyze the effects of antigen concentration, time of cytokine production by CD4⁺ T cells in high vs. low antigen concentration tissue environments, and whether effector cytokines function locally at a particular site or are transported to other distal sites. Nonetheless, the results reported for the tracking and function of conventional CD4⁺ T cells *in vivo* have facilitated analyses of the migration and function of NKT cells *in vivo*.

Imaging of NKT Cell Recirculation, Migration, and Activation

T cell receptor signal strength may determine the cytokine secretion profiles of T cells in a reciprocal manner. That is, the binding of TCRs of type I NKT cells to their antigen ligands can regulate the activity of TCRs on type II NKT cells. In turn, the binding of TCRs of type II NKT cells to their antigen ligands can regulate the activity of TCRs on type I NKT cells. Understanding the basis of how this cross-regulation of NKT cell activation occurs is crucial to develop better strategies to prevent microbial infection (2, 8–12, 48–52).

Such studies require a suitable animal model in which to track NKT cell recirculation and migration *in vivo*. For this reason, heterozygous mice were generated in which the green fluorescent protein (GFP) gene was knocked into a lineage-specific gene enabling certain leukocytes to be fluorescently labeled (53). In mice that express GFP integrated into the Cxcr6 chemokine receptor gene (Cxcr6gfp/+ mice), type I NKT cells traffic to, and become quite abundant in the liver (20–30% of lymphocytes). However, NKT cell migration within the liver is arrested following

interaction with Kupffer cells. The latter interaction occurs within minutes following lipid antigen injection (54–58). In addition, both IL-12 and IL-18 proinflammatory cytokines induced following bacterial infection that suppresses type I NKT cell motility in liver sinusoids of Cxcr6gfp/+ mice via a CD1d-independent mechanism. This block in NKT cell movement is evident within 1 h after exposure to the cytokines and precedes NKT cell activation. Further antigen ligation stabilizes an immune synapse formed between NKT cells and interacting APCs. This synapse potentiates LFA-1/ICAM-1 interactions that enable activated type I NKT cells to remain in the liver. Thus, activated type I NKT cells recirculate less than activated conventional CD4⁺ T cells (59). Identification of the patterns and kinetics of recirculation of type I and type II mouse NKT cells as well as the patterns and kinetics of human type I and type II NKT cells await further study.

Future Challenges

A future goal of studies of human NKT cells is to identify their functional roles in health and disease (1). Determination of how subsets of human NKT cells migrate and recirculate *in vivo* may advance our understanding of the biology and mechanisms of cellular interaction of different human NKT cells with APCs. Current investigations are being performed in two animal models. First, Cxcr6gfp/+ mice are being used to monitor human NKT cell trafficking, localization, and activation *in vivo* (56). Second, the kinetics and dynamics of human CD1d (hCD1d)-restricted NKT cell interactions are being analyzed in hCD1d knock-in mice that express hCD1d in place of mCD1d (59). Subpopulations of mouse type I NKT cells that are similar to human type I NKT cells in phenotype (mouse Vβ8⁺, human Vβ11 homolog⁺, CD4^{low}), tissue distribution, and function (anti-tumor activity) are present in hCD1d knock-in mice. The latter mice serve to model how a lipid antigen induces the migration and function of hCD1d-restricted type I NKT cells and type II NKT cells *in vivo* (59–62). If type I and type II human NKT cells can be differentially activated or inhibited *in vivo*, this may facilitate the design of new immunotherapeutic protocols in the treatment and prevention of infectious diseases.

Additional imaging studies are required to delineate whether, in addition to NKT cells regulation at mucosal surfaces, commensal bacteria also regulate NKT cells at other sites, e.g., the skin where microbiota are in close contact with NKT cells and CD1a-restricted, lipid-reactive T cells (63–65). Future work may also establish potential species-specific and antigen-specific effects of microbiota on NKT cells and the roles of viruses and fungi in this process. Finally, it is of major clinical interest to develop therapeutic strategies that may induce changes in the function of type I NKT cells at mucosal surfaces that will promote and/or preserve mucosal homeostasis and antimicrobial immunity.

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Intravital imaging – dynamic insights into natural killer T cell biology

Pei Xiong Liew and Paul Kubes*

Snyder Institute for Chronic Diseases, Cumming School of Medicine, University of Calgary, Calgary, AB, Canada

Natural killer T (NKT) cells were first recognized more than two decades ago as a separate and distinct lymphocyte lineage that modulates an expansive range of immune responses. As innate immune cells, NKT cells are activated early during inflammation and infection, and can subsequently stimulate or suppress the ensuing immune response. As a result, researchers hope to harness the immunomodulatory properties of NKT cells to treat a variety of diseases. However, many questions still remain unanswered regarding the biology of NKT cells, including how these cells traffic from the thymus to peripheral organs and how they play such contrasting roles in different immune responses and diseases. In this new era of intravital fluorescence microscopy, we are now able to employ this powerful tool to provide quantitative and dynamic insights into NKT cell biology including cellular dynamics, patrolling, and immunoregulatory functions with exquisite resolution. This review will highlight and discuss recent studies that use intravital imaging to understand the spectrum of NKT cell behavior in a variety of animal models.

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António Gil Castro,
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Vasileios Bekiaris,
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*Correspondence:

Paul Kubes,
Snyder Institute for Chronic Diseases,
Cumming School of Medicine,
University of Calgary, HRIC 4AA16,
3330 Hospital Drive NW, Calgary, AB
T2N 4N1, Canada
pkubes@ucalgary.ca

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Introduction

Modern advances in technology have provided a plethora of *in vitro* and *ex vivo* methods to investigate the molecular systems and cellular functions of immune cells. These advances have resulted in significant insights into biological processes at the cellular level and deciphered multiple complex signaling pathways. Nevertheless, the most relevant experimental conditions in which to observe and document these biological processes remain the live animal. The use of intravital microscopy (IVM) provides such a view into the lives and dynamic interactions of diverse immune cell populations in various tissues and organs. Importantly, IVM is performed under experimental conditions which closely resemble the natural environment. As cellular functions and behaviors are influenced by several factors such as shear forces, anatomical location, and extracellular components, absence of these factors could result in tremendously different outcomes in *in vitro* versus *in vivo* settings.

Historically, IVM was first employed in the nineteenth century with brightfield microscopy to visualize leukocyte trafficking in translucent tissues (1). In the last two decades, brightfield-based IVM has brought about important discoveries especially in molecular and biophysical mechanisms of leukocyte adhesion to endothelial cells (2, 3). However, this basic technique applying visible light could only visualize uniformly colorless cells sufficiently slowed by adhesion, which allowed them to be distinguished from rapidly flowing cells (4). The advent of fluorescence-based intravital imaging with modern optical imaging agents and equipment now opens up exciting possibilities for biological observations. Many immune cells can now be tagged with fluorescent probes to visualize their behavior in real time in a live animal. Other important additions to fluorescence-based IVM are the

different varieties of confocal microscopes, which provide deep tissue imaging and better subcellular resolution by excluding out-of-focus light via point illumination and pinhole apertures (5, 6). For example, spinning disk confocal intravital imaging systems provide rapid image acquisitions at the expense of deep tissue imaging, and are extremely competent for dynamic observations of immune behavior and cell-cell interactions particular within the vasculature (7–9). In contrast, multiphoton microscope systems, which employ a pulsed infrared laser excitation to generate fluorescence, have allowed deep tissue imaging of cell-cell interactions up to 500 μm depth (10, 11).

In recent years, fluorescence-based confocal IVM systems have been employed to visualize immune cells in almost all types of tissues to address a variety of immunological questions. Natural killer T (NKT) cells are credited with modulatory roles in a wide variety of diseases, and there is great interest in employing these cells for therapy in diseases or as biomarkers for prognostic purposes. In this review, we will focus on how IVM as a tool has revealed novel insights into NKT cell dynamics and biology.

NKT Cells – A Quick Primer

The name “NKT cell” was first conceived about 25 years ago, and was used to broadly define a subset of murine T lymphocytes that shared functional and phenotypic characteristics with the natural killer cell, including the NK1.1 (NKR-P1 or CD161c) surface marker (12, 13). Although the term NKT cell is now accepted and applied to these cells in both mice and humans, this definition is inaccurate and possibly misleading as NKT cells in certain mouse strains do not express NK1.1 due to the allelic divergence of NK1.1 genes (14, 15). To further complicate this classification, some conventional T cells have been described to spontaneously express NK1.1 after activation (16).

Around the time when NKT cells were identified, a novel process of presenting lipid antigens was discovered (17, 18). This antigen presentation process occurred through the MHC class I-like molecule designated as CD1 (cluster of differentiation 1) that includes CD1a–CD1e (19, 20). All of these CD1 molecules present lipids instead of peptides as antigens. While humans express all five CD1 genes, mice express only CD1d. In mammals, CD1d is highly conserved (21). Further studies in mice subsequently demonstrated that CD1d molecules presented lipids to invariant T cell receptor (TCR)-bearing cells, which also expressed NK1.1 (22–24). This finding led to the realization that NKT cells were reactive to CD1d, and that the invariant TCR α -chain and CD1d were essential for the development of NKT cells. These unique phenotypic characteristics are now used to define NKT cells. An excellent review highlights the detailed timeline of discoveries that contributed to the identification of NKT cells (12).

The discovery of the compound α -galactosylceramide (α GalCer) in 1997 contributed greatly to the understanding of NKT cells (25). This potent and specific lipid antigen, isolated from a marine sponge sample (likely from an infecting proteobacterium), was the first identified antigen for a specific population of NKT cells termed Type I NKT cells or invariant NKT (iNKT) cells. Through the use of CD1d tetramers loaded with α GalCer, iNKT cells in mice were discovered to express

the invariant V α 14–J α 18 TCR α -chain paired with a β -chain biased toward V β 2, V β 3, and V β 8 (26, 27). More than 80% of NKT cells were found to express these invariant chains. A similar TCR limited repertoire was found in human iNKT cells, which expressed V α 24–J α 18 paired with the V β 11 chain (28). Due to large structural and functional similarities between the TCRs expressed by human and mice iNKT cells, α GalCer can bind to and activate iNKT cells from both species (29). In fact, this property has been taken advantage of by researchers to develop multimeric molecules with loaded synthetic α GalCer to identify iNKT cells *ex vivo* (30). These synthetic loaded tetramers are used in conjunction with anti-CD3 or anti-TCR β antibodies to identify and enumerate iNKT cells in multi-parameter flow cytometry. In addition to α GalCer, a considerable number of exogenous ligands have been identified to activate iNKT cells (31). Further, self-derived endogenous lipids as well as the cytokines interleukin (IL)-12 and IL-18 have also been described to activate iNKT cells (32, 33). As iNKT cells can be activated by a range of exogenous and endogenous antigens and diverse inflammatory stimuli (Figure 1), they were found to be more important than initially realized in a variety of diseases (34, 35). Apart from Type I NKT cells, another subset of NKT cells has also been described (36–38). These Type II NKT cells recognize lipid antigens but express diverse TCR α - and β -chains (39, 40) and do not recognize α GalCer (41, 42). As this group of NKT cells cannot be identified through α GalCer-loaded CD1d tetramers, they are comparatively less characterized and understood (as compared to iNKT cells). This review shall focus mainly on findings discovered in mouse and human iNKT cell studies.

Although iNKT cells develop in the thymus, they are generally categorized as innate lymphocytes because iNKT cells exist in a poised effector state when they mature. Accordingly, mature iNKT cells are able to rapidly release large quantities of pro-inflammatory T helper type 1 (T_H1) [(for example, interferon- γ (IFN- γ) or T helper type 2 (T_H2) (IL-4 and IL-10) cytokines within hours of activation (43, 44). In mice, resting iNKT cells contain preformed mRNA for both IFN- γ and IL-4 to allow swift cytokine production (45). These cytokines are able to transactivate other immune cells including neutrophils, NK cells, dendritic cells, and macrophages during an immune response (15, 46). Because iNKT cells are able to rapidly release substantial quantities of cytokines that can polarize the immune response, they are hypothesized to be important orchestrators of immunity. For example, activation of iNKT cells during infection results in the secretion of pro-inflammatory cytokines, which stimulates the developing immune response to fight off microbial invaders (47–49). A similar protective effect of iNKT cells is also observed during cancer (50, 51). On the other hand, iNKT cells can strengthen immuno-suppressive pathways during autoimmunity or ischemia-reperfusion injuries such as stroke (52, 53). In a mouse stroke model, there is increased sympathetic drive which induces iNKT cells to make more IL-10 and less IFN- γ (8). This leads to overall immuno-suppression but places individuals at a greater risk to infections. iNKT cells are therefore pivotal in shaping immune responses during diverse pathological states. An ongoing challenge is to unravel the factors that determine if iNKT cells facilitate or suppress an immune response.

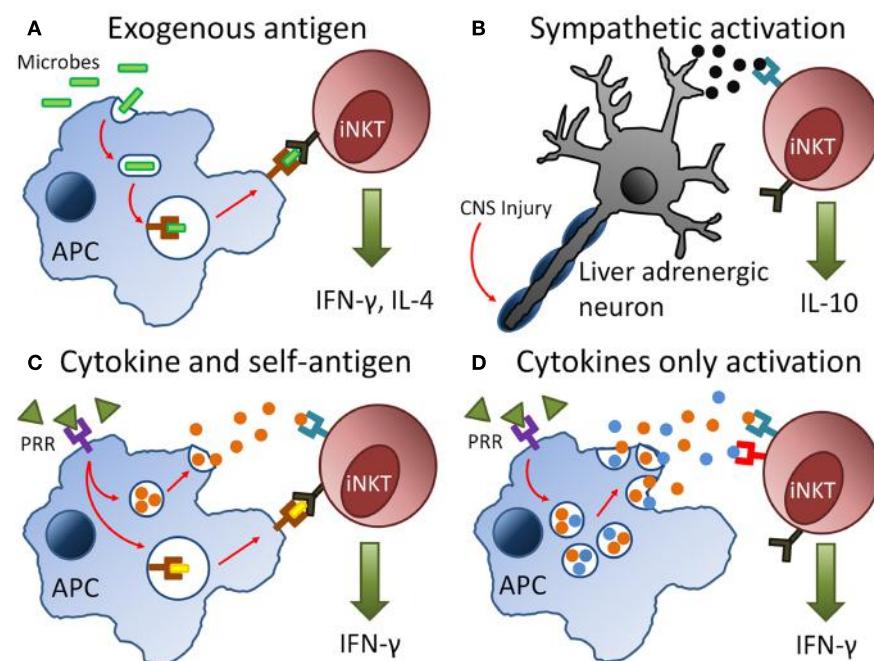


FIGURE 1 | Pathways of invariant natural killer T (iNKT) cell activation during infection and injury. **(A)** Antigen-presenting cells (APCs) engulf invading microbes during infection and present exogenous antigens via CD1d molecules directly to the T cell receptor (TCR) on iNKT cells. **(B)** Injury to the central nervous system (CNS) results in signals transmitted via neurones and release of neurotransmitters such as noradrenaline. These neurotransmitters bind to adrenergic receptors on iNKT cells resulting in their activation.

(C,D) During infection and injury, pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) are released. These PAMPs and DAMPs bind to pattern-recognition receptors (PRRs) on APCs, which result in the production of inflammatory cytokines such as interleukin-12 and presentation of self-antigens on CD1d to the TCR of iNKT cells. The synergy of cytokine or self-antigen presentation contributing to iNKT cell activation depends on the type of injury or microbe involved during infection.

Thus far, iNKT cells have been described to produce IL-2, IL-5, IL-6, IL-10, IL-13, IL-17, IL-21, tumor necrosis factor- α , transforming growth factor- β , and granulocyte monocyte-colony-stimulating factor (15, 54, 55). How does a single population of cells produce such a large variety of cytokines? The type and quantity of cytokine produced is influenced by several non-mutually exclusive factors. First, the quality of TCR signal (i.e., antigen signal strength and CD1d-binding kinetics) affects the cytokine profile. For example, use of different α GalCer analogs have been described to result in different ratios of IFN- γ /IL-4 produced (56–58). A similar phenomenon should occur with endogenous antigens compared to relevant foreign antigens. Second, targeting of antigen to different antigen-presenting cells will alter the pattern of cytokines made by iNKT cells (59, 60). Finally, functionally different subsets of iNKT cells have been described based on tissue localization and cell surface phenotype, which may promote different outcomes when iNKT cells are activated (15, 61).

Imaging iNKT Cells

There are a multitude of publications that describe the activation and cytokine production profiles of iNKT cells in mice and humans. However, their tissue distribution and dynamic behavior have only been brought to light recently. The capacity to visualize and observe iNKT cell behavior relies considerably on the labeling method. To date, no lineage-specific fluorescent antibody has been able to label iNKT cells. Isolating iNKT cells and staining them *ex*

vivo with a fluorescent dye (for example, carboxyfluorescein diacetate succinimidyl ester) for adoptive transfer provides a manner to observe their behavior in an organ (62). However, this opens the possibility that cellular behavior may be altered by the potential artifact of cell isolation. So far, the best avenue is the use of genetically engineered knock-in mice where fluorescent proteins are inserted into a lineage-specific gene locus (63). Both mouse and human iNKT cells express high levels of the Cxcr6 chemokine receptor, which has been demonstrated to mediate the survival of iNKT cells in the liver (64–66). To image iNKT cells in a live animal, a mouse containing enhanced green fluorescent protein (GFP) inserted into the Cxcr6 gene (*Cxcr6*^{Gfp/+}) was generated (67). iNKT cells have been found to account for 75–80% of all GFP^{bright} cells in the liver. For the first time, the dynamic behavior of iNKT cells in different tissues and organs could be observed. Using IVM, hepatic iNKT cells were seen to crawl along the luminal side of liver sinusoidal endothelial cells without directional bias with an average speed of 10 $\mu\text{m}/\text{min}$ (Figure 2A) (9, 67). This distinct behavior is unlike leukocyte behavior observed in post-capillary venules where leukocytes roll along continuous endothelium (3, 68). Detailed analysis of iNKT cell behavior in the liver demonstrated that iNKT cell crawling was random and independent of blood flow (67).

Resident iNKT cells are enriched in the liver, comprising up to 30% of all lymphocytes as compared to the thymus, lung, colon, bone marrow, spleen, lymph nodes, and blood (44). The cause for the higher frequency of resident iNKT cells in the liver is

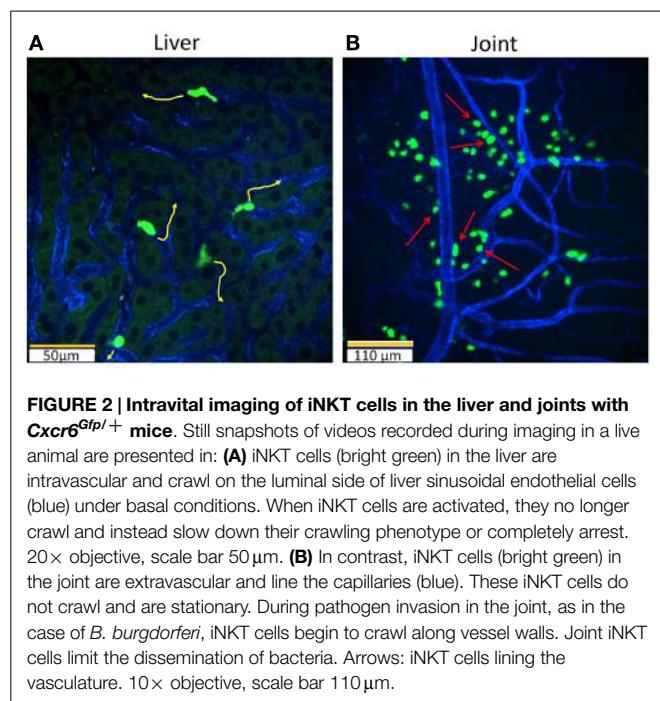


FIGURE 2 | Intravital imaging of iNKT cells in the liver and joints with *Cxcr6^{Gfp/+}* mice. Still snapshots of videos recorded during imaging in a live animal are presented in: **(A)** iNKT cells (bright green) in the liver are intravascular and crawl on the luminal side of liver sinusoidal endothelial cells (blue) under basal conditions. When iNKT cells are activated, they no longer crawl and instead slow down their crawling phenotype or completely arrest. 20× objective, scale bar 50 μm. **(B)** In contrast, iNKT cells (bright green) in the joint are extravascular and line the capillaries (blue). These iNKT cells do not crawl and are stationary. During pathogen invasion in the joint, as in the case of *B. burgdorferi*, iNKT cells begin to crawl along vessel walls. Joint iNKT cells limit the dissemination of bacteria. Arrows: iNKT cells lining the vasculature. 10× objective, scale bar 110 μm.

not completely clear; however, the adhesion molecule leukocyte function-associated antigen-1 (LFA-1, CD11a) has been suggested to be important in retaining iNKT cells in the liver (69). A tandem blockade of LFA-1 and its corresponding ligand, intercellular adhesion molecule 1 (ICAM-1), created a substantial rise in iNKT cells in blood and a reciprocal decrease in their number in the liver. Furthermore, LFA-1-deficient mice have notably reduced numbers of iNKT cells in the liver (70). Although we observed that the crawling phenotype of iNKT cells in liver sinusoids was not affected by LFA-1 and ICAM-1 antibodies, they did detach in collecting venules after treatment with blocking antibodies (71). Taken together, these data indicate that LFA-1 and ICAM-1 were perhaps necessary for interactions in larger vessels but not for crawling in sinusoids.

Previous studies have demonstrated that T cells arrest their movement when they encounter cognate antigen (72, 73). iNKT cells in the liver exhibit a similar behavior; when αGalCer was injected intravenously, crawling GFP^{bright} iNKT cells became stationary within an hour (67). Other studies have shown that hepatic iNKT cell arrest was correlated with iNKT cell activation (8, 9). Activation of iNKT cells via various mechanisms including CD1d, cytokines, or even neurotransmission all induce cell arrest within liver sinusoids. For example, synergistic effects between the inflammatory cytokines IL-12 and IL-18 resulted in the arrest of hepatic iNKT cells (74). In a mouse stroke model, norepinephrine release by the sympathetic nervous system during stroke caused rapid arrest of iNKT cells in the liver (8). Hepatic iNKT cells have been shown to express adrenergic receptors to receive neural signals (75). When iNKT cells are activated through cytokines or noradrenergic receptors, blockade of CD1d had no effect on the arrest of iNKT cells which suggests that classical antigen presentation through CD1d did not play a major role in arrest and activation in these situations.

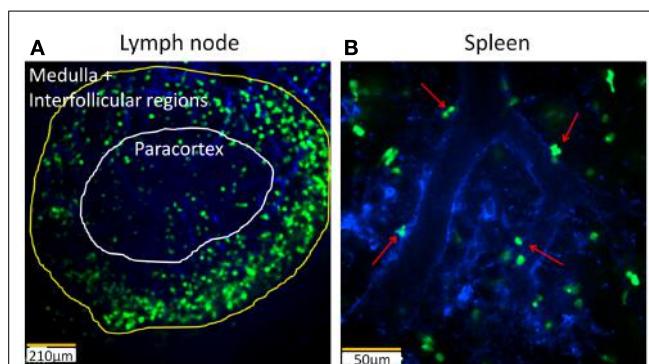


FIGURE 3 | Intravital imaging of iNKT cells in the lymph node and spleen of *Cxcr6^{Gfp/+}* mice. **(A)** Still image demonstrating distribution of iNKT cells (bright green) in different regions of a lymph node. Vasculature of the lymph node is labelled with PECAM-1 (blue). The medullar and interfollicular regions are outlined in yellow while the paracortex is outlined in white. 10× objective, scale bar 210 μm. **(B)** Still snapshot of splenic iNKT cells (bright green) in red pulp of spleen reveals that these cells are located outside the vasculature (blue). Arrows: extravascular iNKT cells. 20× objective, scale bar 50 μm.

Despite the fact that the frequency of iNKT cells in other organs than the liver is low, various studies have highlighted the importance of iNKT cells in these organs in response to blood-borne pathogens (76–78). The *Cxcr6^{Gfp/+}* mouse has been employed to study the spatial organization, behavior, and functional roles of iNKT cells in several organs including the joints, lymph node, spleen, and lung. In distinct contrast to the liver, intravital imaging of iNKT cells in joints revealed dramatic localization of these cells around the joint blood vessels but not inside the vessels (Figure 2B) (78). Joint iNKT cells also exhibited different behavior under basal conditions as they were stationary and non-motile. In the lymph node, iNKT cells are located in the medulla and the interfollicular region but mainly absent in the deep paracortex where naïve T cells reside (Figure 3A) (79). These iNKT cells are highly motile in the lymph node and actively communicate with resident subcapsular sinus macrophages. During systemic infection, resident macrophages produce IL-18 and complementary cytokines, which elicit an innate IFN-γ response from lymph node iNKT cells. On the other hand, iNKT cells were found to be widely distributed throughout the spleen, including B and T cell follicles in the periarteriolar lymphoid sheath, the marginal zone (MZ), as well as the red pulp (69, 76, 80). Dissimilar to the liver, iNKT cells were observed to be crawling outside the vasculature in the spleen (Figure 3B) (71, 76). Interestingly, the localization of iNKT cells changes in the spleen during infection or in the presence of cognate lipid antigens. Under these conditions, iNKT cells slow down or arrest, and are confined to the MZ where antigen-rich MZ macrophages and dendritic cells reside (76, 80).

Although there is evidence demonstrating that iNKT cells are important in allergy and airway inflammation, information on the anatomical distribution of iNKT cells in lung, their mechanism of activation, and role in lung diseases remain scarce (77, 81). Recent studies have attempted to address these questions. Two-photon fluorescence microscopy was employed to examine the localization of iNKT cells in a harvested lobe of a murine lung (82). They

revealed that pulmonary iNKT cells mainly resided in the lung microvasculature. Upon exposure to aerosolized lipid antigen, iNKT cells mobilized and extravasated into lung tissue. Thomas et al. (69) showed that there was an approximate 10- to 20-fold enrichment of iNKT cells in blood drawn from the right or left ventricles of the heart as compared to peripheral blood. Another study suggests that some pulmonary iNKT cells can be long-lived (83). Using parabiotic mice, Bendelac and colleagues demonstrated that pulmonary iNKT cells do not recirculate between parabiotic mice pairs even after 30 days (69).

Interactions Between the Host and Pathogens

Although α GalCer was extracted from a marine sponge sample, the presence of this iNKT cell ligand in marine sponges was not linked to any physiological relevant function. This highly reactive glycolipid likely originated from a bacterium present inside the sample rather than the sponge itself as marine sponges are commonly colonized by α -proteobacteria such as *Sphingomonas* spp. (84, 85). Indeed, the physiologically relevant α GalCer-related compounds, α -glycuronylceramide, and α -galacturonosylceramide which are found in the cell wall of *Sphingomonas*, are strong and potent activators of iNKT cells (48, 49, 86, 87). In addition to ceramide-based compounds from *Sphingomonas*, glycerol-based lipids have been described to potently activate iNKT cells. These include α -galactosyldiacylglycerol from *Borrelia burgdorferi* and α -glucosyldiacylglycerol from *Streptococcus pneumoniae* (88, 89).

Borrelia burgdorferi is a spirochete pathogen that continues to spread in North America (90). This pathogen induces Lyme-disease, and delayed or inadequate treatment typically leads to disabling symptoms as the bacteria invade the joints, heart, and central nervous system (91). The liver functions as an important organ that is positioned to intercept disseminating pathogens in the blood (92). This interception is mediated by liver-resident intravascular macrophages (Kupffer cells), which ensnare pathogens from the blood stream. Visualizing iNKT cell activity in the liver showed dramatically altered iNKT cell behavior after *B. burgdorferi* infection (9). Instead of crawling through the liver sinusoids, iNKT cells formed clusters and arrested next to Kupffer cells that had captured *B. burgdorferi*. This clustering occurred as early as 4 h after exposure and could be inhibited by blocking the Cxcr3 receptor. Anti-CD1d antibody blocked the firm adhesion of iNKT cells to Kupffer cells as well as the activation of iNKT cells, which suggests that Kupffer cells were responsible for presenting antigens to activate iNKT cells. Intravital imaging during this process revealed that Kupffer cells phagocytose *B. burgdorferi* from blood for antigen presentation to iNKT cells, which then produce IFN- γ and other inflammatory cytokines. This activated the local hepatic innate immunity system to prevent bacteria dissemination.

The absence of iNKT cells in mice caused a remarkable 25-fold increase in *B. burgdorferi* burden in joints, but other organs did not have a similar burden, which indicated that iNKT cells in the joint microenvironment had a unique feature. This unique *in vivo* observation led investigators to question the functional significance of joint iNKT cells. Intravital imaging of the joint

during *B. burgdorferi* infection revealed that extravascular iNKT cells interact directly with the spirochetes at joint blood vessel walls (78). This joint iNKT cell behavior was in distinct contrast to iNKT cells in the liver, which were oblivious to the pathogen in the absence of Kupffer cells. During this interaction, joint iNKT cells were no longer stationary but actively crawled along vessel walls toward the pathogen, perhaps due to complement activation. *B. burgdorferi* that interacted with iNKT cells subsequently died, which suggests that joint iNKT cells limit the dissemination of this pathogen into the joint. Indeed, absence of iNKT cells led to a large number of motile spirochetes outside the vasculature in the joint cavity of mice. It is worth noting that human joints had far fewer iNKT cells, and perhaps this may lead to the susceptibility of humans, but not mice, to *B. burgdorferi*-induced Lyme arthritis.

Streptococcus pneumoniae infection is a leading cause of morbidity and mortality in adults and children (93, 94). This encapsulated bacteria typically resides on the mucosal surface of the upper respiratory tract or the nasopharynx of humans and appears to be asymptomatic (95). However, if *S. pneumoniae* gains access to the sterile lower respiratory tract, it causes a potent inflammatory response that result in severe disease. In this situation, pulmonary iNKT cells are important in the protection of the host against an infection by *S. pneumoniae* (96). If iNKT cells are absent (in $J\alpha 18^{-/-}$ mice) following infection by *S. pneumoniae*, lower cytokine levels, less neutrophils, and increased bacteria burden were found in the lung. The iNKT cell-deficient mice also had increased mortality following *S. pneumoniae* infection. A recent paper demonstrated that this protective effect was dependent on recognition of a *S. pneumoniae* glycolipid (89). The behavior of iNKT cells in the lung under basal and *S. pneumoniae* infection has not been fully elucidated due to challenges of imaging the lung. Moreover, it is unclear which immune cells are presenting *S. pneumoniae* glycolipids to iNKT cells. Clearly, it would be of benefit to examine the dynamics of iNKT cells in the lung under these different conditions.

Other naturally occurring microbial antigens including cholesterol ester from *Helicobacter pylori* (97), lipopeptidophosphoglycans from *Leishmania donovani* (98), and *Entameba histolytica* (99) can activate iNKT cells, but the antigenicity of these lipids are not well characterized and direct evidence of significant contribution of these lipid antigens during infection and disease remain elusive. Further study is necessary to determine their contribution to the activation of iNKT cells during these specific infections.

The Balance Between Regulating Inflammation After Tissue Injury Versus Host Defense

The inflammatory response is critical for host defense against invading pathogens. Known as sterile inflammation, inflammation also occurs when self tissue is damaged in the absence of infection (100). Akin to inflammation induced by microbes, sterile inflammation also results in recruitment of neutrophils, monocytes, and macrophages, and the release of chemokines and pro-inflammatory cytokines such as IL-1 (101). Sterile inflammation has been identified to underlie many medical afflictions such as burn injuries or ischemia-reperfusion injury in the heart, liver, and brain (102). Following the initial trauma, the outcome of these

afflictions are immunosuppression and susceptibility of the host to subsequent infection. Some medical examples of these complications include patients with acute myocardial infarction, stroke, or major burn injuries. Systemically inhibiting inflammation in these conditions can lead to adverse infectious complications. With the ability to react to self or invasive pathogens, iNKT cells are the linchpins which can determine a favorable or detrimental outcome during inflammation in these conditions.

Able to respond to “self” lipid antigens, iNKT cells are able to regulate inflammation during tissue injury (46, 103). Several endogenous lipids have been proposed to activate iNKT cells, although identification of the primary endogenous lipid antigen is a subject of intense research (104–106). Nevertheless, during tissue injury and cell death, endogenous antigens can serve as danger signals to activate iNKT cells in the absence of exogenous ligands. The functional role of iNKT cells have been investigated in mouse models of burn injury. In a cauterization-induced corneal inflammation model, iNKT cell-deficient mice had increased neutrophil accumulation and higher levels of pro-inflammatory cytokines in the cauterized eye (107). In addition, lack of iNKT cells led to greater corneal edema and opacity. In this model, iNKT cells played an important role in curbing inflammation and maintained corneal clarity. A similar immunoregulatory effect was observed in a dorsal burn injury model where iNKT cells were found to mediate T cell proliferation after injury by producing IL-4 (108). Production of IL-4 by iNKT cells suppressed antigen-specific T cell delayed-type hypersensitivity after dorsal burn injury.

During stroke, intravital imaging revealed that norepinephrine release rapidly arrested and activated iNKT cells in the liver (8). Interestingly, this increased sympathetic drive induced activated iNKT cells to produce increased levels of anti-inflammatory cytokines such as IL-10, which led to post-stroke immunosuppression. This effect likely protects the brain from inflammatory damage (109) but also leaves the patient open to infection, which is a major cause of post-stroke death (110). In contrast, activating iNKT cells with the potent agonist α GalCer reduced bacterial infection after stroke (8). Collectively, these findings suggest that iNKT cell activation was not the determining factor that mediated immunosuppression after stroke but rather the adrenergic activation and modulation of iNKT cells resulted in a shift from pro-inflammatory to anti-inflammatory cytokine production. This also raises the possibility of therapeutically targeting iNKT cells in the liver to quench detrimental neuro-immunosuppression as long as it does not enhance inflammation in the brain.

Concluding Remarks

There is no doubt that iNKT cells have a pivotal function in directing innate and adaptive immunity during diseases where their diverse effector repertoire can lead to varied outcomes ranging from promoting inflammation to immunosuppression. Despite recent advances in unraveling mechanisms of iNKT cell activation and a greater understanding of iNKT cell biology, more research to elucidate the interactions between iNKT cells and other leukocytes is still needed. Traditionally, visualizing the spatial distribution of iNKT cells and understanding the role of iNKT cells in context of other immune cells were through static

snapshots of tissue sections. However, technological advances in fluorescence microscopy and maturation of IVM technology have revolutionized the iNKT cell research field, allowing us to image the behavior of these cells in different organs under basal and inflammatory conditions at high resolution.

Although the ability to accurately visualize cells in a live animal at microscopic scale provides exciting opportunities for biological observation, caution is still needed at this stage. Fluorescence IVM is dependent on labeling cell types, and current technology is restricted by an inability to label all cell types, structural components, and chemical mediators at the same time. Therefore, only visible cell-cell interactions can be observed. In addition, the reporter mice that are presently available for lineage specific cell types including the *Cxcr6*^{Gfp/+} mice are not entirely specific and in some organs like the intestines, the percentage of GFP-positive cells that are iNKT cells is <50%, which makes it impossible to specifically track these cells. Further, current limitations of IVM technologies do not allow high-resolution imaging of all tissues and for those that can be visualized, it may not be possible to image deep into the tissue. Ongoing improvements to IVM technology such as the use of multiphoton microscopes, far-red probes, and longer wavelength lasers would address some of these issues (5, 10). Finally, current IVM techniques do not allow large areas of the tissue to be scanned at quick speeds; this limits imaging to relatively slower dynamic processes for observation if macroscopic levels are desired. Nevertheless, a thorough understanding of the spectrum of iNKT cell behavior and mechanisms of action will occur as IVM technology improves. Understanding iNKT cell biology will ultimately determine our ability to successfully target iNKT cells for clinical applications.

Outstanding Questions

- What factors determine the outcome, inflammation versus immunosuppression, of iNKT cell activation during diverse pathological states?
- What are the roles of iNKT cells in the context of other immune cells under basal and inflammatory states?
- How does the location and behavior of iNKT cells in the liver differ from other organs (such as the spleen, lung, and intestine)? Are there any similarities?
- How can an enhanced understanding of the spectrum iNKT cell biological behaviors be utilized to manipulate their function for clinical settings?
- Are iNKT cell counts and roles altered during pathological states? Are they reversible and does it affect the therapeutic ability of iNKT cells?

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Type II NKT cells in inflammation, autoimmunity, microbial immunity, and cancer

Idania Marrero, Randle Ware and Vipin Kumar*

Laboratory of Immune Regulation, Department of Medicine, University of California San Diego, La Jolla, CA, USA

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Edited by:

S. M. Mansour Haeryfar,
Western University, Canada

Reviewed by:

Masaki Terabe,

National Institutes of Health, USA

Susanna Cardell,

University of Gothenburg, Sweden

Jay A. Berzofsky,

National Institutes of Health, USA

***Correspondence:**

Vipin Kumar,

Laboratory of Immune Regulation,

Department of Medicine, University of

California San Diego, 9500 Gilman

Drive, La Jolla, CA 92037, USA

vckumar@ucsd.edu

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Natural killer T cells (NKT) recognize self and microbial lipid antigens presented by non-polymorphic CD1d molecules. Two major NKT cell subsets, type I and II, express different types of antigen receptors (TCR) with distinct mode of CD1d/lipid recognition. Though type II NKT cells are less frequent in mice and difficult to study, they are predominant in human. One of the major subsets of type II NKT cells reactive to the self-glycolipid sulfatide is the best characterized and has been shown to induce a dominant immune regulatory mechanism that controls inflammation in autoimmunity and in anti-cancer immunity. Recently, type II NKT cells reactive to other self-glycolipids and phospholipids have been identified suggesting both promiscuous and specific TCR recognition in microbial immunity as well. Since the CD1d pathway is highly conserved, a detailed understanding of the biology and function of type II NKT cells as well as their interplay with type I NKT cells or other innate and adaptive T cells will have major implications for potential novel interventions in inflammatory and autoimmune diseases, microbial immunity, and cancer.

Keywords: CD1d, innate immunity, inflammation, autoimmunity, cancer, sulfatide, lipids, neutrophils

Introduction

Natural killer T (NKT) cells are innate-like T cells that recognize both exogenous and endogenous lipid antigens presented by CD1d, a major histocompatibility (MHC) class I-like antigen-presenting molecule. They comprise two main subsets, type I and type II, based upon differences in the nature of their T cell receptors (TCRs) (1–3). The well-studied type I NKT cell subset that uses a semi-invariant TCR α chain is more prevalent than the type II subset in the mouse, while the less explored type II NKT cell subset that utilizes a more diverse TCR repertoire is predominant in humans (4–6). Both subsets require signaling lymphocytic activation molecule-associated protein (SAP) and promyelocytic leukemia zinc finger (PLZF) for their development and effector program (3, 7, 8). After antigenic activation, NKT cells secrete large amounts of cytokines, such as interferon- γ (IFN γ), interleukins IL-4, IL-10, IL-13, IL-17, and IL-22, tumor necrosis factor- α (TNF α), and granulocyte-macrophage colony-stimulating factor (GM-CSF), which modulate immune responses triggered by other innate NK cells and adaptive T and B cells (3–6). Both subsets appear to modulate immune responses involved in autoimmunity, inflammation, infections, and cancer (4–7, 9, 10). This review primarily focuses on lipid-reactive CD1d-restricted TCR $\alpha/\beta+$ type II NKT cells and their potential role in immunity.

Antigen Recognition, TCR Repertoire, and Activation of Type II NKT Cells

Type I NKT cells respond to a strong agonist α -galactosylceramide (α GalCer) as well as microbial and self-lipids. By contrast, type II NKT cells are not reactive to α GalCer and recognize self-glycolipids and self-phospholipids (5, 6, 11). A major subset of type II NKT cells recognize a naturally occurring self-glycolipid, sulfatide, which is enriched in several membranes, including myelin in the central nervous system (CNS), β -cells in the pancreas, kidney, and liver (12, 13). Recently, other self-lipids, including β -D-glucopyranosylceramide (β -GlcCer), β GalCer, lysophosphatidylethanolamine (lyso-PE), lysophosphatidylglycerol (lyso-PG), or cardiolipin and lysophosphatidylcholine (lyso-PC) have been shown to be involved in the activation of liver type II NKT cells (5, 6, 8, 12–17). Lyso-PC and β -glucosylceramide and glucosyl-sphingosine lipids were reported to activate human type II NKT cells as well (18, 19).

The mechanism of binding of type II NKT TCRs to antigens uses features of TCR binding shared by both type I NKT cells and conventional T cells, but also is distinct from both (12, 20, 21). Thus, type II NKT TCRs contact their ligands primarily via their β chain rather than α chain, suggesting that the TCR V β chain contributes significantly to antigen fine specificity (20). Sulfatide-reactive type II NKT cells express oligoclonal TCRs with a limited number of V α - and V β -chains (V α 3/V α 1 and V β 8.1/V β 3.1). In contrast to the germline-encoded TCR α chain in type I NKT cells, only about 14% of TCR V α and 13–27% of TCR V β chains in type II NKT cells are encoded by germline gene segments (12). A prevalent type II NKT cell subset expressing V α 3.2–V β 8 TCR has also been described (22). It remains to be seen whether other type II NKT cells will also use a similar mechanism of lipid recognition to sulfatide-reactive T cells. It appears that type II NKT cells are mostly reactive to self-lipid ligands, but they can also recognize structurally similar microbial-derived lipids because of their TCR degeneracy or promiscuity.

Type I NKT cells can be activated either directly through TCR stimulation by exogenous microbial lipid antigens or indirectly by stimulatory self-lipids presented by CD1d and/or cytokines (IL-12, IL-18, or type I IFN) produced through Toll-like receptor (TLR)-mediated signaling (23, 24). Thus, different self-lipids as well as cytokines present at elevated levels during inflammation can potentially stimulate type I NKT cells. Recent studies suggest that type I NKT cells can be activated in response to bacteria, as well as viruses, without antigen receptor stimulation (25). By contrast, type II NKT cells are mainly stimulated by direct recognition of lipid/CD1d complexes by their TCR. It has been consistently found that stimulation of type II NKT hybridomas with phospholipids and glycolipids requires lipid uptake, intracellular processing, and presentation to TCR but not TLR signaling (15, 26). In many experimental conditions wherein type I NKT cells are activated, type II NKT cells remain inactivated suggesting that type II NKT cells may not be easily activated by cytokine/TLR signaling but require self-lipid recognition.

It is becoming clear that the TCR recognition by type II NKT cells can be highly specific or promiscuous. For example, sulfatide-reactive type II NKT cell hybridomas XV19 or 19.3 can

recognize sulfatide or lyso-PC effectively but not so efficiently all other phospholipids or glycolipids (12–15, 17, 26). Consistent with this, at the polyclonal level, some lyso-PC-reactive NKT cells are distinct from sulfatide-reactive NKT cells (17) and in 4get mice, type II NKT cells are reactive to several self-lipids but not sulfatide (8, 16). Similarly, some lyso-PG-reactive type II NKT hybridomas can recognize both self and microbial lipids derived from *Mycobacterium tuberculosis* or *Corynebacterium glutamicum* and others are non-responsive to these lipids (15). These findings identify some redundancy as well as overlapping TCR repertoires among type II NKT cells that recognize self-lipids.

Immune regulatory activity of NKT cells can be mediated by cytokines secreted by NKT cells themselves or following their interaction with other immune cells, including DCs, NK cells, Tregs, monocytes, and B cells. Activation of NKT cell subsets can result in the deviation of a cytokine secretion profile in MHC-restricted CD4 $^{+}$ /CD8 $^{+}$ T cells toward either a pronounced Th1-, Th2-, or Th17-like response.

It is noteworthy that in inflamed target tissues, such as in pancreas in non-obese diabetic (NOD) mice that spontaneously develop type 1 diabetes (T1D) and in the CNS during experimental autoimmune encephalomyelitis (EAE), both type I and type II NKT cells accumulate (13, 27). However, activation of type II NKT cells following sulfatide or lyso-PC administration leads to a rapid accumulation of type I NKT cells into liver in an IL-12 and macrophage inflammatory protein 2 (MIP2)-dependent fashion. But these recruited type I NKT cells are neither activated nor do they secrete cytokines, and consequently they are anergic, leading to decreased levels of IFN γ followed by reduced recruitment of myeloid cells, NK cells, and protection from liver damage (28, 29). In contrast to the activation of lyso-PE-reactive type II NKT cells in an infectious model of HBV, hepatic type I NKT cells are not anergized but stimulated to secrete cytokines (16). This difference in type I NKT stimulation may relate to the differential milieu in liver during sterile versus infectious immunity.

A Novel Type II NKT Cell-Mediated Immune Regulatory Pathway

Sulfatide-mediated type II NKT cell stimulation *in vivo* results in the activation of predominantly hepatic plasmacytoid DCs (pDC) but not conventional DC (cDC) and ultimately induction of anergy in hepatic type I NKT cells. This unique immune regulatory pathway not only involves cross-regulation of type I NKT cells but also inhibition of pathogenic Th1/Th17 cells through tolerization of hepatic cDC and tissue-resident antigen-presenting cells (APCs), such as microglia in the CNS (28, 30). By contrast, activation of type I NKT cells following α GalCer administration predominantly activates hepatic cDC (28, 29). We are currently investigating the molecular mechanism of these NKT-DCs interactions.

It has been shown that this immune regulatory pathway effectively controls EAE, T1D, inflammatory liver diseases, and systemic lupus erythematosus (SLE) (17, 27, 28, 30–32) (Halder, unpublished). A recent study has suggested that the ICOS and PD-1 ligand pathways are required for the regulation of T1D in NOD mice by CD4 $^{+}$ type II NKT cells (33). Sulfatide-mediated type II NKT cell activation can also result in IL-10 secretion and,

consequently, inhibition of type I NKT cells and diabetogenic or encephalitogenic CD4⁺ and CD8⁺ T cells (13, 27). Furthermore, activation of type II NKT cells also induces alterations in other innate cells, including myeloid-derived suppressor cells (MDSCs), CD11b⁺Gr-1⁺ cells, and neutrophils (17, 28, 30–32). Accordingly, MDSCs have been shown to protect mice from EAE (34). CD11b⁺Gr-1⁺ cells and neutrophil alterations can also protect from inflammatory liver diseases (31, 32, 35). Additionally, activation of type II NKT cells by PD-L1-deficient DCs increases the IL-4 and IL-13 levels and, consequently, decreases the numbers of IFN γ and IL-17-secreting pathogenic T cells (36). Thus, targeting type II NKT-mediated inhibition of the effector functions of Th1/Th17 cells and APCs in peripheral organs as well as in affected target tissues offers a potent strategy for intervention in autoimmune and inflammatory diseases (30).

Type II NKT Cells in Autoimmune and Inflammatory Diseases

The activation of type II NKT cells with sulfatide controls both antigen-induced and spontaneously arising autoimmune diseases. Additionally, sulfatide-mediated immune regulation inhibits inflammatory liver diseases elicited by type I NKT cells (13, 17, 27, 28, 30–32, 35). Sulfatide-reactive type II NKT cells also have been shown to abrogate ischemic–reperfusion injury in mice and in patients with acute tubular necrosis (37). Interestingly, during EAE and T1D progression, sulfatide-reactive type II NKT cells accumulate in the target tissue and in the draining of lymph nodes, respectively. This greater abundance of type II NKT cells in the CNS inverts the usual ratio of type II/type I NKT cells (type II NKT cells, 3–4%, and type I NKT cells, 0.6–0.9%) (13, 27). Thus, administration of brain-derived sulfatides or synthetic cic-tetracosanoyl or tetracosanoyl sulfatide affords protection from EAE and diabetes (13, 27, 30). In both cases, it is the sulfatide with the longer fatty acid chain that is able to efficiently activate type II NKT cells and prevent autoimmunity. These data suggest that sulfatide analogs should be examined in clinical studies in multiple sclerosis (MS) and T1D. Our preliminary studies also suggest that activation of type II NKT cells following administration of sulfatide significantly inhibits development of lupus nephritis in (NZB X NZW) F1 mice, further indicating a regulatory role of type II NKT cells (5) (Halder, unpublished).

Recent studies have indicated key mutual interactions among NKT cells, CD1d⁺ cells, and commensal microbiota in the intestine (38). Evidence from several animal models of inflammatory bowel disease (IBD) demonstrates that type I NKT cells can be either protective or pathogenic (39). Interestingly, type II NKT cells seem to promote intestinal inflammation and mediate a pathogenic response when both CD1d expression and the frequency of IL-13 producing type II NKT cells are increased in mice as well as patients with ulcerative colitis (40–42). It is noteworthy that type II NKT cells involved in ulcerative colitis in humans are also reactive to lysosulfatide, but in contrast to the liver type II NKT cells, they secrete IL-13 and not IFN γ (41). This suggests that there are subsets of type II NKT cells that may have different TCR repertoires as well as different cytokine secretion patterns in different tissues, just as there are different subsets of type I NKT cells.

Type II NKT Cells in Metabolic and Liver Disorders

Both NKT cell subsets have been shown to be involved in adipose-tissue inflammation, diet-induced obesity, and glucose metabolism (43). Roles for eosinophils, macrophages, and innate lymphoid type 2 cells (ILC2) have also been suggested in metabolic disorders (44, 45). More recently, type II NKT cells induced by both IL-25 and sulfatide treatments have been shown to be involved in the regulation of inflammation in adipose tissue and prevention of high fat diet-induced obesity in mice. Transfer of type II NKT cells into obese mice induced a greater and prolonged weight loss and improved glucose tolerance (44).

In inflammatory liver diseases, type I and type II NKT cells have been shown to play opposing roles (35). Earlier, it was shown that following liver injury after ischemic–reperfusion or ConA administration, a rapid activation of IFN γ -secreting type I but not type II NKT cells takes place (28, 31). Activation of type I NKT cells generates a cascade of events that contributes to liver inflammation and damage. The secretion of pro-inflammatory cytokines, such as IFN α , and chemokines leads to accumulation of CD11b⁺ Gr-1⁺ cells as well as other myeloid cells resulting in the destruction of hepatocytes. By contrast, sulfatide-activated type II NKT cells inhibit the cascade of pro-inflammatory events through a mechanism that includes activation of pDC resulting in tolerization of cDC, anergy in type I NKT cells and consequently protection from liver injury (28, 31, 35). In a mouse model of chronic alcohol liver disease (ALD), we have found that type I, but not type II, NKT cells are activated, leading to recruitment of inflammatory neutrophils and liver damage (46, 47). Inhibition of type I NKT cells following a novel direct mechanism involving all-trans retinoic acid (48) and its receptor (RAR- γ) signaling, or an indirect mechanism mediated by sulfatide-activated type II NKT cells significantly blunts ALD (46). Consistent with this, accumulation of activated type I NKT cells in patients with NAFLD has recently been shown (49–51). Currently, clinical studies are being carried out to examine the potential use of a RAR- γ analog for the treatment of alcohol- and non-alcoholic liver disease. We are presently investigating in humans the role of both type I and type II NKT cells in the promotion as well as regulation of inflammatory immune responses in liver and gut. The identification of the role of these cell subsets in liver disorders could potentially lead to the development of novel therapeutics.

Type II NKT Cells in Infectious Diseases

Natural killer T cells contribute to the early immune response against a broad range of microbial pathogens, playing either a beneficial role in some infections or a negative role in others (52). Frequently, type I and type II NKT cells can have opposing roles in microbial immunity. For example, in the case of *Trypanosoma cruzi* infections, type II NKT cells were shown to promote inflammation and mortality and reduced titers of pathogen-specific antibodies, whereas type I NKT cells led to reduced inflammation and improved mortality and antibody titers (53). By contrast, during murine *Schistosoma mansoni* infection, type II NKT cells led to

increased Th2 cytokine secretion and decreased IFN γ production, while type I NKT cells reinstated IFN γ levels (54).

Sulfatide-activated type II NKT cells have also been shown to affect the course of infectious diseases. Previously, we showed that sulfatide-activated type II NKT cells inhibit HIV-1 replication and enhance multi-lineage hematopoiesis in a SCID-Hu (Thy/Liv) HIV model (55). We hypothesized that sulfatide-mediated activation of type II NKT cells and pDC results in the induction of anergy in type I NKT cells. It was shown that peripheral CD4 $^{+}$ type I NKT cells are depleted in early HIV infection and that the remaining cells in the circulation during HIV infection are functionally impaired in IFN γ expression (56). Sulfatide-mediated activation of type II NKT cells also has a protective effect in a *Staphylococcus aureus* murine model of sepsis and is associated with a decrease in pro-inflammatory cytokines, such as TNF α and IL-6 (57). This beneficial outcome was found to depend on CD1d but not on type I NKT cells.

Recent studies in HBV infection in animal models and humans have shown that NKT cells contribute to the initiation of antiviral immune responses against HBV. An early activation of type I and type II NKT cells was found following infection, mainly in the liver, which correlated with IFN γ -dependent suppression of viral replication; but also NKT cells contribute to HBV-induced hepatitis (16, 58). Using a mouse model of infection with HBV-expressing adenoviral particles (Ad-HBV), it was demonstrated that $\text{J}\alpha 18$ -deficient (lacking type I NKT cells) and CD1d-deficient (lacking all NKT cells) mice exhibited a significant decrease in NK, B, CD4 $^{+}$, and CD8 $^{+}$ T cell activation and hepatic immune infiltration, supporting the idea that NKT cells play a role in the immune response to HBV (16). More importantly, it was shown that HBV infection induces production of modified ER self-lipids, including phosphatidylethanolamine (PE) and lysophosphatidylethanolamine (lyso-PE), direct activation of liver type II NKT cells, and downstream cytokine-dependent activation of type I NKT cells. Type II NKT activation required hepatocyte expression of microsomal triglyceride transfer protein (MTP) and CD1d (16). In a murine acute hepatitis B transgenic model, NKG2D-dependent activation of type II NKT cells has been shown to result in liver damage (59).

Type II NKT Cells in Tumor Immunity

Similar to the immune responses in liver, type I and type II NKT cells have been shown to play opposing roles in tumor immunity

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(10, 60). Type I NKT cells are usually associated with the promotion of tumor immunity, whereas type II NKT cells are associated with its suppression (61). Thus, type I NKT cells were found to induce lysis of tumor cells directly via a perforin/granzyme-dependent mechanism or indirectly by induction of Th1 cytokine secretion and activation of NK and DC cells. By contrast, type II NKT cells have shown immunosuppressive activity down-regulating tumor immunosurveillance (60, 62–64). Type II NKT cell-induced tumor suppression can be mediated by IL-13 secretion resulting in the activation of TGF- β -secreting MDSCs that inhibit tumor-specific CD8 $^{+}$ T cells or type I NKT cells. In humans, Chang et al. have also shown an increase in IL-13-secreting lyso-PC-reactive type II NKT cells in multiple myeloma patients (18). Interestingly, type I NKT cells are decreased in these patients, suggesting opposing roles, as their increased frequency is associated with better prognosis.

Future Studies and Challenges

Availability of stable reagents for analysis of type I NKT cells has resulted in characterization of changes in their frequency and phenotype in humans in different disease conditions, including autoimmune and infectious diseases and cancer. A detailed characterization of type II NKT cell repertoires and their ligands in humans is required for a broader understanding of their physiological role in health and in disease. A recent study suggesting a role for lyso-glucosylsphingosine (lyso-GL1)-reactive type II NKT cells in Gaucher disease is an important development (19). Together, all of these observations indicate that it may be possible in the future to differentially activate or inhibit type I and type II NKT cells for the development of novel immunotherapeutic protocols in altering the course of cancer and both infectious and autoimmune diseases.

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The extended family of CD1d-restricted NKT cells: sifting through a mixed bag of TCRs, antigens, and functions

Elodie Macho-Fernandez and Manfred Brigi*

Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA

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Thierry Mallevaey,
University of Toronto, Canada

Reviewed by:

Vipin Kumar,
University of California San Diego,
USA

Sebastian Joyce,
Vanderbilt University School of
Medicine, USA

*Correspondence:

Manfred Brigi,
Department of Pathology, Brigham
and Women's Hospital, Harvard
Medical School, 77 Avenue Louis
Pasteur, Harvard New Research
Building, Boston, MA 02115, USA
mbrigl@rics.bwh.harvard.edu

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Natural killer T (NKT) cells comprise a family of specialized T cells that recognize lipid antigens presented by CD1d. Based on their T cell receptor (TCR) usage and antigen specificities, CD1d-restricted NKT cells have been divided into two main subsets: type I NKT cells that use a canonical invariant TCR α -chain and recognize α -galactosylceramide (α -GalCer), and type II NKT cells that use a more diverse $\alpha\beta$ TCR repertoire and do not recognize α -GalCer. In addition, α -GalCer-reactive NKT cells that use non-canonical $\alpha\beta$ TCRs and CD1d-restricted T cells that use $\gamma\delta$ or $\delta/\alpha\beta$ TCRs have recently been identified, revealing further diversity among CD1d-restricted T cells. Importantly, in addition to their distinct antigen specificities, functional differences are beginning to emerge between the different members of the CD1d-restricted T cell family. In this review, while using type I NKT cells as comparison, we will focus on type II NKT cells and the other non-invariant CD1d-restricted T cell subsets, and discuss our current understanding of the antigens they recognize, the formation of stimulatory CD1d/antigen complexes, the modes of TCR-mediated antigen recognition, and the mechanisms and consequences of their activation that underlie their function in antimicrobial responses, anti-tumor immunity, and autoimmunity.

Keywords: CD1d, antigen presentation, lipid antigens, glycosphingolipids, phospholipids, inflammation, infection, cancer

Introduction

Traditionally, the immune system has been separated into innate and adaptive immunity. However, in an unconventional way, innate T cells such as CD1d-restricted natural killer T (NKT) cells, MR1-restricted mucosal-associated invariant T (MAIT) cells, $\gamma\delta$ T cells, and some CD1a, b, c-restricted T cells share features of both innate and adaptive immune cells, allowing them to form a critical bridge between the two arms of the immune system (1–4). NKT cells recognize lipid antigens presented by the monomorphic MHC class-I-like molecule CD1d and are highly conserved in humans and mice. In response to a wide range of self- and foreign antigens, NKT cells are activated rapidly and exhibit both pro-inflammatory and immunoregulatory characteristics, resulting in either protective or harmful roles in numerous pathological states in mice and humans, including microbial infection, autoimmune disease, allergic disease, and cancer (5–10). Therefore, NKT cells represent an immunotherapeutic target with broad clinical potential. CD1d-restricted T cells can be divided into four main subsets based on their T cell receptor (TCR) usage and antigen specificities (Table 1). Type I (or invariant) NKT cells constitute the first and best characterized subset, and use an invariant

TABLE 1 | Classification of CD1d-restricted T cells.

	Type I NKT cells	V α 24 $^{-}$ (h) and V α 10 $^{+}$ (m) NKT cells	Type II NKT cells	$\gamma\delta$ and $\delta/\alpha\beta$ T cells
TCR repertoire	m: invariant (j)V α 14J α 18, V β 8, V β 7 or V β 2 h: V α 24J α 18, V β 11	m: iV α 10J α 50, V β 8 h: limited α chains (V α 10, V α 2 or V α 3), V β 11	m: diverse with oligoclonal V α 3.2-J α 9/V β 8 and V α 8/V β 8 h: diverse?	m: V δ 4 $^{+}$ h: V δ 1 $^{+}$, V δ 3 $^{+}$
α -GalCer reactivity	all	all	no	some
Sulfatide reactivity	no	no	some	some
Other antigens	Microbial α -GSL, α -GDAG Mammalian β -GSL, phospholipids, lysophospholipids, plasmalogens	Microbial α -GSL Mammalian β -GSL and β -GlcSph	Mammalian and microbial phospholipids (PG, DPG, PI) Mammalian lysophospholipids (LPC, LSM, LPE, β -GlcSph)	Mammalian and pollen phospholipids (PE, PC, DPG)
Function	NKT1 (IFN- γ , IL-2) NKT2 (IL-4, IL-9, IL-10, IL-13) NKT17 (IL-17A, IL-21, IL-22) NKT $_{FH}^{+}$ -like (IL-5, IL-6, IL-10, IL-17) Cytotoxicity (perforin, granzyme)	T H 1-like (IFN- γ) T H 2-like (IL-4, IL-13) T H 17-like (IL-17)	T H 1-like (IFN- γ , IL-2) T H 2-like (IL-4, IL-10, IL-13) T H 17-like (IL-17) Cytotoxicity (perforin, granzyme) T FH -like (IL-5, IL-6, IL-10, IL-17)	T H 1-like (IFN- γ) T H 2-like (IL-4)
Phenotype	Activated/memory	Activated/memory some naïve?	Activated/memory some naïve?	Activated/memory

α -GalCer, α -galactosylceramide; α -GSL, α -glycosphingolipid; α -GDAG, α -glyco(Gal/Glc)diacylglycerol; β -GlcSph, β -glucosylsphingosine; DPG, diphosphatidylglycerol; h, human; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LSM, lysosphingomyelin; m, mouse; PG, phosphatidylglycerol; NKT, natural killer T cell; TCR, T cell receptor.

TCR α chain (V α 14-J α 18 in mice and V α 24-J α 18 in humans). In addition, there are CD1d-restricted T cells that use a more varied TCR repertoire, including V α 24 $^{-}$ and V α 10 $^{+}$ NKT cells in mice and humans, respectively, diverse or type II NKT cells, as well as T cells expressing $\gamma\delta$ or $\delta/\alpha\beta$ TCRs. In this review, while using type I NKT cells as comparison, we will focus on type II NKT cells and the other non-invariant CD1d-restricted T cell populations and discuss our emerging understanding of their TCR usage, antigen specificities, mode of antigen/CD1d recognition, innate-like mechanisms of activation, and their immunological functions.

The CD1d-Restricted T Cell Family

Type I NKT Cells

The discovery of the first CD1d-presented antigen, α -galactosylceramide (α -GalCer), by Kawano and colleagues in 1997 enabled several important steps forward in our understanding of NKT cell biology, particularly of type I or invariant NKT (iNKT) cells (11). Type I NKT cells express an invariant V α 14J α 18 TCR α -chain in mice and V α 24J α 18 in humans, paired with a limited repertoire of TCR β -chains (V β 8, V β 7, V β 2 in mice and exclusively V β 11 in humans) (Table 1). Type I NKT cells are highly autoreactive even at steady state and display an activated/memory phenotype with high surface levels of the activation markers CD69, CD44, and CD122 (IL-2R β -chain) and low expression of CD62L, a marker expressed by naïve T cells that home to lymph nodes (12, 13). The use of mice that are deficient in CD1d (lack type I and type II NKT cells) or selectively deficient in type I NKT cells (J α 18 $^{-/-}$), administration of α -GalCer to activate type I NKT cells *in vivo*, and the ability to track type I NKT cells with fluorescent CD1d/ α -GalCer tetramers has allowed the elucidation of many functions of type I NKT cells *in vivo*. Type I NKT cells play critical roles in local and systemic immune responses and are essential for controlling

tumor development and antimicrobial immune responses. They can also exert detrimental effects in the pathogenesis of autoimmune and allergic disorders. The biology of type I NKT cells has been extensively covered in several excellent recent reviews (2, 3, 14–16), including their roles in microbial infections (2, 7, 17–20), autoimmunity and inflammation (9, 21, 22), and tumor immunity (23). This broad range of type I NKT cell functions relies on their rapid secretion of copious amounts of various cytokines, including IFN- γ , IL-2, IL-4, IL-9, IL-10, IL-13, IL-17, IL-21, and GM-CSF (24–26), and their interactions with other immune cells (2, 16). Based on the differential expression of cytokines, transcription factors and surface markers, several functionally distinct type I NKT cell subsets have been described in humans and mice (Table 1). Human type I NKT cells expressing CD4 produce Th2-type cytokines whereas both CD4 $^{+}$ and CD4 $^{-}$ subsets can generate Th1-type cytokines and secrete cytotoxic molecules such as perforin and granzyme (24, 27). In C57BL/6 mice, Th1-like type I NKT cells (also referred to as NKT1 cells) represent the majority of type I NKT cells in liver and spleen, are characterized by the Th1-associated transcription factor T-bet, mostly express NK1.1 and IL-12 receptor (IL-12R) and their pronounced production of IFN- γ is critical for their function during various immune responses, including anti-tumor immunity (24, 28, 29). Th2-like type I NKT cells (NKT2 cells) are the most abundant type I NKT cell subset in BALB/c mice and are enriched in lung and intestine of C57BL/6 mice. NKT2 cells have been reported to play an important role in Th2-mediated diseases through the secretion of IL-4, IL-9, IL-10, and IL-13 (30, 31). Th17-like type I NKT cells (NKT17 cells) are primarily found in lung, skin and peripheral lymph nodes, produce IL-17A and IL-22 (26, 32, 33), and express the retinoic acid receptor-related orphan receptor γ t (ROR γ t) transcription factor (34). Finally, a small number of type I NKT cells with developmental, phenotypical, and functional features

of follicular helper (35) T cells secrete IL-21, support the formation of germinal centers (36–38) and require the transcription factor Bcl-6 for their development (36). Despite their varied functional differentiation, most type I NKT cell subsets express the transcription factors PLZF, which is fundamental for their development, and GATA3 (39). However, instead of PLZF, a distinct type I NKT cell population, newly referred to as NKT10 cells, express the transcription factor E4BP4 which confers their regulatory properties (40, 41). Thus, the remarkable functional versatility of type I NKT cells during various immune responses can at least in part be explained by the existence of functionally distinct subsets, while organ-specific functions and plasticity of type I NKT cell subsets have not been adequately investigated.

α-GalCer-Reactive V α 24 $^{-}$ and V α 10 $^{+}$ NKT Cells

Recognition of α-GalCer by type I NKT cells was thought to be highly correlated with expression of the invariant V α 14-J α 18 or V α 24-J α 18 TCR α-chains in mice and humans, respectively. However, α-GalCer-reactive, CD1d-restricted NKT cells that use different TCR α-chains have subsequently been described in both mice and humans. In humans, one study using α-GalCer-loaded CD1d tetramers found V α 24-negative T cell populations expressing V α 10, V α 2, or V α 3 joined to J α 18 and paired with V β 11 (42) whereas another study found a diversity of V α 24-J α 18 $^{-}$ /V β 11 $^{-}$ TCRs that stained with α-GalCer/CD1d tetramers (Table 1) (42–44). Despite their equivalent reactivity to α-GalCer or bacterial α-linked glycosphingolipids (GSLs), V α 24-negative subset displayed preferential antigen specificities for α-GlcCer (42). Surprisingly, a V α 24 $^{-}$ /CD1d-α-GalCer $^{+}$ population was found to predominantly display a naïve phenotype and low or intermediate expression level of PLZF (45). So far, data regarding the function of these NKT cells in humans are limited.

Similarly, in mice, CD1d-restricted NKT cells that recognize α-GalCer but do not use the canonical V α 14-J α 18 TCR α-chain have also been described, and predominantly express a semi-invariant V α 10-J α 50 TCR α-chain paired with a V β 8 $^{+}$ TCR β-chain (V β 8.1/0.2 or V β 8.3). V α 10 $^{+}$ NKT cells have been identified in thymus, spleen, and liver of J α 18 $^{-/-}$ mice by CD1d-α-GalCer tetramer staining, and displayed a CD44 $^{\text{high}}$ CD69 $^{\text{int}}$ pre-activated phenotype, similar to type I NKT cells. Like other NKT cell populations, their development required expression of CD1d. In addition to α-GalCer, V α 10 $^{+}$ NKT cells preferentially recognize other glucose-based glycolipids such as α-GlcCer, GSL-1 from *Sphingomonas* or α-GlcA-DAG from *Mycobacterium smegmatis*, and in response to stimulation with these glycolipids produce large amounts of cytokines including IFN-γ, IL-4, IL-13, and IL-17 (46). Thus, recognition of CD1d/α-GalCer complexes by NKT cells in humans and mice is not uniformly restricted to the use of V α 24-J α 18/V β 11 or V α 14-J α 18 TCRs, respectively, but can be mediated by a range of V α and V β domains, highlighting the variation in antigen recognition among CD1d-restricted α-GalCer-reactive TCRs. Furthermore, the different antigen specificities of some of these non-canonical α-GalCer-reactive NKT cells may correlate with distinct functional capabilities, and it remains to be explored if this subset harbors NKT cells that can be expanded *in vivo* and form memory responses.

Type II NKT Cells

CD1d-restricted T cells that do not express the V α 14-J α 18 rearrangement and do not recognize α-GalCer were first described in MHC II-deficient mice among the remaining CD4 $^{+}$ T cells (47). From then called diverse NKT (dNKT), type II NKT, or variant NKT (vNKT) cells, this NKT cell population, found in both mice and humans, exhibits a more heterogeneous TCR repertoire (Table 1). For example in mice, the type II NKT cells that have been described use V α 1, V α 3, V α 8, or V α 11 TCR α-chains paired with V β 8 or V β 3 TCR β-chains, or V α 4 paired with V β 5 or V β 11, and appear to contain oligoclonal V α 3.2-J α 9/V β 8 and V α 8/V β 8 TCR families (48–50). Currently, no direct and specific tools exist to identify the entire type II NKT cell population *in vivo*, but different approaches have been developed to study these cells in mice. The first is to compare the immune responses between J α 18 $^{-/-}$ mice (lacking only type I NKT cells) and CD1d $^{-/-}$ mice (lacking both type I and type II NKT cells). It should be noted that J α 18 $^{-/-}$ mice exhibit lower TCR repertoire diversity due to deficits in rearrangements of several J α segments (51). This raises the possibility that defects described in J α 18 $^{-/-}$ mice are not solely due to type I NKT cell deficiency and a more specific type I NKT cell-deficient mouse model is needed. The second tool used to study type II NKT cell function is 24αβ TCR transgenic mice that were generated by overexpressing the V α 3.2/V β 9 TCR from the type II NKT cell hybridoma VIII24 (52). A third approach is the use of J α 18-deficient IL-4 GFP (J α 18 $^{-/-}$ 4get) reporter mice. This model is based on the finding that some type II NKT cells spontaneously express IL-4 mRNA transcripts, similar to type I NKT cells (53, 54). However, this approach does not identify all type II NKT cells since, for example, GFP $^{+}$ type II NKT cells from J α 18 $^{-/-}$ 4get mice do not respond to sulfatide, an antigen that is recognized by a significant number of type II NKT cells. The fourth approach to identify type II NKT cells is the use of lipid antigen-loaded CD1d tetramer reagents, similar to the use of α-GalCer/CD1d tetramers that are used to detect type I NKT cells. The discovery of sulfatide as a potent type II NKT cell ligand led to the generation of sulfatide-loaded CD1d tetramers and revealed an oligoclonal TCR repertoire among sulfatide-specific type II NKT cells with predominant use of V α 3/V α 1-J α 7/J α 9 and V β 8.1/V β 3.1-J β 2.7 genes (50, 55, 56). However, sulfatide/CD1d tetramers appear to be more difficult to use compared to α-GalCer/CD1d tetramers, likely due to difficulties in loading the antigen and/or greater instability of sulfatide/CD1d complexes and, moreover, not all type II NKT cells recognize sulfatide (50, 54). Similar to sulfatide, β-glucosylceramide (β-GlcCer)- or glucosylsphingosine β-GlcSph-loaded CD1d tetramers stained a subset of human and mouse type II NKT cells (57). Furthermore, the recent discovery of several microbial antigens recognized by different type II NKT hybridomas enables the design of antigen-loaded CD1d tetramers that are likely to be useful to characterize type II NKT cells *in vivo* (58, 59). Another approach to study type II NKT cells is the use of dNKT hybridomas that were initially identified by their recognition of CD1d-expressing APC and their use of TCR α-chains different from V α 14-J α 18 (47–49, 60, 61). These dNKT hybridomas were used to characterize the TCRs expressed by type II NKT cells and continue to be used to

identify self- and microbial lipid antigens that are recognized by type II NKT cells.

Using the approaches described above, many type II dNKT cells appear to share phenotypic and functional features with type I NKT cells such as high autoreactivity (62), PLZF- and SAP-dependent thymic development (54, 63), constitutive expression of IL-4 mRNA (54), and the ability to secrete a wide range of cytokines rapidly after stimulation, including IFN- γ , IL-2, IL-4, IL-10, IL-17, GM-CSF, and cytolytic mediators such as perforin (54, 63). Furthermore, many type II NKT cells have a CD44^{high} CD69⁺ CD122⁺ activated/memory phenotype, whereas CD62L is more or less expressed dependent on which transgenic mouse model is used, and can be divided into different subsets depending on CD4 and NK1.1 expressions (54, 63–65). However, several studies suggest that type II NKT cells exist that are phenotypically and functionally distinct from type I NKT cells. For example, most of the T cells stained with sulfatide/CD1d tetramers in C57BL/6 mice did not express the early activation marker CD69 (50). Moreover, in 24 $\alpha\beta$ TCR transgenic mice on the non-obese diabetic (NOD) background, the majority of DN 24 $\alpha\beta$ NKT cells were CD44^{int}, CD45RB^{high}, CD62L^{high}, CD69^{-low}, similar to conventional T cells, whereas the majority of CD4⁺ 24 $\alpha\beta$ NKT cells exhibited the typical type I NKT CD44^{high}, CD45RB^{low}, CD62L^{low}, CD69^{high} activated/memory phenotype (66). In addition, in both humans and mice, type II NKT-T_{FH} populations have recently been described that recognized β -GlcCer or β -GlcSph (57). The human type II NKT-T_{FH} population utilized V α 24⁻/V β 11⁻ TCRs with diverse V β chains and displayed a naïve CD45RA⁺, CD45RO⁻, CD62^{high}, CD69^{-low} phenotype. The majority of these cells expressed a T_{FH}-like phenotype in mice and humans (CXCR5⁺, PD-1^{high}, ICOS^{high}, Bcl6^{high}, FoxP3⁻, IL-21⁺) at steady state and mainly secreted IL-5, IL-6, IL-10, and IL-17 following activation. Their T_{FH} properties were associated with the induction of GC B cells and lipid-specific antibodies *in vivo* in a CD1d-dependent manner.

In humans, CD1d-restricted type II NKT cells appear to be much more frequent than type I NKT cells. In human bone marrow, approximately 25% of CD3⁺ T cells expressed CD161 and half of the CD161⁺CD3⁺ cells recognized CD1d. Interestingly, the majority of these CD1d-restricted T cells used V α 24⁻/V β 11⁻ TCRs (67). In PBMC of healthy individuals, approximately 0.5% of CD3⁺ lymphocytes stained with β -GlcCer/CD1d tetramers, similar to numbers in Gaucher's disease patients, whereas 1–2% of CD3⁺ lymphocytes in these patients stained positive with β -GlcSph/CD1d tetramers, compared to 0.2% in healthy individuals (57). In myeloma patients, lysophosphatidylcholine (LPC)-loaded CD1d dimers stained on average 0.6% of T cells in PBMC, several fold higher than type I NKT cell numbers determined with α -GalCer-loaded CD1d dimers (68), whereas in healthy controls, both LPC- and α -GalCer-loaded CD1d dimers stained approximately 0.05% of PBMC.

Thus, type II NKT cells can be distinguished from type I NKT cells by their use of more diverse TCRs and their distinct antigen specificities. Many type II NKT cells are phenotypically and functionally similar to type I NKT cells, however, some type II NKT cells appear to have a naïve T cell phenotype and further studies are required to test if these cells are capable to

form antigen-specific memory responses. In addition, similar to type I NKT cells, type II NKT cells may also harbor functionally distinct subsets, and it will be important to determine to what extent functional differentiation correlates with antigen specificities.

CD1d-Restricted $\gamma\delta$ and $\delta/\alpha\beta$ T Cells

In addition to the use of $\alpha\beta$ TCRs, CD1d-restricted T cells expressing $\gamma\delta$ TCRs have recently been described in both mice and humans (Table 1). According to their V δ -chain expression, human $\gamma\delta$ T cells can be divided into two major populations: V82⁺ and "non-V82" subsets, the latter comprise V81⁺ $\gamma\delta$ T cells and the less prevalent V83⁺ $\gamma\delta$ T cells (69, 70). V81⁺ $\gamma\delta$ T cells are mainly tissue-resident and are found in the skin and at mucosal surfaces, whereas V82⁺ $\gamma\delta$ T cells are predominant in human blood. Compared with $\alpha\beta$ T cells, the types of antigens recognized by $\gamma\delta$ T cells and the role and function of antigen presentation in $\gamma\delta$ TCR recognition are much less clear. Interestingly, some $\gamma\delta$ T cells have recently been found to directly recognize CD1d-presented lipid antigens (71). Indeed, Spinazzo and colleagues showed that only $\gamma\delta$ T cells, but not $\alpha\beta$ T cells, from peripheral blood and nasal mucosa of cypress pollen-sensitive subjects were activated in a CD1d-dependent manner by phospholipids extracted from pollen grains (72–74). Up to now thought of as type I NKT cell-specific ligands, α -GalCer and some of its derivatives including OCH were recognized by a subset of circulating human V81⁺ $\gamma\delta$ T cells (75). Similarly, sulfatide-reactive CD1d-restricted V81⁺ $\gamma\delta$ T cells were found in human blood and among gut T cells (76, 77). In addition, V83⁺ $\gamma\delta$ T cells found in the liver of patients with leukemia or chronic viral infection recognized CD1d molecules and killed CD1d⁺ cells (78). In mice, development of myocarditis following infection with coxsackievirus B3 (CVB3) relied on CD1d up-regulation and CD1d-dependent activation of V γ 4⁺ $\gamma\delta$ T cells (79). Furthermore, α -GalCer reactive, CD1d-restricted T cells using TCRs in which the V81 gene is fused to J α and C α domains that are paired with an array of TCR β -chains to form a $\delta/\alpha\beta$ TCR have recently been described (80). Thus, lipid-specific $\gamma\delta$ and $\delta/\alpha\beta$ TCRs expand the TCR diversity among CD1d-restricted T cells. Understanding the full range of antigens that are recognized by CD1d-restricted T cells that use TCR γ/δ genes, the extent of permissive TCR diversity and the numbers of these cells under physiologic conditions and during infection and other pathologies constitute important areas for future exploration.

Mechanisms of CD1d-Restricted T Cell Activation

In contrast to the high degree of TCR diversity and antigen specificity of adaptive MHC-restricted T cells, CD1d-restricted T cells utilize a more restricted TCR repertoire and recognize antigens presented by a monomorphic antigen-presenting molecule. Yet, these cells are able to respond to highly diverse infectious agents and become activated in a variety of non-infectious pathological conditions. Recent progress has started to shed light on how the self- and exogenous lipid antigens that are presented by CD1d, the molecular underpinnings of TCR-mediated recognition of

lipid/CD1d complexes, the antigen-presenting pathways that result in formation of stimulatory lipid/CD1d complexes, and the non-TCR signals all contribute to the activation of type II NKT cells and other non-invariant CD1d-restricted T cell populations.

CD1d-Presented Ligands

α -galactosylceramide and numerous related derivatives have helped to define the range of antigen specificity among type I NKT cells and provide powerful agents for their pharmacologic stimulation with various functional outcomes (Figure 1). Furthermore, several α -linked GSLs and diacylglycerols that stimulate type I NKT cells have been isolated from various microbes, confirming that α -linked GSLs are indeed important natural antigens for type I NKT cells. In addition, an intensive search for the self-antigens that are critical for type I NKT cell development has revealed several candidates, including iGb3, β - and α -linked GSLs, and various phospholipids. All of these antigens have been the topic of excellent recent reviews and will not be discussed in detail here (81–84). Similarly, a number of endogenous and exogenous glycerol- or ceramide-based lipid antigens that stimulate non-invariant CD1d-restricted T cells have been identified, but significant differences in antigen specificity exist between the various CD1d-restricted T cell subsets as a result of their varied TCR usage. For example, type II NKT cells display specificities for antigens that are not typically considered to be agonists for type I NKT cells, but some overlap in antigen specificity between these two NKT cell subsets is becoming apparent. In contrast, only subtle differences in antigen specificity have been observed between type I NKT cells and the non-canonical V α 24 $^{-}$ NKT cells and V α 10 $^{+}$ in humans and mice, respectively. Interestingly, CD1d-restricted T cells with γ/δ TCRs have been identified that recognize α -GalCer or sulfatide, antigens that were thought to be specific for type I and type II NKT cells, respectively. Thus, antigen specificity alone is not a reliable criterion to define CD1d-restricted T cell subsets (Table 1; Figure 2).

Self-Ligands

Type II NKT cells were originally identified based on their autoreactivity to CD1d-expressing cells and subsequently individual stimulatory self-lipid antigens were identified. In addition, numerous studies have now demonstrated the activation of non-invariant CD1d-restricted T cells in diseases where foreign lipids are not present, such as autoimmunity, cancer, viral infection, or in response to toll-like receptor agonists, suggesting that the recognition of self-lipids is critical for their peripheral activation.

Sulfatide ($\text{SO}_3\text{-}3\text{Gal}\beta 1\text{Cer}$) is a self-GSL recognized by a subset of type II NKT cells that predominantly express V α 3/V α 1 and V β 8.1/V β 3.1 TCRs (Figure 1) (50, 55). Native sulfatide is a mixture of multiple sulfatide isoforms with different lengths and saturation of their fatty acid chains, and is found in many organs (brain, kidney, pancreatic β -cells) and tumor cells. A major component of the myelin sheets of the central nervous system, the mono-unsaturated C24:1 sulfatide isoform activates XV19 type II NKT cells in a CD1d-dependant manner, unlike its saturated

(C24:0) or shortened (C18:1) isoforms. Although found in the nervous system in only minor quantities, the lysosulfatide (LSF) that lacks the entire fatty acid chain, is the most potent sulfatide isoform described (85). However, the XV19 type II NKT hybridoma exhibits high autoreactivity toward splenocytes from CST $^{-/-}$ mice that lack cerebroside sulfotransferase and therefore cannot generate sulfatide, and sulfatide-reactive NKT cells were still detected in CST $^{-/-}$ mice (55), indicating that sulfatide is not required for type II NKT cell development. Administration of sulfatide to mice has widely been used to study the function of sulfatide-specific type II NKT cells *in vivo*. In humans, the majority of T cells in PBMC that stained with sulfatide-loaded CD1d tetramers were $\gamma\delta$ T cells that use a semi-invariant V $\delta 1^{+}$ $\gamma\delta$ TCR (76).

Another β -linked GSL, β -glucosylceramide (β -GlcCer), has recently been shown to activate type I and type II NKT cells (Figure 1). Stimulation of APC by diverse TLR agonists can modify the GSL biosynthesis pathway by enhancing the expression of several glycosyltransferases and, as a consequence, the accumulation of endogenous GSL, including β -GlcCer, that are then presented by CD1d (86, 87). However, two recent studies have questioned the role of β -GlcCer in the activation of type I NKT cells. The studies attributed the stimulatory activity of synthetic or mammalian β -GlcCer to co-purified α -GlcCer and suggest that mammalian immune cells produce constitutively small quantities of stimulatory α -glycosylceramides under control of catabolic enzymes of the ceramide and glycolipid pathway (88, 89). For type II NKT cells, the β -GlcCer-22:0 isoform and its deacylated product, glucosylsphingosine or β -GlcSph, have been shown to activate type II NKT subsets in humans and mice and CD1d tetramers loaded with these antigens can be used to stain these cells (57). In another example of induced self-lipid-mediated activation, Zhao and co-workers showed that type II NKT cells in the J α 18-deficient IL-4 reporter mouse were activated in response to CpG ODN, a potent synthetic agonist of TLR9, which mimics a hallmark of microbial DNA (54). Similar to what has been observed for type I NKT cells (86), type II NKT cell activation by CpG ODN resulted in production of IFN- γ but not IL-4 or IL-13, and was partially CD1d-dependent. Type II NKT cells isolated from J α 18-deficient IL-4 reporter mice recognized β -GlcCer but not sulfatide or phospholipids. However, the lipids responsible for the activation of type II NKT cells following CpG ODN stimulation were not identified. Several type II NKT hybridomas (i.e., XV19, VIII24, VII57) were unresponsive to α -glycosylceramides such as α -GlcCer (90) and autoreactivity of the XV19 type II NKT cell hybridoma persisted with use of β -GlcCer- or GSL-deficient cell lines, suggesting that glycolipids are not the only self-lipids responsible for the autoreactivity of type II NKT cells (91).

Mammalian phospholipids are a major component of cell membranes and have been identified as natural antigens that are recognized by CD1d-restricted NKT cells. Mouse CD1d-restricted type I and type II NKT cell hybridomas exhibit distinct but overlapping antigen recognition specificities, particularly in response to phosphatidylinositol (PI), phosphatidylethanolamine (PE), and phosphatidylglycerol (PG) (Figure 1) (62). Mammalian phospholipids differ from their

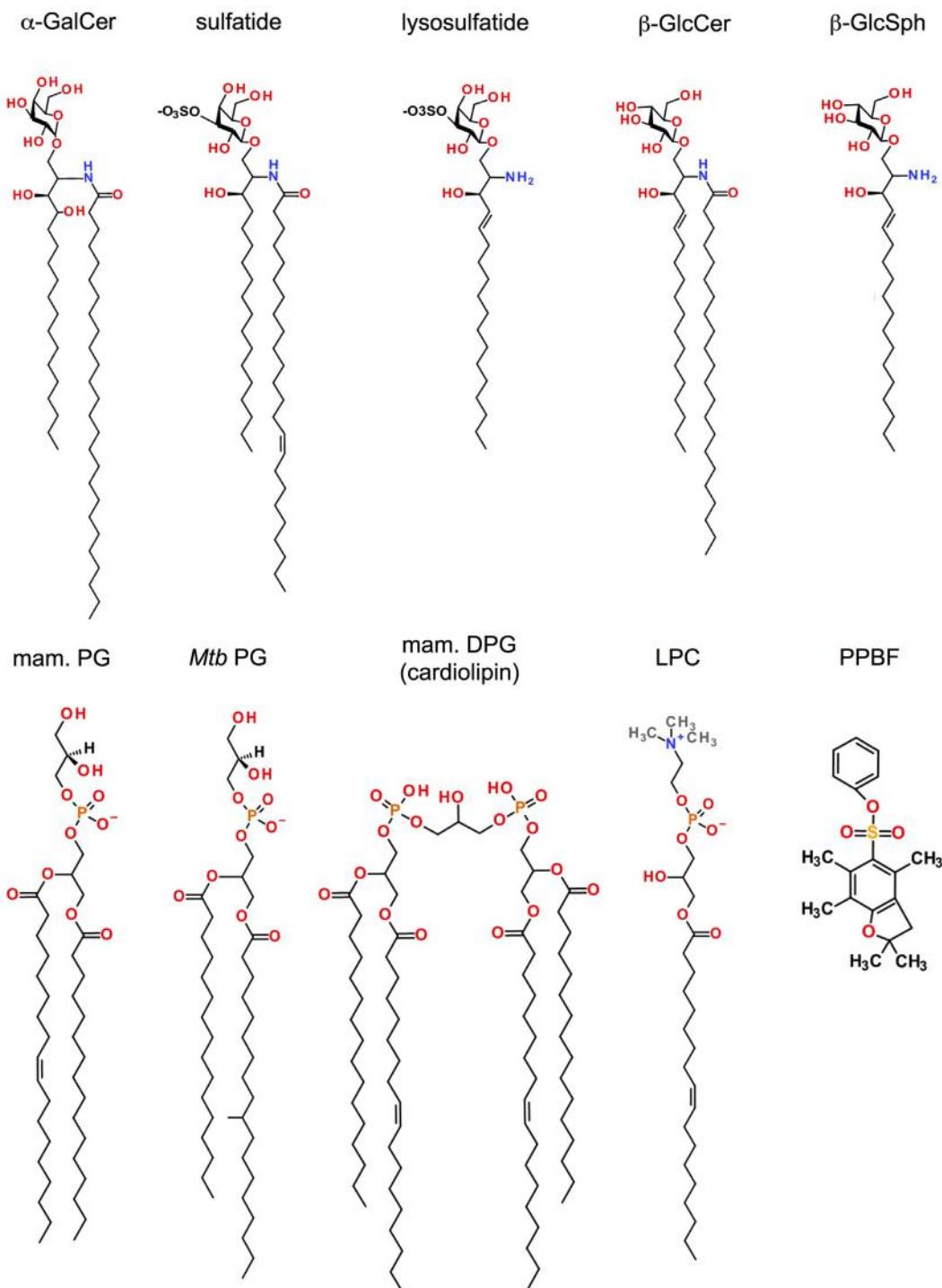
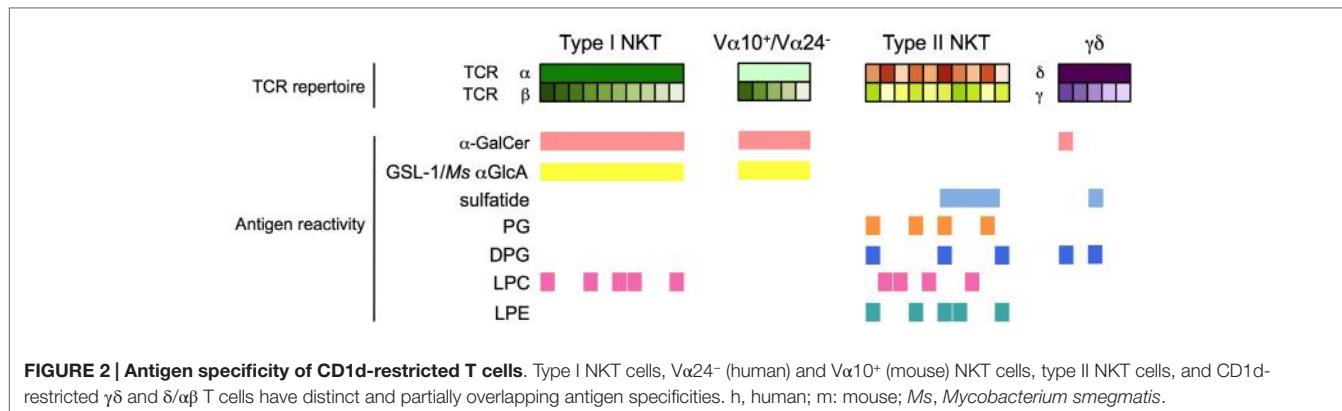


FIGURE 1 | Antigens recognized by CD1d-restricted T cells. Shown are the structures of lipid antigens for type I and/or type II NKT cells: the prototypical type I NKT cell glycolipid α -galactosylceramide (α -GalCer); the lipid self-antigen sulfatide (type II NKT cells); the mammalian phosphatidylglycerol (PG; type I and type II NKT cells) and its microbial counterparts from *Corynebacterium glutamicum* (Cg) or *Mycobacterium*

tuberculosis (Mtb) (type II NKT cells); the mammalian diphosphatidylglycerol (DPG or cardiolipin) whose Mtb counterpart shares the same alkyl chains as Mtb PG (Type II NKT cells); lysophosphatidylethanolamine (lysoPE; type I and type II NKT cells), lysophosphatidylcholine (LyoPC; type I and type II NKT cells), and phenyl 2,2,4,6,7-pentamethylidihydrobenzofuran-5-sulfonate (PPBF, type II NKT cells).



microbial counterparts in the fatty acyl chain composition or the position of the fatty acyl molecules at the *sn*-1 and *sn*-2 positions of the glycerol backbone. Such modifications of the lipid tails can considerably impact type I NKT cell activation (92). Surprisingly, C16:0/18:1 PG isolated from murine skin, bovine DPG mainly composed of (C18:2/C:18:2)₂ species and their respective synthetic version have high potency to activate the 14S.10 type II NKT cell hybridoma in a CD1d-dependent manner (58), suggesting that both self- and microbial phospholipids can be recognized by these T cells.

Lysophospholipids are produced after phospholipid hydrolysis by a phospholipase and act as lipid messengers in many physiological processes. These lipids are found in high concentration at inflammatory sites, suggesting a role in the etiology of disorders such as autoimmune diseases, obesity, atherosclerosis, and cancer. Markedly up-regulated in myeloma patients, LPC species were recognized by human type I NKT cell clones (93) and V α 24 $^{-}$ /V β 11 $^{-}$ type II NKT cells (Figure 1) (68). Chang et al. also reported that human LPC-reactive type II NKT cells predominantly secreted IL-13 in response to LPC, suggesting an immunosuppressive function of these cells (68). LPC isoforms C18:0 and C16:0 were the most potent to activate sulfatide-reactive type II NKT cells compared to the C24:0 isoform (94). Notably, LPC is not recognized by murine type II NKT cells. Similar to LPC, lysosphingomyelin (LSM), which displays the same head group, choline, activated both type I and XV19 type II NKT cells (93, 94). Recently, Zeissig and co-workers showed that lysophosphatidylethanolamine (LPE), which accumulated during hepatitis B virus (HBV) infection, activated type II NKT cells from J α 18-deficient IL-4 reporter mice but not type I NKT cells (53). This activation could be induced by different LPE isoforms, including C16:0, C18:0, and C18:1. Thus, activation of CD1d-restricted T cells in inflammatory contexts where exogenous microbial lipid antigens are not available can result from recognition of inducible self-lipids.

Thus, several self-lipids are recognized by subsets of type II NKT cells and recognition of these lipids is important for type II NKT cell function. Indeed, during microbial infection and non-infectious inflammatory conditions, up-regulation and presentation of self-lipids or stimulatory lyso forms converts innate danger signals into activation of type II NKT cells.

Microbial and Other Exogenous Ligands

Until recently, no microbial antigen had been identified that could be directly recognized by non-iNKT cells. Based on the ability of human CD1b molecules to present lipid antigens from *Mycobacterium tuberculosis* (*Mtb*) to diverse CD1b-restricted T cells (7) and due to the similarities between CD1b and CD1d intracellular trafficking and localization in humans (95), we investigated *Mtb* lipids for antigens that may be recognized by type II NKT cells. Purification, isolation and structural analysis of *Mtb* polar lipids revealed PG species with different acyl chain combinations (including C19:0/C16:0, C19:0/C16:1, C18:1/C16:0, and C18:1/C16:1) to stimulate several type II NKT cell hybridomas (14S.6, 14S.10, TBA7, VII68, and XV19) in a CD1d-dependent manner (Figure 1) (93). Moreover, PG, diphosphatidylglycerol (DPG or cardiolipin) and PI from *Corynebacterium glutamicum* (*Cg*), with C18:1/C16:0, (C18:1/C16:1)₂, and C18:1/C16:0 fatty acid composition, respectively, also activated type II NKT cells, in particular 14S.6, 14S.10, and TBA7 hybridoma cells. In addition, *Mtb* and *Cg* apolar lipids activated type II NKT cell clones in a CD1d-dependent manner, but specific stimulatory lipid species have not yet been identified from this lipid fraction. Similarly, Wolf and colleagues recently demonstrated that PG and DPG isolated from *Listeria monocytogenes* (*Lm*) were also potent ligands for type II NKT clones (TBA7 and 14S.10 especially) (59). Interestingly, whereas in both studies neither PG nor DPG from *Mtb*, *Cg*, and *Lm* succeeded in activating type I NKT cell hybridomas, DPG can be recognized by a subset of CD1d-restricted $\gamma\delta$ T cells. Indeed, hepatic and splenic $\gamma\delta$ T cells secreted IFN- γ and RANTES (CCL5) in response to DPG in a CD1d-dependent manner (74).

Phospholipids are also found in plant, especially in pollen, which are an important source of environmental allergens. Extracted from cypress grains, phospholipids, as PC in particular, stimulated non-V α 24 NKT and $\gamma\delta$ T cells from cypress pollen-sensitive subjects in a CD1d-restricted fashion (72, 73, 96). However, the structure of the acyl chain combination of the stimulatory PC from pollen has not been defined. Interestingly, non-lipidic small molecules can also be associated with CD1d proteins with low affinity. These sulfur-containing molecules, including phenyl 2,2,4,6,7-pentamethylidihydrobenzofuran-5-sulfonate (PPBF) and structurally related compounds (Figure 1), activated human non-V α 24 $^{-}$ CD1d-restricted T cells that expressed a V α 2/

V β 21 TCR (97). Thus, in addition to the prominent recognition of self-lipid antigens, non-invariant CD1d-restricted T cells also recognize a range of microbial and exogenous lipids presented by CD1d.

CD1d/Antigen Recognition

Considering the differences in TCR usage and antigen specificities among the different CD1d-restricted T cell populations, unraveling the molecular mechanisms by which their TCRs recognize CD1d/antigen complexes is central to understanding their functions. Indeed, recent structural analyses have brought to light key differences in antigen/CD1d recognition by the TCRs of type I NKT cells, V α 24 $^{-}$ /V α 10 $^{+}$ NKT cells, type II NKT cells, and $\gamma\delta$ T cells.

The invariant type I NKT cell TCR docks over the F' pocket of CD1d in an orthogonal conformation (Figures 3A,B) (98, 99). Only the α -chain, via the complementary-determining region 1 α (CDR1 α) and CDR3 α , is in contact with the head group of α -linked glycolipids such as α -GalCer, and thus has an important role in specificity for, and recognition of, glycolipids. CDR3 β and CDR2 β loops stabilize the complex by interacting with the CD1d molecule. Variations in TCR β -chain usage cause subtle structural modifications in the conformation of the TCR α -chain, which indirectly contribute to the preferential recognition of some antigens or modulate the affinity for antigen/CD1d complexes (99–101). For the recognition of β -linked ligands, type I NKT TCRs operate through induced-fit molecular mimicry by pushing the β -linked headgroup into a flattened position that is similar to that of the headgroup of stimulatory α -linked lipids (102–104). Therefore, the mode of antigen/CD1d recognition by type I NKT TCRs has been compared to the function of pattern-recognition receptors that interact in an innate-like and conserved manner with ligands that represent microbe-associated molecular patterns or endogenous stress-induced ligands (105, 106).

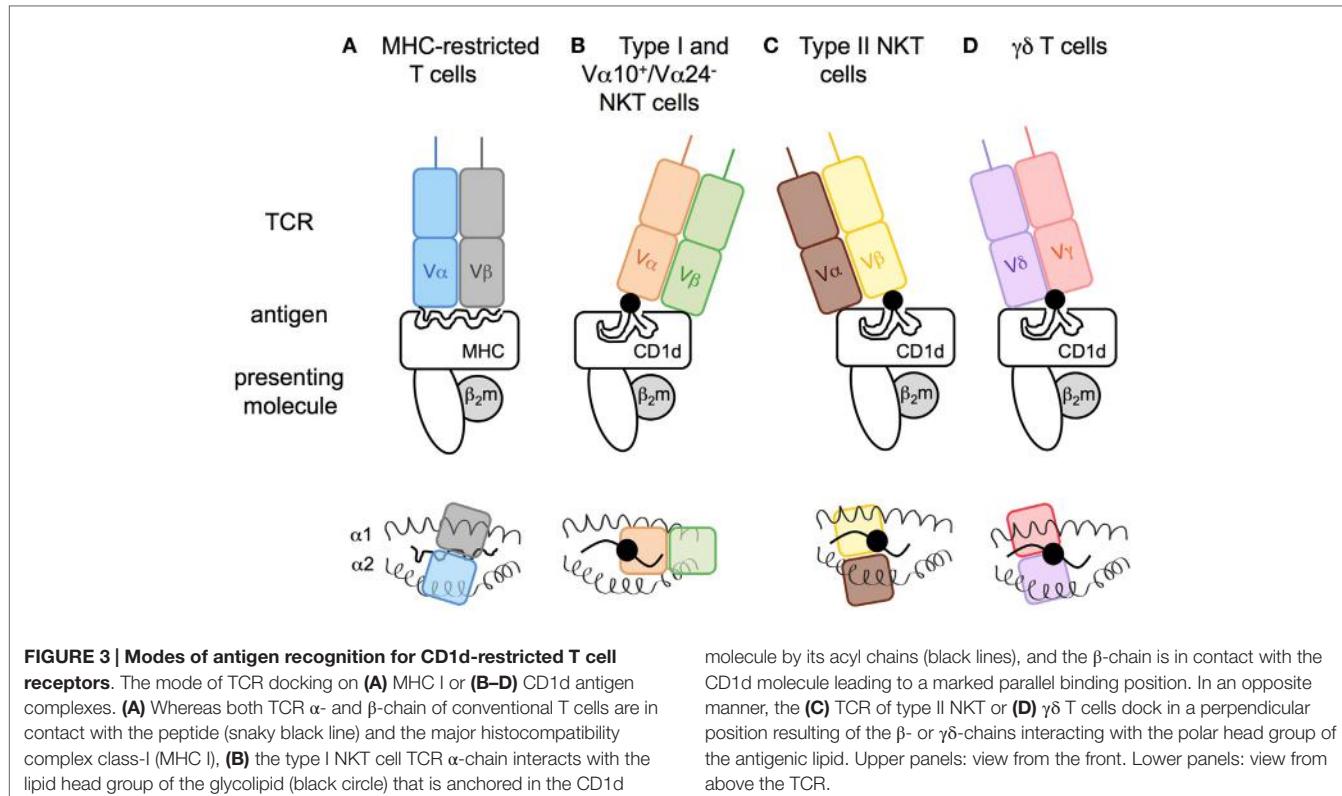
The V α 24 $^{-}$ and V α 10 $^{+}$ NKT TCRs from humans and mice, respectively, display very similar conformation compared with type I NKT TCRs in complex with α -GalCer/CD1d, except that the V α 10 $^{+}$ CDR2 α loop is also in contact with the antigen (46) and that the V α 24 $^{-}$ CDR1 α loop interacts with the galactose headgroup highly contributing to the TCR fine specificity for the antigen (44).

In contrast, structural analysis of the tri-molecular sulfatide/CD1d/XV19 TCR complex showed that this type II NKT TCR docks above the extreme end of the A' pocket and adopts a parallel mode vis-a-vis of the CD1d molecule, similar to the interaction of conventional T cell with MHC molecules (Figures 3A,C). Both TCR α - and β -chains interact with CD1d and CDR1 β and CDR3 β loops confer recognition and discrimination for the antigen (107, 108). Unlike the recognition of β -linked glycolipids by the type I NKT TCR, the type II NKT TCR does not flatten the sulfatide headgroup during ligation. Thus, differences at the TCR-CD1d/antigen interfaces between type I and type II NKT cells provide insights into the molecular basis for the different ligand specificities and highlight how altered TCR use results in a differing docking solution on a monomorphic antigen-presenting molecule. The extent to which the TCR diversity of type II NKT cells results in different docking solutions for distinct TCRs remains to be determined.

Similar to type II NKT TCRs, the TCRs of CD1d-restricted $\gamma\delta$ T cells dock above the A' pocket and all CDR δ loops (CDR1 δ , CDR2 δ , CDR3 δ) are in contact with CD1d (Figure 3D). Notably, the CDR3 δ loop also contacts the antigen. Whereas sulfatide recognition requires a central position of the δ -chain above the lipid portal with no contribution of the TCR γ -chain (77), the interaction with α -GalCer involves γ -chain/CD1d contacts and a docking mode closer to the extreme end of the A' pocket (75). Thus, $\gamma\delta$ T cells can utilize a variety of interactions with their TCR γ - and δ -chains to recognize lipid/CD1d complexes.

CD1d Antigen Presentation

In humans, the CD1 family is composed of five isoforms divided into two groups: CD1a, CD1b, CD1c, and CD1e forming group 1 and CD1d forming group 2. In mice, only the CD1d isoform is expressed. CD1 molecules are transmembrane glycoproteins that display structural similarities to MHC class-I: one heavy chain composed of three domains (α 1, α 2, and α 3) non-covalently linked to β 2-microglobulin via the α 3 domain. The CD1d ligand-binding site is composed of α 1 and α 2 helices, which form two deep hydrophobic pockets, called A' and F'. The pockets accommodate hydrocarbon chains of glycolipid antigens leading to the protrusion of the lipid head group on the surface and thus its accessibility to the TCR of CD1d-restricted T cells (109, 110). The α 3 domain connects the ligand-binding region to a transmembrane domain, followed by a short cytoplasmic tail. The cytoplasmic tail of human and mouse CD1d is critical for its intracellular localization as deletion of a tyrosine-based motif encoded in it interrupts CD1d recycling between the plasma membrane and endolysosomal compartments (Figure 4) (111, 112). Moreover, the internalization of murine CD1d and its subsequent lysosomal localization require the binding of the adaptor protein complex AP-3 to the tyrosine-based motif (113, 114). Interestingly, human CD1d fails to bind the AP-3 complex, unlike human CD1b (113). In humans, CD1d trafficking from ER to cell surface and lysosomes is orchestrated by its physical interaction with MHC II and invariant chain (115, 116). By extension from its role in CD1d trafficking, the tyrosine-based motif in the cytoplasmic tail of CD1d is also critical for type I NKT cell function. Indeed, its deletion impaired type I NKT cell development and activation by endogenous and exogenous antigens such as α -GalCer (60, 112, 117). In contrast, CD1d with a truncated cytoplasmic tail is still capable of activating type II NKT cells and does not impair their development, suggesting that loading of type II NKT cell self-antigens onto CD1d occurs within the ER (60, 111, 112). Recently, Shin and co-workers have shown that three consecutive arginine residues between the transmembrane region and the cytoplasmic tail are involved in the intracellular trafficking of CD1d and the presentation of endogenous glycolipids to both type I and type II NKT cells (118). Selection and editing of CD1d-bound lipids is also influenced by accessory lipid-binding and -loading molecules such as the microsomal triglyceride transfer protein (MTP) and saposins that facilitate the formation of lipid/CD1d complexes in the ER and throughout the endocytic pathway, respectively (Figure 4). The ability of CD1d molecules to differentially stimulate type I versus type II NKT cells based on their distinct intracellular trafficking routes is associated with



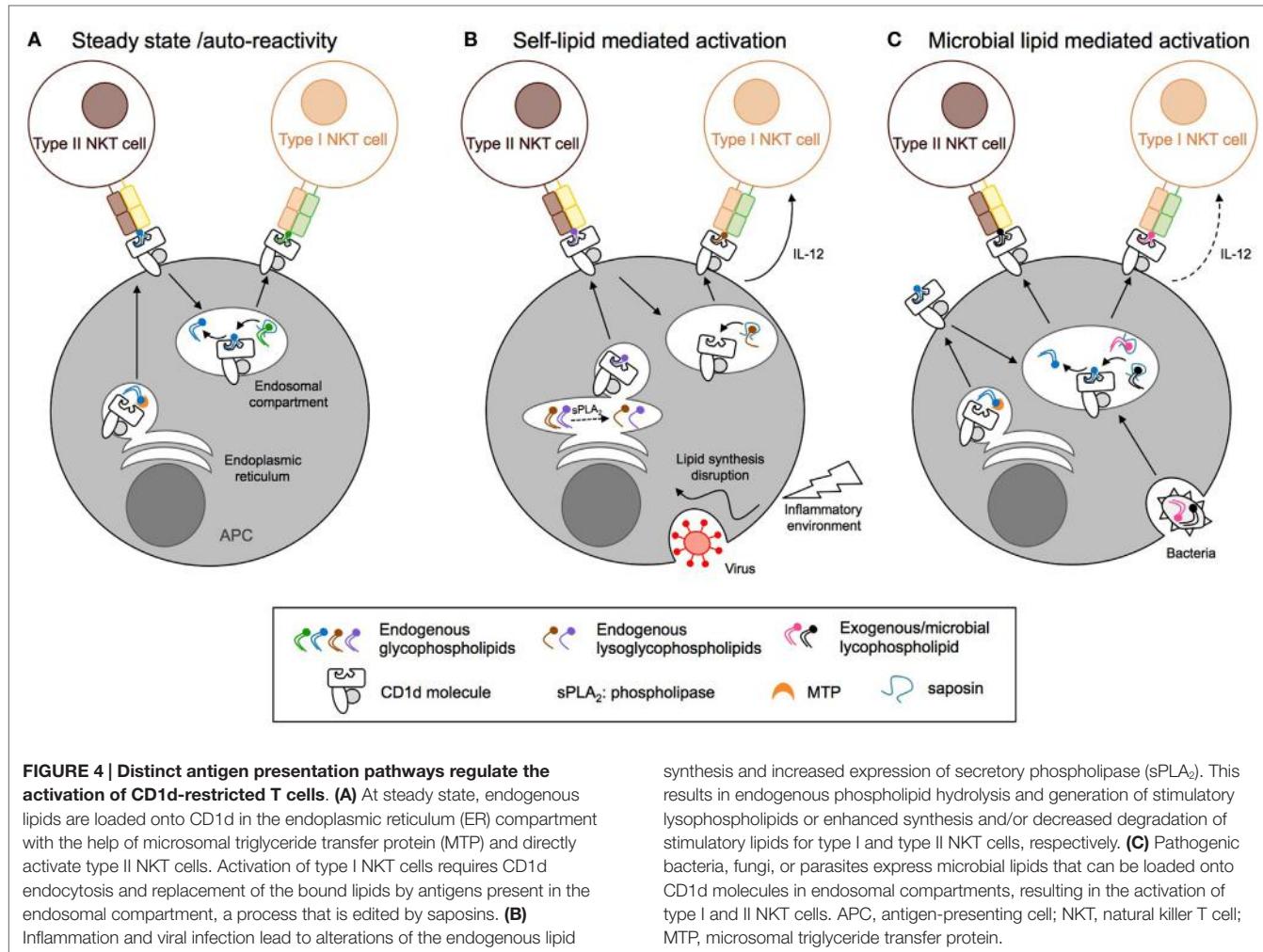
loading of distinct self-lipids onto CD1d. Indeed, using lipid elution and mass spectrometry, Yuan et al. demonstrated that ER-retained CD1d molecules associated with phosphatidylcholine (PC), whereas CD1d molecules that trafficked through the secretory pathway were loaded with sphingomyelin for which terminal synthesis occurs within the Golgi apparatus (119). Interestingly, recycling CD1d molecules carried a combination of both sphingomyelin and PC but also lysophospholipids that result from lipid degradation by phospholipase in lysosomes. In contrast to the presentation of endogenous lipid antigens, activation of type II NKT cells by exogenous self- or microbial lipids requires cellular lipid uptake and intersection of lipid loading and intracellular CD1d trafficking in secretory and/or lysosomal compartments (93, 120). Thus, different intracellular antigen presentation pathways result in formation of stimulatory lipid/CD1d complexes for type I and type II NKT cells.

Cytokine- and NK Receptor-Mediated Activation

Activation of naïve MHC-restricted T cells is controlled by TCR signals that result from highly specific recognition of peptide antigen/MHC complexes in combination with receptor-mediated co-stimulatory signals, whereas the immediate cytokine milieu is critical to guide the differentiation of T cell effector functions. In contrast, innate T cells such as type I NKT cells have adopted a strategy for their activation that integrates TCR-mediated signaling and stimulation by pro-inflammatory cytokines to result in rapid activation (16). Dependent on the context and on the affinity of the CD1d-presented lipid antigens, one signal prevails over the other. For example, a high-affinity lipid antigen such as

a potent induced self-lipid or microbial lipid can result in strong and predominantly TCR-mediated activation with no or only limited need for cytokine stimulation. In contrast, presentation of low-affinity microbial or self-antigens requires stimulation with antigen-presenting cell-derived inflammatory cytokines to result in overt NKT cell activation. This cytokine-mediated stimulation can be so strong that it alone results in overt activation, obviating the need for TCR-mediated stimulation altogether. This mechanism ensures activation of type I NKT cells early in an ensuing inflammatory response during infection even when no microbial CD1d-presented lipids are expressed, as is the case, for example, during viral infections, and similar mechanisms may explain type I NKT cell activation in tumor immunity and autoimmune diseases (121, 122). At steady state, type I NKT cells highly express a wide range of cytokine receptors and have therefore the ability to respond to multiple cytokines like IL-12 (123, 124), IL-18 (125), and IL-23 alone or in combination with IL-1 β (126, 127), IL-25 (30), and IFN type 1 (86). Depending on the cytokines present in the inflammatory environment, type I NKT cells secrete different cytokines such as IFN- γ in response to IL-12, IL-18 or IFN type 1, IL-17 with IL-23, and IL-22 with IL-23/IL-1 β in combination.

Rolf and co-workers have compared by gene expression profiling the expression of cytokine receptors between type I NKT cells and type II NKT cells from 24 $\alpha\beta$ TCR transgenic mice (128). Interestingly, whereas *IL-18r1*, *IL-18rap* (IL-18 receptor associated protein), and *IL2ra* (CD122) were expressed at high and similar levels in both subsets, type I NKT cells present a higher expression of *IL12rb1* (up to 3-fold) and *IL2ra* (CD25) (up to 11-fold). Compared to conventional naïve CD4 $^{+}$



T cells, both type I and type II NKT cells displayed low levels of *IL1r2*, *IL6ra*, and *Ifngr2*. Recently, IL-25 has been shown to activate type II NKT cells *in vivo* and promote their production of IL-13 through IL17RB (129, 130). This suggests that, similar to type I NKT cells, type II NKT cells can respond to stimulation with inflammatory cytokines. Interestingly, during HBV infection activation of type II NKT cells was primarily driven by recognition of CD1d-presented lysophospholipids and largely independent of IL-12 signaling, whereas activation of type I NKT cells was significantly reduced in the absence of IL-12 (53). Whether indirect mechanisms also contribute to the activation of CD1d-restricted $\gamma\delta$ T cells remains to be determined.

Initially named owing to their expression of NK1.1, NKT cells express a multitude of both activating (for example, NKG2D, NK1.1, CD160, NKR-P1A, NKp46) and inhibitory (Ly49c, Ly49G2, NKG2A, 2B4) NK receptors that play an important role in their activation and regulation. For example, stimulation of NK1.1 and NKG2D was sufficient to directly activate type I NKT cells (131, 132), and NKG2D-ligand interactions were critical for type II NKT cell-mediated disease induction in a mouse model of HBV infection (133). At steady state, both NKT cell subsets

highly express *Klrk1* (NKG2D), *Klrbc1* (NK1.1), and *CD160* but only type II NKT cells display high levels of *Klra3* (Ly49c), *Klra7* (Ly49G2), and in a lesser manner *Ncr1* (NKp46). For their part, type I NKT cells express higher levels of *Klrba1* (NKR-P1A) and *Klrc1* (NKG2A) (128).

Thus, in order to overcome the limitations of restricted antigen specificity that result from limited TCR diversity and the recognition of a monomorphic antigen-presenting molecule, both type I and type II NKT cells appear able to integrate stimulatory signals provided by TCR-mediated stimulation and inflammatory cytokines with modulation by NK receptor-signaling, to ensure their rapid activation in various infectious and inflammatory contexts.

Functions of Type II NKT Cells

In contrast to the large body of literature previously mentioned that has documented a role for type I NKT cells in various pathological states, progress in understanding the role of other CD1d-restricted T cell populations has been hampered by the limited ability to track these cells and the lack of models to assess their function *in vivo*, in particular for V α 24 $^{-}$ /V α 14 $^{-}$ NKT cells

and CD1d-restricted $\gamma\delta$ or $\delta/\alpha\beta$ T cells. However, several lines of evidence suggest that type II NKT cells can contribute to and modulate a range of immune responses, occasionally in opposing roles to type I NKT cells.

Microbial Infections

Viral Infections

The first indication that type II NKT cells can contribute to protective immunity during viral infection came from studies in mice using diabetogenic encephalomyocarditis virus-induced pathology (EMCV-D) that is characterized by hind-limb paralysis and impaired glucose-tolerance resulting from virus cytopathic effects on neuronal cells and islet cells, respectively. Whereas EMCV-D infection resulted in similar disease severity in WT and $J\alpha 18^{-/-}$ mice, CD1d $^{-/-}$ mice exhibited a higher incidence and exacerbated severity of the disease, suggesting a protective role of type II NKT cells during this infection (**Figure 5**) (134). However, this protective function was not unique to type II NKT cells since activation of type I NKT cells by α -GalCer provided protection of WT mice during EMCV-D infection. An additional example of the protective potential of type II NKT cells during viral infection comes from studies in humanized SCID mice that were infected with HIV-1, in which administration of sulfatide resulted in reduced viral replication (135). Thus, natural or sulfatide-induced activation of type II NKT cells during viral infections can enhance protective innate and adaptive immune responses (**Figure 5**).

Infection with human HBV is a common cause of acute and chronic liver injury, including cirrhosis and hepatocellular carcinoma. The immune response during HBV infection plays a dual role, by eliciting tissue damage in response to viral antigens and by controlling viral replication. Mice engineered to express HBV envelope proteins in hepatocytes, or a terminally redundant HBV DNA construct that results in intrahepatic HBV replication has been used to study HBV infection in mice, and showed that type II NKT cell responses contributed to acute hepatitis and tissue injury in a CD1d- and NKG2D-dependent manner (133, 136). More recently, Zeissig and colleagues used HBV-expressing adenoviral particles to reproduce HBV infection in mice and uncovered a protective role for type II NKT cells (53). Type II NKT cell activation in response to HBV was dependent on the expression of CD1d and MTP (53) which transfers endogenous phospholipids onto CD1d (137, 138), suggesting an essential role for endogenous lipid presentation. Furthermore, as observed both in patients with viral hepatitis and HBV-infected mice, HBV infection increased the transcription of the secretory phospholipase A₂ (sPLA₂) (139) and expression of one of its substrates, PE. Interestingly, the analysis of microsomal lipids revealed an increase of LPE isoforms able to activate type II NKT cells. Type II NKT cell activation during HBV infection was predominantly based on LPE recognition, as IL-12 neutralization had little effect. By contrast, type I NKT cells did not recognize LPE but required IL-12 and MTP, suggesting that their activation occurred after type II NKT activation and APC maturation. In addition to their critical role in initiating an innate immune response against HBV in this model, type II NKT cells appear to also be involved in modulating the generation of protective adaptive immune

responses. Indeed, HBV-infected CD1d $^{-/-}$ mice (lacking both type I and type II NKT cells) displayed significantly reduced activation of CD4 and CD8 T cells, whereas $J\alpha 18^{-/-}$ mice (lacking only type I NKT cells) showed only reduced activation of CD4 T cells. This type II NKT cell-dependent reduction of adaptive CD8 $^{+}$ T cell responses during HBV infection led to a defect in viral control and resulted in chronic inflammation (53). Thus, in mouse models of HBV infection, type II NKT cells can both contribute to immune-mediated tissue damage and to the innate and adaptive immune responses that control viral replication (**Figure 5**). In humans, type II NKT cells that produced large amounts of IFN- γ , but little IL-13 or IL-4, accumulated in the liver during hepatitis C (HCV) infection, but their role during infection remains to be determined (140, 141).

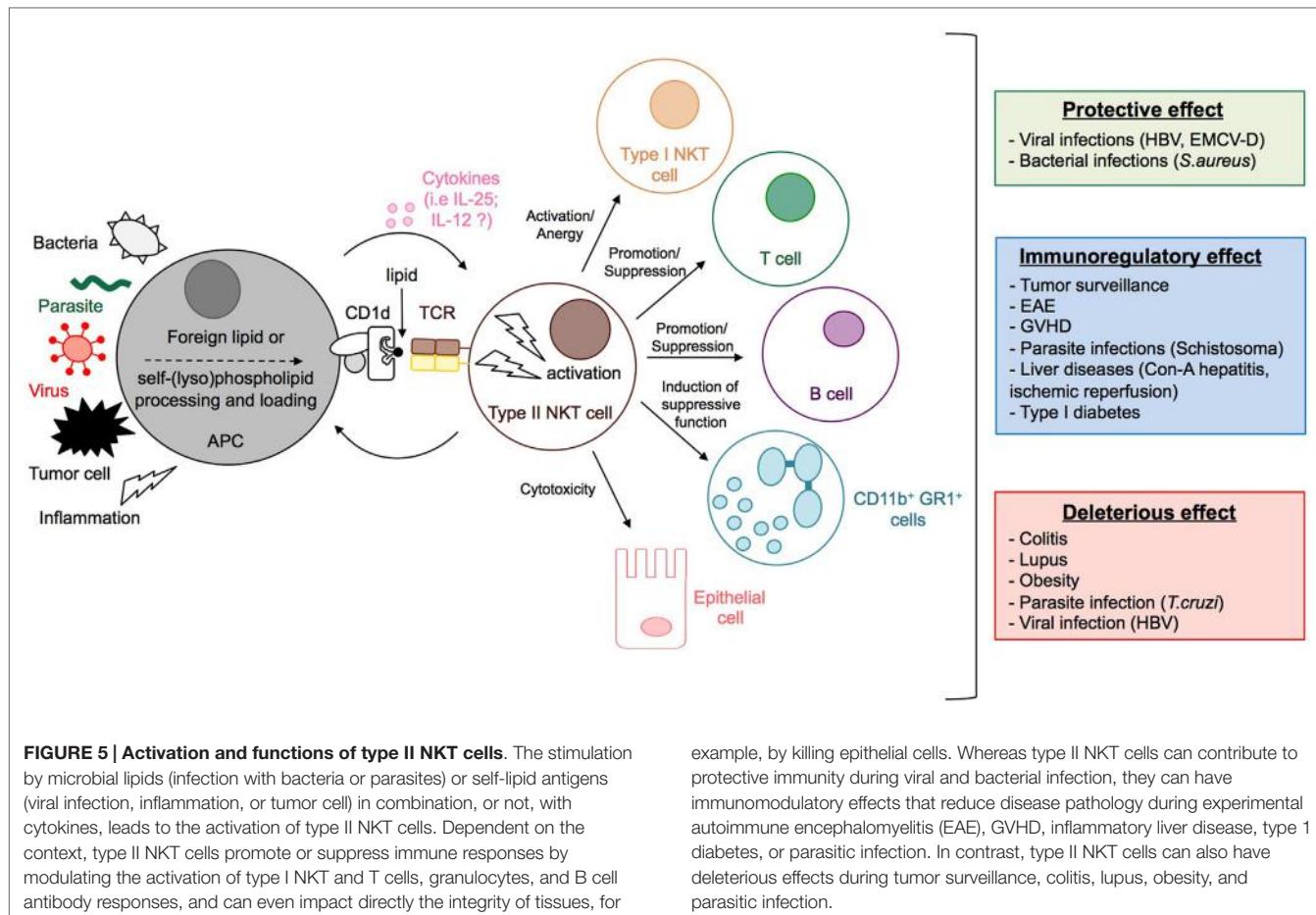
Bacterial Infections

As described above, type II NKT cells are activated in response to diverse bacterial antigens, suggesting a role for these cells in host defense against these bacterial pathogens. During sepsis, the immune response consists of a primary phase characterized by a potentially lethal cytokine burst induced by activated leukocytes, and a secondary latent phase where host defense is reduced. Despite the rapid activation of type I NKT cells (increased CD69 expression, proliferation) after *Staphylococcus aureus* inoculation, $J\alpha 18^{-/-}$, CD1d $^{-/-}$, and WT mice presented comparable rates of mortality, suggesting that neither type I nor type II NKT cells play a significant protective or deleterious role in *S. aureus* sepsis (142). However, administration of sulfatide concomitant with *S. aureus* inoculation improved the survival rate of mice (**Figure 5**). Interestingly, the protective effect of sulfatide was CD1d-dependent and type I NKT cell-independent, suggesting that activation of sulfatide-reactive type II NKT cells is essential and sufficient for this protective effect. Sulfatide treatment was accompanied by a decrease of the inflammatory cytokines TNF- α and IL-6. Thus, activated sulfatide-reactive type II NKT cells reduced the cytokine storm of the primary hyper-reactive phase during *S. aureus* sepsis while maintaining an adequate immune response to limit bacterial growth and clearance.

During *Mtb* infection, type I NKT cells produced IFN- γ through stimulation with IL-12/IL-18 and inhibited intracellular bacterial replication by their CD1d-restricted secretion of GM-CSF (143, 144). As described above, several type II NKT hybridomas were activated by *Mtb*-infected APC through the specific recognition of *Mtb* phospholipids (93), suggesting that, similar to type I NKT cells, type II NKT cells may also contribute to protective immunity during *Mtb* infection. Similarly, given the recognition of *Lm* antigens by type II NKT cells, the increased bacterial burden in CD1d $^{-/-}$ during *Lm* infection as well as the ameliorated *Lm* infection following treatment with blocking anti-CD1d antibodies suggests that type II NKT cells may contribute to protective immunity or have a regulatory function during *Lm* infection (145, 146).

Parasitic Infections

Schistosomiasis is a chronic parasitic disease caused by the extracellular parasite *Schistosoma*, and a strong Th2 response, triggered by parasite eggs, is essential for host survival.



CD1d-deficient mice developed a markedly reduced Th2 response during schistosomiasis (147) indicating an important role of CD1d-restricted T cells in the development of a protective immune response. Whereas type I and type II NKT cells were not essential during the very early phase (1 and 4 weeks) of infection, the two subsets played distinct but complementary roles during the acute phase (7 and 12 weeks) of infection (148). Indeed, type II NKT cells promoted the Th2 response as their lack induced a decrease of Th2 cytokines (IL-4, IL-5, and IL-10) and specific IgG1 production (Figure 5), a feature also described in alum-induced humoral immune response (149). On the other hand, secretion of IFN- γ by type I NKT cells contributed to the production of specific IgG2b, a marker of Th1 responses. Interestingly, schistosome egg-sensitized DC activated type I but not type II NKT cells and this activation required the presentation of self-antigen rather than parasite-derived antigens or TLR2/TLR3 engagement (148). Nonetheless, Magalhaes and co-workers have shown that administration of schistosoma-derived LPC or cercaria induced secretion of IL-5 and IL-13 (150) dependent on TLR2, and lead to the recruitment of eosinophils at the site of infection. The cellular source(s) of Th2 cytokines have not been investigated in this model, but considering that LPC is a potent type II NKT cell antigen, it is likely that these cells are activated during infection.

Natural killer T cell subsets also displayed opposing roles during infection with *Trypanosoma cruzi* (151). *T. cruzi* infection causes a chronic inflammatory disease in which the anti-*T. cruzi* immune response that controls the persistent parasites can also contribute to the inflammatory tissue damage of the myocardium and gastro-intestinal tract that causes Chagas disease. Indeed, $J\alpha 18^{-/-}$ mice were more sensitive to infection with *T. cruzi* compared to WT or CD1d $^{-/-}$ mice as indicated by greater morbidity and mortality. The increased susceptibility to infection in the absence of type I NKT cells was accompanied by an enhanced inflammatory response with infiltrates of activated cells (NK and T cells), B cells and DC in lymphoid organs and greater muscle inflammation and pro-inflammatory cytokine secretion (IFN- γ , TNF- α , nitric oxide). Moreover, the humoral response was impaired as shown by a decreased anti-*T. cruzi* IgG2a antibody titer, compared to WT and CD1d $^{-/-}$ mice. This suggests that during *T. cruzi* infection, type II NKT cells augment the inflammatory anti-parasite response, whereas type I NKT cells limit this response (Figure 5).

Together, these examples show that in a range of viral, bacterial, and parasitic infections type II NKT cells can either promote protective innate and adaptive cell-mediated immune responses, or contribute to infection-induced pathology. Understanding the mechanisms that determine these opposing

functions of type II NKT cells during infection will be important areas of future research.

Anti-Tumor Immunity

Type I NKT cells critically contribute to natural anti-tumor responses, as demonstrated by the prompt growth of spontaneous tumors in type I NKT cell-deficient $\text{J}\alpha 18^{-/-}$ mice compared to WT mice (152–154). Furthermore, the activation of type I NKT cells by α -GalCer provides potent effects against hematologic malignancies and solid tumors through their IFN- γ -production and the subsequent activation of DC and NK cells (155, 156). By contrast, sulfatide-activated type II NKT cells repress anti-tumor immunity (**Figure 5**) (157–159) by abrogating type I NKT activation in response to α -GalCer, in terms of cytokine secretion and expansion (160). Moreover, their IL-13 production, in combination with TNF- α , led to up-regulation of TGF- β secretion by myeloid-derived suppressor cells (MDSC), and resulted in decreased cytotoxic T cell activity (161). Interestingly, in contrast to their notable immunoregulatory role in anti-tumor responses, two recent studies have highlighted the ability of type II NKT cells to contribute to anti-tumor immunity in response to CpG in a B16 melanoma model (54), and by their ability to directly kill lymphoma cells *in vitro* (63). Thus, type II NKT cells can suppress anti-tumor immunity, counteracting the anti-tumor activity of type I NKT cells, but can also contribute to defense against tumor growth. Understanding the factors that determine the role of type II NKT cells in tumor immunity will be critical to harness their potential in novel anti-tumor strategies.

Autoimmunity and Tolerance

Type II NKT cells help maintain tolerance to self-antigens and thereby prevent autoimmune disease. On the other hand, they also can mediate tissue damage and play a pathogenic role in autoimmunity.

Experimental Autoimmune Encephalomyelitis

An important role for type II NKT cells has been shown in experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis. During EAE, sulfatide-mediated activation of type II NKT cells induced tolerogenic DC polarization, abrogated activation of both type I and type II NKT cells, class-II MHC effector myelin protein reactive T cells, and microglial cells and suppressed autoimmunity (**Figure 5**) (55, 162).

Type 1 Diabetes

NOD mice, which spontaneously develop type 1 diabetes, were protected from disease following sulfatide administration that led to type II NKT cell activation and secretion of IL-10 by DC, resulting in inhibition of type I NKT cells and activation and expansion of diabetogenic T cells (**Figure 5**) (163). In contrast, in another study using a different experimental approach, sulfatide failed to protect NOD mice from diabetes (164). A non-sulfatide-reactive type II NKT cell subset was also able to protect NOD mice from diabetes by regulating diabetogenic T cells through PD-1/PD-L1 and ICOS/ICOSL pathways (66, 165).

Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by antinuclear autoantibodies (IgG2a particularly) and multiorgan injury such as proteinuria and immune complex glomerulonephritis, which is dependent on IFN- γ (166, 167). Whereas α -GalCer administration exacerbated the disease by inducing a strong Th1 response, CD1d-blocking slowed down the development of the disease mediated by a decrease of IgG2a production (167). Interestingly, transfer of splenic CD4 $^{+}$, CD8 $^{+}$, or DN V α 4.4/V β 9 type II NKT cells, purified from transgenic mice generated with CD1d-reactive T cells initially derived from irradiated mice, promoted the disease by producing large amount of IFN- γ . By contrast, NKT cells derived from bone marrow and displaying a DN phenotype prevented SLE by secreting large amounts of IL-4 (166).

Colitis

Type II NKT cells exposed *in vivo* to high levels of CD1d expression could directly contribute to the spontaneous development of colitis in mice (168). Their harmful role was mediated through their IL-13 secretion induced by IL-25 and their cytotoxic activity against epithelial cells (**Figure 5**) (56, 129, 169).

Liver Diseases, GVHD, and Obesity

Halder and co-workers have shown that presentation of sulfatide by hepatic plasmacytoid DC (pDC) to type II NKT cells led to the recruitment of anergic type I NKT cells, in a IL-12- and MIP-2-dependent manner and prevented concanavalin A-induced hepatitis (170). The same group has also shown that type II NKT cells neutralized the pathogenic role of type I NKT cell during hepatic ischemic reperfusion disease (50) and alcoholic liver disease (94). Indeed, activation of type II NKT cells after sulfatide administration suppressed IFN- γ production by hepatic type I NKT cells and inhibited the recruitment of myeloid cells in the liver that have been shown to enhance injury (**Figure 5**) (171).

In bone-marrow (BM) transfer, both host-residual and donor-derived NKT cells exert protective functions that are particularly well described in graft-versus-host disease (GVHD). Whereas protection by host-residual T cells was provided by the type I NKT subset, type II NKT cells played critical roles in donor-derived protective effects (172–175). Indeed, on the one hand, adoptive transfer of type I NKT cells or the administration of α -GalCer attenuated GVHD in recipient mice due to the vigorous secretion of IL-4 by type I NKT cells and the subsequent Th2 polarization of the immune response (172–175). On the other hand, donor BM type II NKT cells produced IL-4, like type I NKT cells, but also IFN- γ which induced apoptosis of donor CD4 $^{+}$ and CD8 $^{+}$ T cells in a Fas-dependent manner (174, 176). Moreover, Exley and co-workers have shown that human CD161 $^{+}$ CD1d-reactive BM-derived non-invariant T cells specifically suppress mixed-lymphocytes reaction (MLR) and could induce tolerance to allografts (67).

In obesity, activation of type II NKT cells by lipid excess initiated adipose tissue and liver inflammation leading to obesity (177). By contrast, Hams and colleagues found that type II NKT cells activated by IL-25 regulated weight and glucose homeostasis

by recruiting to visceral adipose tissue eosinophils and activated macrophages that are found in lean individuals (130).

Thus, in a context-dependent manner, type II NKT cells provide protective or deleterious effects in a number of diverse diseases including microbial infection, anti-tumor immunity, and autoimmunity (**Figure 5**), suggesting that these cells may constitute promising therapeutic targets for a broad range of diseases.

Summary and Future Directions

Significant progress has been made in defining the diversity among CD1d-restricted T cells that, in addition to the widely studied type I NKT cells, include α -GalCer-reactive NKT cells that use non-canonical TCRs, type II NKT cells that use a more diverse repertoire of $\alpha\beta$ TCRs, as well as T cells that use $\gamma\delta$ or $\delta/\alpha\beta$ TCRs. Critical differences distinguish the members of the CD1d-restricted T cell family beyond their use of different TCRs, including their antigen specificities and modes of antigen/CD1d recognition. A major hurdle in unraveling the biology of the CD1d-restricted T cell subsets that do not recognize α -GalCer is the difficulty to reliably detect and analyze these cells in humans and mice *ex vivo* and *in vivo*. Discovery of new self- and foreign antigens in combination with strategies and tools to directly detect these cells such as antigen-loaded CD1d tetramers, and availability of suitable small animal models will be necessary to characterize the entire repertoire and to determine the degree of diversity versus oligoclonality among all CD1d-restricted T cell subsets. Furthermore, it is currently unknown to what extent the differences in TCR usage and antigen specificity of these subsets correlate with phenotypic attributes or immunologic functions that are distinct from those of type I NKT cells. For example, in anti-tumor immunity and in response to microbial infection, type II NKT cells have been shown to have opposing functions to those of type I NKT cells. In addition, while many non-invariant CD1d-restricted T cells appear to display a type I NKT cell-like activated/memory phenotype, several studies have indicated that CD1d-restricted T cells exist that are more similar to conventional MHC-restricted T cells. Importantly, it remains to be determined if truly naïve CD1d-restricted T cells exist that can mount antigen-specific memory T cell responses following primary stimulation and expansion. Given their ability to recognize bacterial antigens including ones expressed by *Mtb*, this would provide a rationale to target these cells in

vaccine strategies. In analogy to the functionally distinct type I NKT cell subsets that comprise NKT1, NKT2, NKT17, and NKT_{FH} cells, the Th2-bias observed among some human type II NKT cells and following sulfatide administration, and the discovery of T_{FH}-like type II NKT cells in humans and mice suggest that functionally distinct subsets may also exist among non-invariant CD1d-restricted T cells. Correlation of such functional differences with antigen specificity would allow therapeutic targeting of these cells with activating or inhibitory ligands.

Several examples illustrate that non-invariant NKT cells, in particular type II NKT cells, critically contribute to the immune responses in a wide range of diseases, including microbial infection, anti-tumor immunity, and autoimmunity. Central to our understanding of the varied roles of CD1d-restricted T cells is unraveling the mechanisms that regulate their activation in these diverse conditions. During infection, recognition of inducible self-lipid antigens in combination with stimulation by the inflammatory milieu through cytokines, or direct recognition of microbial antigens results in the rapid activation of CD1d-restricted T cells. How these T cells distinguish between self- and microbial lipids, whether activation by antigens with different affinities results in distinct functional outcomes, and whether recognition of inducible self-antigens extends to non-infectious conditions such as tumor immunity or autoimmunity are currently not known. Defining the context-dependent mechanisms of activation, decrypting the disease-specific balance of protective and potentially harmful functions of the different CD1d-restricted T cell populations and a better understanding of their effector functions and interactions with other immune cells present significant ongoing challenge and will ultimately determine the success of targeting CD1d-restricted T cells for preventive or therapeutic interventions.

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MR1-restricted mucosal-associated invariant T cells and their activation during infectious diseases

Lauren J. Howson, Mariolina Salio and Vincenzo Cerundolo*

MRC Human Immunology Unit, Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, UK

MR1-restricted mucosal-associated invariant T (MAIT) cells recognize vitamin B metabolites, which are generated by a broad range of bacteria, from *Escherichia coli* to *Mycobacterium tuberculosis* and BCG. MAIT cells have been described as innate sensors of infection as they accumulate early in infected tissues. MAIT cells maintain an activated phenotype throughout the course of infections, secrete inflammatory cytokines, and have the potential to directly kill infected cells, playing an important role in shaping the host response. In this review, we will discuss the current knowledge regarding the molecular mechanisms that underline MAIT cells activation in sterile and non-sterile inflammatory conditions.

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Marcelo B. Sztein,
University of Maryland School of
Medicine, USA
Olivier Lantz,
Institut Curie, France

*Correspondence:

Vincenzo Cerundolo,
MRC Human Immunology Unit,
Weatherall Institute of Molecular
Medicine, John Radcliffe Hospital,
Headley Way, Oxford, OX3 9DS, UK
vincenzo.cerundolo@imm.ox.ac.uk

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Introduction

Innate-like lymphocytes, such as invariant natural killer T (iNKT) cells, have gained great interest since their discovery, as these cells lie at the interface between innate and adaptive immune responses. iNKT cells are memory cells bearing a semi-invariant T cell receptor (TCR) through which they recognize self- and bacterial-derived lipid antigens presented by CD1d molecules. During pathogen infection, iNKT cells facilitate adaptive immune responses by inducing dendritic cell maturation (1). Over the last few years, in addition to iNKT cells, another innate-like lymphocyte cell subset has been the focus of much research: mucosal-associated invariant T (MAIT) cells, recognizing a unique family of bacterial-derived metabolites in the context of the major histocompatibility complex (MHC) class I-like molecule MR1.

MR1 Structure and Function

The MR1 gene was first described in 1995 in a genome screen aimed at identifying novel MHC-related genes (2). The human MR1 gene is located on chromosome 1, like CD1 genes, and despite a similar intron/exon organization to classical MHC molecules, it is non-polymorphic. MR1 is highly conserved among mammalian species, with 90% sequence homology between human and mouse MR1-ligand binding domains and a high level of functional cross-reactivity, a feature reminiscent of CD1 molecules (3, 4). Similar to MHC class I molecules, MR1 associates with β_2 -microglobulin and interacts with the peptide-loading complex during biosynthesis in the endoplasmic reticulum (5, 6). In 2003, the connection between MR1 and MAIT cells was made by demonstrating the central role of MR1, along with commensal flora and B cells, for MAIT cell development (7). Although MR1 is ubiquitously transcribed, its physiological expression at the cell surface has been difficult to demonstrate, with the exception of B cell subsets in the intestinal mucosa (8). A study of mouse MR1 reported that only the folded form of MR1 was able to activate MAIT cells and certain mutations in

the putative binding groove disrupted MAIT cell activation, through presentation of yet unknown antigens (9). These results led to great interest in determining the identity of the antigens presented.

The observation that MAIT cells have anti-microbial activity provided important insights into the identification of the MAIT agonists presented by MR1. Kjer-Nielsen et al. (10) showed that, similar to other MHC class I and MHC class I-like molecules, MR1 was unable to fold correctly in refolding buffer alone. This suggested that it required the presence of its ligand in order to stabilize its structure. Refolding was attempted in a number of conditions using RPMI-1640 as a control. Surprisingly, it was found that MR1 molecules were able to fold in this cell culture media, which was known to contain a number of vitamins produced by microbes, but not present in mammals. This observation-based approach led Kjer-Nielsen and colleagues to test the ability of vitamins to bind and stabilize MR1, and ultimately led to the discovery of a new class of antigens: vitamin B metabolites (10). The first crystal structure of the MR1-ligand complex was with the folic acid (vitamin B9) metabolite 6-formylpterin (6-FP), which was shown to be unable to activate MAIT cells (10). To determine the identity of MAIT stimulatory ligands, MR1 molecules were refolded in the presence of the supernatant from *Salmonella typhimurium* bacterial cultures, as it was known that *Salmonella*-infected cells strongly activate MAIT cells (11, 12). By high-resolution mass spectrometry (MS) [ESI-TOF-MS], MR1 was shown to associate with a compound at *m/z* 329.1100, with an atomic composition matching that of several riboflavin (vitamin B2) derivatives: 6,7-dimethyl-8-D-ribityllumazine (RL-6,7-diMe), 7-hydroxy-6-methyl-8-D-ribityllumazine (RL-6-Me-7-OH), and reduced 6-hydroxymethyl-8-D-ribityllumazine (rRL-6-CH₂OH). In functional assays, these compounds were stimulatory and induced CD69 expression as well as interferon-gamma (IFN γ) and tissue necrosis factor-alpha (TNF α) production by MAIT cells (10).

In order to understand the origin of the above MAIT cell ligands, *Lactococcus lactis* mutants of the four-gene operon, which controls riboflavin biosynthesis, were studied (13). These results confirmed that riboflavin is necessary and sufficient to generate natural MAIT cell ligands. Furthermore, these results pointed to the compound 5-amino-6-D-ribitylaminouracil (5-A-RU) as an intermediate necessary for MAIT cell activation. This intermediate does not bind to MR1, but forms MAIT-stimulating ligands through a non-enzymatic condensation with glyoxal or methylglyoxal species, which can be of bacterial or host cell origin (such as byproducts of glycolysis). The yields of these condensation reactions are the unstable, yet potent intermediates 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU) and 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU). These molecules can be captured and stabilized through Schiff bases with Lys43 in the MR1 groove. Furthermore, these compounds are the substrates for conversion to RL-6,7-diMe. Mass spectrometry analysis of MR1 refolded in the presence of culture supernatants from *S. typhimurium*, *E. coli* (DH5 α), or rRL-6-CH₂OH also revealed species with matching properties to the synthetic 5-OP-RU, raising the possibility that the compound initially identified as MR1 ligand was indeed 5-OP-RU (10, 13).

These results provide evidence that MAIT cells are able to sense a wide range of bacteria through detection of vitamin metabolites, including transitory intermediates, presented by MR1 molecules.

MAIT Cell Phenotype

Mucosal-associated invariant T cells were identified based on their surface phenotype and mucosal tissue localization. The MAIT cell invariant TCR V α 7.2-J α 33/12/20 in humans was first described in 1993 as one of the few preferentially used TCRs in the double-negative T cell compartment (14). This finding was the first evidence of a new subset of T cells possibly recognizing a limited set of antigens in the context of non-polymorphic antigen presenting molecules. It was not until 1999 that the MAIT cell subset was defined as a conserved subpopulation distinct from MHC class I- and CD1-restricted cells with an activated/memory phenotype (15). They also reported that the human MAIT cell TCR β chain usage was primarily TRBV6 or TRBV20. Initial research into MAIT cells has been hampered by the lack of specific reagents; however, the generation of a monoclonal antibody specific for the V α 7.2 TCR chain (16) and MR1 tetramers (17) have recently enabled the functional and phenotypic analysis of MAIT cells and brought them to the forefront of innate-like lymphocyte research. MAIT cells are defined as CD3 $^{+}$ V α 7.2 $^{+}$ CD161 $^{++}$ and either CD8 $^{+}$ or double-negative T cells (12, 16). MR1-loaded tetramer experiments have also identified a small subset of MAIT cells that are CD4 $^{+}$ (17). All human MAIT cell subsets express the transcription factor PLZF, known to direct the effector program of the iNKT cell lineage (18). However, murine MAIT cells do not express PLZF (16), thus the functional relevance of this observation is currently unclear. MAIT cells can also be defined based on co-expression of interleukin (IL)-18R (12) and CD26 (19). Furthermore, in adults, peripheral blood MAIT cells have an effector memory phenotype defined as CD45RO $^{+}$, CD62L lo , CD95 hi CD122 int , CD127 int , and they express tissue-homing chemokine receptors: CCR5, CCR6, CXCR6, and CCR9 (20). By contrast, MAIT cells do not express CCR7 that is a marker for homing to lymph nodes. The distinct memory phenotype and peripheral location of these cells are linked to their unique developmental pathway.

MAIT Cell Development

Mucosal-associated invariant T cells develop and undergo selection in the thymus. Like iNKT cells, MAIT cells are selected by CD4/CD8 double-positive (DP) thymocytes (21). MR1 expression on DP thymocytes is essential, as MR1-deficient mice do not develop T cells expressing the MAIT cell TCR at detectable levels (7). Although thymic selection occurs independently of B cell and commensal flora (16), B cells are essential for MAIT cell peripheral expansion and memory phenotype acquisition. However, the endogenous antigen(s) capable of selecting MAIT cells in the thymus to date remain unclear. Furthermore, unlike iNKT cells, human MAIT cells egress the thymus as naïve cells, that in the periphery acquire a memory phenotype prior to birth (22).

In mice, the interaction of MAIT cells with B cells in the periphery drives the acquisition of the MAIT cell memory phenotype

(16). In humans, a recent study analyzed MAIT cell development in second trimester fetal tissues, and reported acquisition of innate-like anti-microbial activity already *in utero* (22). This study suggested that MAIT cell maturation begins in the secondary lymphoid organs, where they reported V α 7.2 $^{+}$ CD161 $^{+}$ cells acquire expression of the transcription factor PLZF, CD62L, and CD45RO. In fetal liver and mucosal tissues, V α 7.2 $^{+}$ CD161 $^{+}$ cells expressed high levels of PLZF, IL-18R α , CD62L, and CD45RO and were capable of responding to bacterial antigen stimulation both through proliferation and production of IFN γ and IL-22. While murine studies have revealed the importance of the microbiota in shaping MAIT cell differentiation, as they are absent in germ-free mice (7), this study suggests that human MAIT cells develop prenatally, before establishment of the commensal microflora, although the factors driving this maturation remain to be identified (22). However, it remains unclear whether V α 7.2 $^{+}$ CD161 $^{+}$ T cells described by Leeansyah and colleagues are MR1-restricted MAIT cells, as MR1-tetramer staining or MR1-blocking antibody experiments were not performed (22).

The interaction of MR1 expressing B cells and MAIT cells in the periphery is necessary for MAIT cell expansion in mice (16). Likewise in humans, individuals with mutated Bruton's tyrosine kinase, and thus with disrupted B cell development, have reduced MAIT cell TCR transcripts in the blood compared to healthy controls (7). These results are consistent with the observation that both primary B cells and B cell lines can efficiently present bacterial antigens to MAIT cells in an MR1-dependent manner (23).

Together, the results from these studies suggest that MAIT cells are selected and undergo maturation prior to exposure to commensal bacteria. It can be speculated that peripheral expansion of MAIT cells may occur via presentation of an endogenous antigen present at mucosal sites or through maternally derived commensal flora antigen, possibly carried in the amniotic fluid.

MAIT Tissue Distribution

Mucosal-associated invariant T cells are primarily located in the mucosal compartments and blood. The expression of chemokine receptors CXCR6 and CCR9 at the surface of MAIT cells suggested that they traffic to the tissues, particularly the intestine, lung, and liver (20). Indeed, these tissues have a higher proportion of MAIT cells, composing 20–40% of liver T cells and 4–10% intestinal T cells (20, 24). MAIT cells compose 1–8% of T cells in the blood. Analysis of the V α 7.2-J α 33 and V α 7.2-J α 12 transcripts in different tissues revealed that MAIT cells are also present in the kidney and at a lower frequency in the tonsils and lymph nodes (25). They also reported variable expression of these transcripts in the prostate and ovary (25). Analysis of the α chains revealed a predominance of V α 7.2-J α 33 in the blood and V α 7.2-J α 12 in tissues indicating that these two MAIT subsets might differ in their tissue-homing properties (25). Further investigation is needed to determine whether these two subtypes are functionally distinct in terms of their antigen/pathogen specificity or are related to the infection history of the individual.

In humans, circulating MAIT cells are more abundant than iNKT cells, while in mice they are far fewer (15, 16, 20). Studies assessing the influence of age and gender of MAIT cells found that MAIT cells decrease with age, but are not significantly different

between males and females (26–28). The MAIT cell phenotype also changes according to the tissue compartment. For example, liver MAIT cells have a more activated phenotype compared to blood MAIT cells and express higher levels of CD69, CD38 and HLA-DR, possibly reflecting continuous antigen exposure (24).

MR1-Dependent and -Independent MAIT Cell Activation

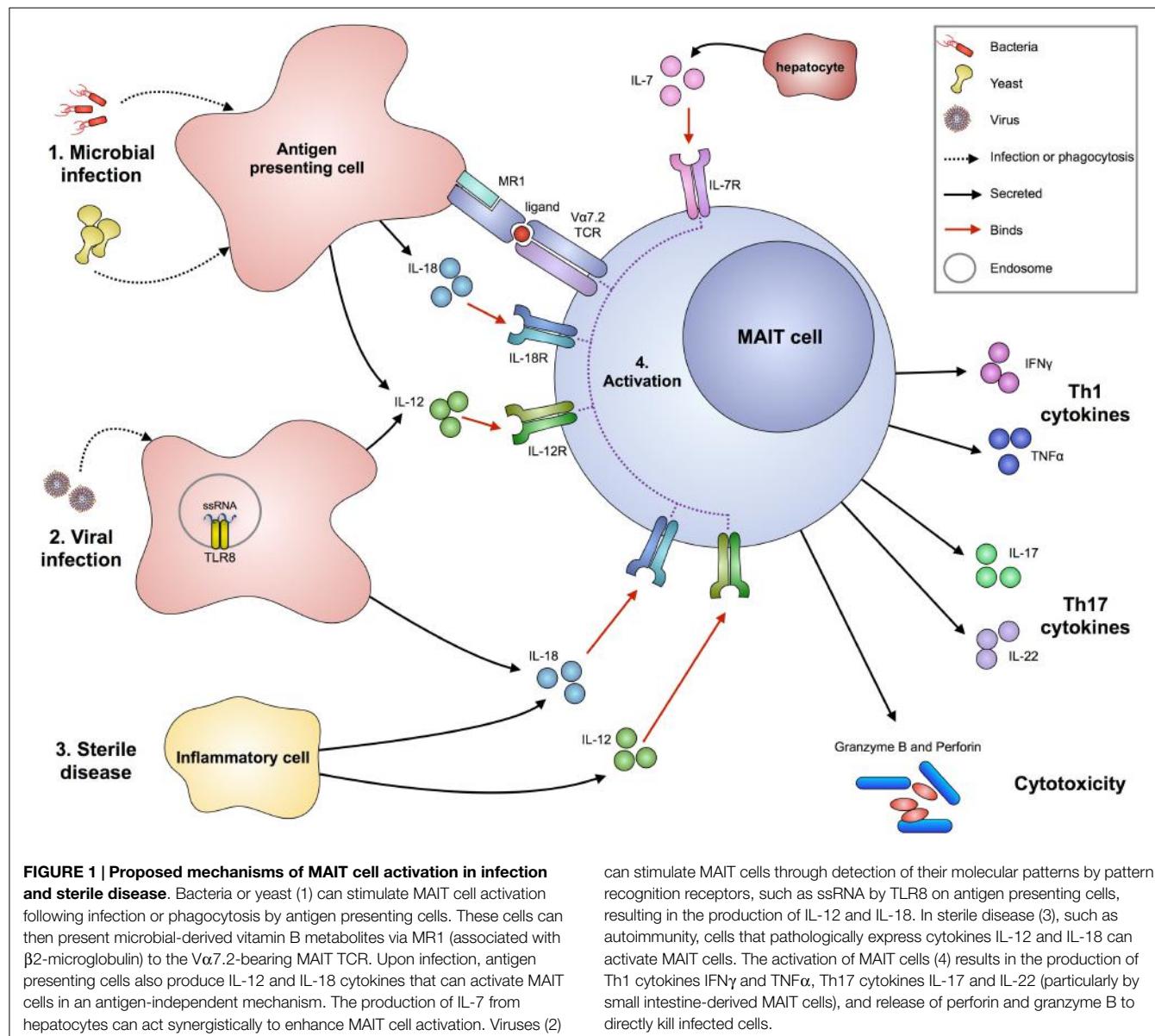
In 2010, two publications described a wide range of phylogenetically diverse bacteria and yeast that were able to activate MAIT cells. These microbes included: *E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Lactobacillus acidophilus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Saccharomyces cerevisiae*, *Candida glabrata*, and *Candida albicans* (11, 12). Conversely, viruses and few bacterial species such as *Enterococcus faecalis* and *Streptococcus* group A did not elicit MR1-dependent MAIT cell activation. The later discovery of the MAIT cell ligand offered the explanation for the lack of response as these bacteria lack the vitamin B metabolites that bind MR1 molecules and stimulate MAIT cells (10).

Cytokine Production

Mucosal-associated invariant T cells can produce pro-inflammatory cytokines upon activation (Figure 1). Stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin, as well as bacteria, showed that human MAIT cells are capable of producing IFN γ , TNF α , IL-2, and IL-17 but not IL-10 (20, 24). *Ex vivo* multiparametric mass cytometry in three donors recently confirmed that there are distinct subsets of circulating MAIT cells releasing different cytokine combinations, most frequently MIP1 β , IFN γ , TNF α , and IL-2, and to a lesser extent IL-4 and IL-10 (25). Furthermore, MAIT cells from the small intestine are capable of producing IL-22 in response to *E. coli*, but this was not observed in MAIT cells from the liver or lung (22). Thus, MAIT cells have a mixed Th1/Th17 cytokine profile with differences according to their peripheral location, possibly reflecting local environmental imprinting.

Cytotoxicity

An important property of MAIT cells is their ability to kill infected cells (Figure 1). Cytotoxicity occurs primarily via granule exocytosis and relies on the effector cell containing components that enable destruction of a target cell. These include perforin and granzymes that, in humans, consist of five isoforms with different substrate specificities: granzyme (Gr) A, GrB, GrH, GrK, and GrM (29). GrB and perforin have been directly linked to the cytotoxic activity of CD8 $^{+}$ T cells (30). It has been shown that MAIT cells are able to lyse bacteria-infected epithelial cells (31) and *E. coli*-infected THP1 cells (32) in an MR1-dependent manner. Interestingly, human MAIT cells have a unique cytotoxic profile, characterized by low perforin expression, but high GrA and GrK, contained in CD107 $^{+}$ lytic granules (32). Upon antigen recognition, an MR1-dependent GrB upregulation and an MR1-independent perforin upregulation was observed, suggesting that antigen pre-exposure licenses MAIT to acquire a stronger cytotoxic phenotype (32). The ability to kill infected target cells suggests that MAIT cells could have an important role



in controlling infections by intracellular bacteria, such as *Shigella* and *Salmonella*, although the latter may escape MAIT cell detection by preventing vacuole-lysosomal fusion (33). Indeed, it has been shown that, while HeLa cells infected with *Shigella flexneri* can be readily killed by MAIT cells, HeLa cells infected with *S. typhimurium* failed to be detected or lysed by MAIT cells (31).

MR1-Independent Activation

Like other innate-like cells, MAIT cells can also respond to infection in an MR1-independent manner. For example, the *in vitro* MAIT cell response to cells infected with *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG) is largely MR1-independent (34). The high expression of IL-18R on MAIT cells suggested that IL-18 could be one of the cytokines able to activate MAIT cells (20, 35). Therefore, cytokine-dependent activation of MAIT cells was explored in more detail and it was reported that MAIT cells could produce IFN γ when their TCR is blocked and they are

cultured with IL-18 and IL-12 (36). Furthermore, cytokines can synergize with TCR stimulation to enhance MAIT cell activation. For example, liver-resident MAIT cells express high levels of IL-7R and IL-7 potentiates TCR-dependent secretion of Th1 cytokine and of IL-17A (24). These results suggest that multiple factors could shape MAIT cells responsiveness during bacterial infection, and their ability to respond to cytokine stimulation, independent of TCR cross-linking, may account for their response to microbes not containing MR1 ligands, such as viruses and some bacterial species (Figure 1).

MAIT Cells in Bacterial Infections

Murine infection models and natural human infections have been researched in order to further our understanding of MAIT cells and their activation during pathological conditions.

In a mouse model of lung infection with live *Francisella tularensis* (LVS), it was demonstrated that MAIT cells colonize the lung in both early and intermediate phases of the infection, reach their peak expansion in the late clearance phase of infection, and persist following clearance of the bacteria (37). From day 8 of infection onward, MAIT cells in the lung produced anti-bacterial cytokines (IFN γ , TNF α , and IL-17A). The evidence for the importance of MAIT cells in controlling infection came from MR1 knockout mice, which showed a delay in clearance of the bacteria in the lungs (but not in the spleen or liver) and delayed appearance of adaptive immune responses in the lung (37). A similar increase in bacterial load in comparison with wild type mice was found when mice lacking MAIT cells were infected with *K. pneumonia* or *M. bovis* BCG (34, 38). Together, these studies indicate that the role of MAIT cells in controlling infection is not redundant, and is vital for timely mounting of effective adaptive immune responses.

Tuberculosis

In humans, the contribution of MAIT cells to anti-bacterial immunity has been studied mostly during *Mycobacterium tuberculosis* infection. MAIT cells have been shown to be able to respond *in vitro* to *M. tuberculosis* infected primary lung epithelium and dendritic cells (11, 39). In individuals with active *M. tuberculosis* infection, it was found that circulating MAIT cells were in lower absolute numbers and proportions compared to healthy individuals (11, 12). This suggested that MAIT cells may migrate to inflamed tissues, where they can have a localized response to infection. There is also evidence that MAIT cells are enriched in the lung of healthy donors and therefore could respond early during the course of infection (11).

To assess whether the reduced number of MAIT cells is accompanied by an altered functionality, a study analyzed the cytokine production by MAIT cells from individuals with tuberculosis (TB) in response to different stimuli. Despite lower frequencies, a higher proportion of MAIT cells in individuals with active TB secreted IFN γ and TNF α in response to BCG stimulation as compared to healthy controls (although the overall response was very low) (40). More convincingly, a lower proportion of MAIT cells responded to *E. coli* in the same cohort of infected individuals compared to healthy controls. These results suggest that there may be an overall enrichment of MAIT cells specific for BCG compared to *E. coli* upon *in vivo* antigenic exposure.

This concept of expansion of pathogen-specific MAIT T cell clones is suggested by a recent paper reporting that heterogeneity in the TCR β chain usage by MAIT cell clones was directly related to their ligand specificity, and thus, to responses to different microbes (41). While this is a very attractive possibility, this observation requires further confirmation as the MAIT cells assessed in the above study were defined as V α 7.2 $^{+}$ CD8 $^{+}$ cells that produced TNF α in response to bacterial stimulation. Without using MAIT specific markers, such as CD161, IL-18R, or CD26, this strategy could have included a proportion of conventional V α 7.2 $^{+}$ T cells. Furthermore, no MR1 blocking experiments were included to demonstrate differential microbe recognition. Nevertheless, a contribution of the TCR β chain sequence to antigen binding has been also suggested by a second study (42), where six MAIT TCRs with differing β chains were refolded and their

biophysical parameters were measured. This study showed that the CDR3 β loop usage directly impacts MAIT cell recognition of ligands through altering the TCR flexibility and contact with MR1 and the ligand (42); thus, suggesting a role of the TCR CDR3 β loop in fine-tuning MAIT cells' specificity.

These findings open up an exciting area of research, suggesting that this innate-like lymphocyte subset may be capable of forming memory-like responses upon selective expansion of MAIT cell clones following lifetime microbe exposure. The ability to form memory responses could have implications for the therapeutic use of MAIT cells.

Other Bacterial Infections

Decreased numbers of circulating MAIT cells have also been reported in individuals with cystic fibrosis during infection with the opportunistic pathogen *P. aeruginosa* (43). In these individuals, the lower MAIT cell numbers also correlated with lung disease severity, systemic inflammation, and clinical status. However, whether the reduction in numbers was a consequence of their localization to the airway mucosa, or by itself influenced colonization and disease progression, was not addressed.

The relevance of MAIT cells in a model of invasive enteric infection was addressed in a study investigating the efficacy of an attenuated strain of *Shigella dysenteriae* (31). A specific reduction in circulating MAIT cells was observed at day 11 in subjects who received bacteria compared to controls. Individuals who responded to the vaccine, producing a specific IgA response, also had a trend toward a higher baseline proportion of MAIT cells and at day 11 had an increased proportion of activated MAIT cells as determined by HLA-DR upregulation.

Mucosal-associated invariant T cells have also been investigated in cases of severe infection with *Vibrio cholerae*. It was reported that MAIT cells activation, as measured by CD38 expression, peaks 7 days after the onset of infection (44). In adults, the proportion of MAIT cells did not change upon infection, but interestingly, in children a significant and persistent decrease in MAIT cell proportion was observed. The reason for the adult/child discrepancy is not clear and requires further investigation. Although *V. cholerae* is known to have a riboflavin biosynthetic pathway, *in vitro* experiments aimed at demonstrating the role of vitamin B2 metabolites in this recognition were not performed.

A specific decrease in MAIT cell counts (but not iNKT or $\gamma\delta$ T cells) was also observed in severe infections. Critically ill patients admitted to the intensive care unit (ICU) with sepsis had lower circulating levels of MAIT cells compared to healthy controls and non-septic critically ill individuals (45). Patients with lower MAIT numbers were also more susceptible to hospital-acquired infections. Of interest, it was reported that streptococcal infections (which do not stimulate MAIT cells *in vitro*) induced a less pronounced decrease in MAIT cell numbers than non-streptococcal infections, although there was a large overlap between groups that prevented statistical significance.

MAIT Cells in Viral Infection

Although it has been established that virally infected cells do not directly activate MAIT cells (12), several publications have investigated the numbers and activation of MAIT cells in human

immunodeficiency virus (HIV) infection. The rationale behind investigating MAIT cells in the context of HIV is due to the effects this virus has on increasing the permeability of intestinal epithelia. This alteration causes translocation of microbial products from the gastrointestinal tract, resulting in systemic immune activation (46, 47).

Human Immunodeficiency Virus

Two groups reported that circulating MAIT cells are reduced early in HIV infection compared to healthy controls (48, 49). This trend was also observed in individuals with chronic HIV, and numbers could not be restored following anti-retroviral therapy (49). Consistent with these findings, staining with MR1 tetramers loaded with 5-OP-RU confirmed that the tetramer-positive MAIT population is reduced during HIV infection (50).

The activation and function of circulating MAIT cells in HIV were also assessed, and it was found that they express lower levels of CD69 and produce less IFN γ and TNF α in response to *ex vivo* bacterial stimulation compared to healthy controls (49). Interestingly, after anti-retroviral therapy, a partial restoration of cytokine production was observed. However, this impaired activation and function of MAIT cells were not confirmed in a separate study (50).

The authors also investigated MAIT cells in the rectal and colon mucosa of HIV infected individuals, which were reduced, like circulating MAIT cells. However, mucosal numbers were better preserved and could be restored after anti-retroviral therapy (49, 51).

The decline and possible impaired activation of MAIT cells in patients infected with HIV are likely to affect the MAIT cells' ability to control bacteria and yeast infections and, as a result, leave the host extremely vulnerable and contributing to acquired immune deficiency syndrome (AIDS) pathogenesis.

Hepatitis B and C

As previously discussed, a large proportion of liver-resident T cells are MAIT cells. These liver-resident immune cells are of great interest as viruses, such as hepatitis B virus (HBV) and hepatitis C virus (HCV), can establish persistent infections in the liver. To determine whether liver MAIT cells could respond to innate signals upon viral infection, Toll-like receptor (TLR) agonists were used to stimulate liver intra-sinusoidal cells. It was found that TLR8 agonist ssRNA40 was able to activate liver MAIT and NK cells to produce large amounts of IFN γ (52). The response was MR1-independent and driven by IL-12 and IL-18 released by TLR8-stimulated monocytes. A similar activation of hepatic MAIT cells was observed in individuals infected with HBV and HCV. This preservation of anti-viral activity of MAIT cells in diseased livers could provide a possible therapeutic target for the treatment of viral infection.

MAIT Cells in Sterile Disease

The capacity of MAIT cells to respond to cytokine stimulation, as described above, raises the possibility that these cells may also play a role in sterile inflammation such as autoimmune disorders and cancer.

Multiple Sclerosis (MS)

The role of MAIT cells in MS has been extensively researched. MAIT cells' TCR was identified in central nervous system MS lesions using single-strand conformation polymorphism analysis in autopic specimens (53). Normal frequency of MAIT cells was found in the blood of MS patients and MAIT cells were also detected in the cerebrospinal fluid of relapsing patients. This suggested a role for MAIT cells in the progression of this disease, which was functionally explored by a study using a mouse model for MS, experimental autoimmune encephalitis (EAE). In transgenic mice overexpressing the MAIT cell TCR, MAIT cells protected against EAE through suppression of Th1 cytokine production and increased production of IL-10 primarily by B cells (54). It was also observed that, in MR1 knockout mice, EAE was exacerbated, suggesting a new regulatory role for MAIT cells.

Conflicting results have emerged from studies assessing the distribution of circulating MAIT cells in MS patients. A study of an Italian cohort assessing MAIT cells of monozygotic twins with discordant disease states showed a greater number of circulating CD8 $^{+}$ CD161 $^{++}$ cells in MS patients compared to their healthy twin (55). By contrast, a study of a Japanese cohort showed decreased circulating MAIT cells in MS patients compared to healthy controls (56). This discrepancy between the two studies findings underlines the difficulties of studies with human subjects and suggests the variation in the commensal flora or lifetime infection burden may have affected MAIT cell frequencies in the two populations.

Psoriasis

It has previously been reported that IL-17A is a vital component in the etiology of psoriasis (57). As MAIT cells are able to produce this cytokine, it was thought that they might be the CD8 $^{+}$ cells previously observed in psoriatic lesions. It was found that MAIT cells are indeed present in the psoriatic lesions along with Th17 cells (58). This provided evidence that MAIT cells may contribute to psoriasis, however, their activation in this setting is yet to be explored.

Rheumatic Diseases

Mucosal-associated invariant T cells have also been studied in the context of rheumatic diseases. It was found that individuals with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) had lower circulating levels of MAIT cells (59). Furthermore, MAIT cells have been detected in the synovial fluid of RA patients in higher proportions than in the blood. Individuals with SLE had MAIT cells with impaired activation, as they had a lower production of IFN γ when challenged with bacteria or stimulated with PMA and ionomycin. This impaired function was more prominent in individuals with lower MAIT cell numbers, suggesting a correlation between MAIT cell numbers and function. Conversely, in RA patients, the IFN γ secretion was preserved. To assess whether the inhibitory molecule PD-1 correlated with MAIT cell dysfunction, its expression on MAIT cells was assessed and found to be higher on MAIT cells in individuals with SLE compared to individuals with RA or healthy controls. However, after PD-1 blockade, MAIT cell activation was only partially

restored, indicating other negative regulation mechanisms may be contributing to MAIT cell dysfunction in SLE.

Inflammatory Bowel Disease and Celiac Disease

The location of MAIT cells in the small intestine mucosa makes them of great relevance in the pathogenesis of intestinal inflammatory disorders, such as inflammatory bowel diseases (IBD): Crohn's disease and ulcerative colitis. A study reported that the number of circulating MAIT cells was reduced in individuals with Crohn's disease and ulcerative colitis compared to healthy controls (60). A similar observation was reported with circulating MAIT cell numbers in individuals with celiac disease (61). It was also reported that MAIT cells were higher in number in inflamed compared to healthy tissues (60). Functional assessment of MAIT cells from individuals with IBD showed increased IL-17 production upon stimulation along with increased IL-22 in ulcerative colitis but decreased IFN γ secretion in individuals with Crohn's disease. This first demonstration of activated MAIT cells in inflamed gut tissues warrants further investigation as to their potential pathogenic role.

Cancer

A less explored area is the potential role of MAIT cells in cancer. A publication has suggested MAIT cells may be present in renal and brain cancers through detection of their TCR in these tissues (62). This presence correlated with the presence of pro-inflammatory cytokines in the tumor tissue, suggesting they could have anti-neoplastic functions. However, this has not been explored any further.

MAIT Cell as a Potential Therapeutic Target

With their ability to alter the cytokine microenvironment to pro-inflammatory, direct killing of infected cells, potential to enhance adaptive immune responses, and the recent discovery of their activating ligands, MAIT cells may be an ideal target to manipulate for enhancing immune responses. An understanding of the potential of these cells could aid the designing of more targeted vaccines and effective immunotherapies.

The use of bacteria as adjuvants to enhance immune responses was first explored in 1891 when William Coley conducted intra-tumoral injections of *S. pyogenes* and *Serratia marcescens* to cause inflammation and subsequent destruction of the tumor cells (63). This form of immunotherapy, in which bacteria are used to stimulate anti-tumor immune responses, has not been highly successful with the notable exception of the intra-vesical instillation of BCG, which is currently the gold standard treatment of non-invasive bladder cancer. Although successful, it is still not well understood what the immune principles are that underlie its success.

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It has previously been discussed that MAIT cells are able to respond to BCG-infected cells *in vitro* (34, 40). Understanding whether MAIT cells are key players in mediating the success of BCG immunotherapy may help in designing more targeted therapies and also opens up the potential for MAIT cell-targeted immunotherapies for other cancers.

Outstanding Questions

A number of questions still need to be addressed in the field of MAIT cell research. First, the full spectrum of MAIT-activating ligands remains to be explored. Whether other possible vitamin metabolites or small organic compounds could bind MR1 and stimulate MAIT cell responses is still an open question. Also, a priority will be identification of endogenous ligand(s) involved in shaping MAIT cell selection and maturation during development.

Second, although MAIT cells can respond to yeast species *in vitro*, the role of MAIT cells during yeast infections *in vivo* remains to be addressed.

Third, it is still not clear what factors determine the relative contribution of antigen-dependent versus cytokine-dependent MAIT cell response during an infection. Early findings suggest that the presence of the ligand, mode of infection, and subcellular localization of the bacteria may all impact the way MAIT cells respond during infection.

Finally, it is still not known whether the MAIT cell subset could be an effective therapeutic target. The identification of potent antigens for MAIT cells could provide a possible adjuvant in vaccine and immunotherapies targeting both infections and also non-sterile diseases.

Concluding Remarks

The role of MAIT cells during infection is only beginning to be established, with their early infiltration into infected tissues, production of pro-inflammatory cytokines, and role in assisting an effective adaptive immune response. The effector functions of MAIT cells in sterile disease conditions are also being explored. Such an integral role in the progression of infection, and possibly sterile diseases, makes these cells an ideal target for enhancing immune responses.

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The role of mucosal associated invariant T cells in antimicrobial immunity

Ruth J. Napier¹, Erin J. Adams², Marielle C. Gold^{1,3,4} and David M. Lewinsohn^{1,3,4*}

¹ Pulmonary and Critical Care Medicine, Oregon Health & Science University, Portland, OR, USA, ² The University of Chicago, Chicago, IL, USA, ³ VA Portland Health Care System (VAPORHCS), Portland, OR, USA, ⁴ Molecular Microbiology and Immunology, Oregon Health & Science University, Portland, OR, USA

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Munich, Germany

***Correspondence:**

David M. Lewinsohn,
Pulmonary and Critical Care
Medicine, Oregon Health & Science
University, 3181 SW Sam Jackson
Park Road, Mail Code VA R&D
11, Portland, OR 97239, USA
lewinsohd@ohsu.edu

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Mucosal associated invariant T (MAIT) cells are an innate-like T cell subset prevalent in humans and distributed throughout the blood and mucosal sites. Human MAIT cells are defined by the expression of the semi-invariant TCR α chain *TRAV1-2/TRAJ12/20/33* and are restricted by the non-polymorphic major histocompatibility complex (MHC) class I-like molecule, MHC-related protein 1, MR1. MAIT cells are activated by small organic molecules, derived from the riboflavin biosynthesis pathway of bacteria and fungi, presented by MR1. Traditionally, MAIT cells were thought to recognize a limited number of antigens due to usage of an invariant TCR α chain and restriction by a non-polymorphic MHC molecule. However, recent studies demonstrate that the TCR repertoire of MAIT cells is more heterogeneous, suggesting there is a more diverse array of MR1 antigens that MAIT cells can recognize. In response to infected cells, MAIT cells produce the pro-inflammatory cytokines, IFN- γ and TNF, and are cytolytic. Studies performed in MR1-deficient mice suggest that MAIT cells can provide anti-bacterial control within the first few days post-infection, as well as contribute to enhanced adaptive immunity in murine models of respiratory infections. In humans, the role of MAIT cells is unclear; however, evidence points to interplay between MAIT cells and microbial infections, including *Mycobacterium tuberculosis*. Given that MAIT cells are pro-inflammatory, serve in early control of bacterial infections, and appear enriched at tissue sites where microbes interface and gain access to the body, we postulate that they play an important role in antimicrobial immune responses. In this review, we discuss the most recent studies on the function and phenotype of MAIT cells, including their TCR diversity and antigenic repertoire, with a focus on the contribution of human MAIT cells in the immune response to microbial infection.

Keywords: MR1, mucosal associated invariant T cells, antimicrobial, immunity

Introduction

The immune system is conceptually divided into two general categories: innate and adaptive. Innate immunity is considered the first line of defense and is mediated largely by epithelial cells and phagocytes that detect and kill foreign microbes through many mechanisms, including the use of germ-line encoded receptors, referred to as pattern recognition receptors (PRRs). PRRs allow innate immune cells to quickly detect and respond to infections by binding to molecules associated with

pathogens, called pathogen associated molecular patterns (PAMPs). Adaptive immunity consists of T and B lymphocytes that use rearranged antigen recognition receptors to detect a wide range of antigens. The antigen recognition receptor on T cells, the T cell receptor (TCR), recognizes foreign antigens only when they are bound to major histocompatibility complex (MHC) molecules, which are expressed on the cell surface of host cells. In general, activation through the TCR results in clonal expansion of T cells specific for a particular antigen, acquisition of effector functions, and the development of long-lived T cells, which provide immunological memory, resulting in long-term protection against subsequent re-infection.

Mucosal associated invariant T (MAIT) cells, a subset of non-classically restricted T cells, share characteristics of classical MHC-I- and MHC-II-restricted T cells, yet have unique properties that have lead to their description as “innate-like.” Like conventional T cells, MAIT cells undergo TCR rearrangement and positive selection in the thymus (1–3). However, unlike conventional T cells that remain naïve until antigenic-stimulation in the periphery, MAIT cells gain effector capacity prior to exiting the thymus (4, 5). Thus, MAIT cells have an inherent capacity to detect and respond to infection. In addition to the acquisition of innate-like features in the thymus, MAIT cells can expand and adapt following egress into the circulation (5).

In stark contrast to conventional TCR $\alpha\beta$ T cells that recognize peptide antigens presented by MHC-I or MHC-II molecules, MAIT cells become activated by small molecules presented by the non-polymorphic MHC class I-related molecule, MR1(6). MAIT cells function similarly to conventional CD8 $^{+}$ effector T cells by secreting pro-inflammatory cytokines and cytotoxic molecules in response to microbial infections *ex vivo* (7, 8). Moreover, MAIT cells have been shown to play a role in host antibacterial responses *in vivo* (9–11). In this review, we will present compelling evidence suggesting MAIT cells serve as sentinels of infection at the mucosal surface, where they may (i) contribute to immediate protection against microbes, (ii) augment induction of adaptive immunity, and (iii) potentially provide immunological memory.

MAIT Cells at a Glance

TRAV1-2 expressing T cells were originally described in 1993 by Porcelli et al. as a population of TCR $\alpha\beta$ T cells, enriched in the CD4 $^{-}$ CD8 $^{-}$ (double negative) T cell subset of human blood, expressing the invariant TCR α chain *TRAV1-2* paired with *TRAJ33* (V α 7.2/J33) (12). The authors suggested that these invariant TCR sequences were indicative of restriction by a non-polymorphic MHC molecule potentially presenting a limited family of antigens. Tilloy et al. further described this population of *TRAV1-2* expressing T cells as a TAP-independent and β 2-microglobulin-dependent T cell subset (13). A decade after their original description, Treiner et al. described the non-polymorphic non-classical MHC molecule, MR1, as the antigen-presenting molecule for *TRAV1-2/TRAJ33*-expressing T cells (1). Furthermore, they described these cells as being enriched at mucosal tissues, including the small intestine, and thereby named them MAIT T cells.

Within this seminal study, Treiner et al. presented data demonstrating for the first time that there was a relationship between MAIT cells and microbes. MAIT cells were found to be absent in germ-free mice, indicating their expansion in the periphery depended on microbial ligands (1). In 2010, MAIT cells were shown to have a physiological role in their capacity to detect bacteria and fungi and secrete pro-inflammatory cytokines (7, 8). Consistent with this finding was the discovery of the first set of MR1 ligands, which were identified as small molecule vitamin B metabolites derived from microbes (6). Cumulatively, these studies suggested that MAIT cells could serve as innate-like T cells in the antimicrobial immune response. This hypothesis was supported by mouse models where deletion of MR1, and hence MAIT cells, rendered mice more susceptible to bacterial infections (9–11).

MAIT Cells are Antimicrobial

In 2003, Treiner et al. found that MAIT cells were absent in germ-free mice; yet, they could be reconstituted by the oral inoculation of single bacterial species, we now know to be MAIT-activating, including *Enterobacter cloacae* or *Lactobacillus acidophilus* (1, 8). By contrast, iNKT cell frequencies were unaltered in germ-free mice (14). These data suggested that MAIT cells, but not iNKT cells, required microbial ligands for expansion in blood and tissue. A decade later, two simultaneous studies presented definitive evidence that MAIT cells were reactive to antigens produced by bacteria and fungi presented by MR1(7, 8).

The first study was predicated on the observation that a significant proportion of CD8 $^{+}$ T cells from the blood of humans who had no prior exposure to Mtb could produce IFN- γ when co-cultured overnight with dendritic cells (DCs) infected with Mtb (15, 16). Given that responses to known protein ligands were limited to those individuals with evidence of previous infection with Mtb, we postulated that these responses could either reflect previous exposure to antigens derived from ubiquitous environmental mycobacteria or could be non-classically restricted T cells.

Direct demonstration of the presence of mycobacteria-reactive, non-classically restricted CD8 $^{+}$ T cells came from a study of human thymocytes, a population of antigen-inexperienced T cells (4). In this work, Mtb-reactive CD4 $^{-}$ thymocytes with the ability to produce IFN- γ directly *ex vivo* were readily detected in all donors tested. Furthermore, the functional capacity of these cells was not altered in the presence of W6/32, a pan-HLA (HLA-A, B, C) blocking antibody, suggesting that these thymocytes were restricted by a non-classical MHC molecule.

In an effort to characterize the MHC restriction of human Mtb-reactive CD8 $^{+}$ T cells, limiting dilution analysis (LDA) cloning was used to isolate CD8 $^{+}$ T cell clones from the blood of either uninfected individuals or individuals infected with Mtb (15). The majority (64%) of the Mtb-reactive CD8 $^{+}$ T cell clones generated from subjects with active TB were classically restricted T cells, as they responded uniquely to antigen-presenting cells (APCs) from HLA-matched donors but not those that were HLA-mismatched. By contrast, 85% of the CD8 $^{+}$ T cell clones generated from uninfected individuals with no previous exposure to Mtb detected antigen that was non-classically restricted, consistent with their

ability to respond to HLA-mismatched APCs. Expansion of non-classically restricted T cells from all donors allowed for more detailed analysis of the restricting allele. While the addition of either W6/32 or anti-CD1 antibodies did not result in diminished T cell activation, the addition of an anti-MR1 antibody resulted in complete inhibition. Furthermore, these MR1-restricted clones expressed the invariant TCR α chain, TRAV1-2, consistent with their characterization as MAIT cells (7). In addition to Mtb, the MAIT cell clones could be activated by DCs infected with bacteria and fungi, including *Salmonella enterica* serovar *typhimurium*, *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, and *Saccharomyces cerevisiae* in an MR1-dependent manner. Taken together, these data indicated that MAIT cells were present in humans with no previous exposure to Mtb, and that these cells recognized cells infected with bacteria and fungi.

In a parallel study, Le Bourhis et al. also showed that purified CD3 $^+$ TRAV1-2 $^+$ CD161 $^+$ MAIT cells from humans could be activated by monocytes infected with *E. coli* or *Mycobacterium abscessus* in an MR1-dependent manner (8). Using TCR-transgenic mice expressing TRAV1 TRBV6-transgenic T cells, they demonstrated that MAIT cells were activated by DCs infected with multiple bacterial and fungal species, including *E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *L. acidophilus*, *S. aureus*, and *S. epidermidis*, *C. albicans*, *C. glabrata*, and *S. cerevisiae*. Importantly, these studies suggested that the bacteria *Listeria monocytogenes*, *Streptococcus group A*, and *Escherichia faecalis*, and as shown in both studies, all viruses tested, were not able to activate MAIT cells (7). For the first time, these studies demonstrated that specific microbes activated MAIT cells in an MR1-dependent manner and proposed a role for MAIT cells in antimicrobial immunity.

MAIT Cell Development

Mucosal associated invariant T cells, like other TCR $\alpha\beta$ T cells, depend on the thymus for their development (1–3). In the thymus, MAIT cells undergo TCR rearrangement through somatic recombination followed by positive selection (1–3). However, while classically restricted T cells are positively selected on thymic epithelial cells expressing MHC-I or MHC-II, murine MAIT cells are selected on CD4 $^+$ CD8 $^+$ double positive hematopoietic cells that express high levels of MR1(2, 3). In humans, MR1 is also expressed on human hematopoietic CD4 $^+$ CD8 $^+$ thymocytes, suggesting human MAIT cells are selected in a similar manner to mouse MAIT cells (5). Unlike classically restricted thymocytes, human MAIT thymocytes produce TNF and IFN- γ in response to bacterially infected cells (4, 5). This thymically acquired functional capacity is distinct from that of naïve classically restricted T cells that require antigen-dependent activation in the periphery followed by division and differentiation, a process that delays acquisition of effector function.

While thymic MAIT cells have the functional capacity to serve as “innate” effector T cells, their cell surface phenotype is similar to that of a naïve T cell. Following thymic egress, MAIT cells in the periphery expand and acquire a cell surface memory phenotype indicative of antigen-experience (7). To this extent, human CD8 $^+$ TRAV1-2 $^+$ MAIT cells appear to be enriched within the CD8 $^+$

T cell subset of the blood compared to the thymus (4, 5, 17). These data imply that while MAIT cells gain effector capacity in the thymus, they proliferate and expand in the periphery, presumably due to exposure to exogenous antigen.

Human MAIT Cell Phenotype in Peripheral Blood and Tissues

Mucosal associated invariant T cells egress from the thymus into the blood where they circulate the lymphatic system and acquire an effector memory phenotype, indicated by cell surface expression of CD45RA $^-$ CD45RO $^+$ CD95 hi CCR7 $^-$ CD62L lo (5, 18). MAIT cells are abundant in human blood and in a number of tissues, including the small intestine, lungs, and liver (1, 7, 8, 18–21). Indeed, MAIT cells received the name “mucosal-associated invariant T cells” based on early data suggesting their enrichment in the lamina propria of the small intestine (1). Although their frequencies and role at mucosal tissues are largely unknown, these data indicate that MAIT cells accumulate at sites where foreign microbes have the potential to gain access to the body.

Human MAIT cells were originally defined as double negative or CD8 $^+$ T cells that expressed TRAV1-2 mRNA transcripts by PCR (12). However, given that classically restricted T cells and CD4 $^+$ CD1b-restricted germline-encoded mycolyl lipid (GEM) T cells also express TRAV1-2, TRAV1-2 expression alone is not sufficient for the definition of a MAIT cell. MAIT cells can also be characterized by high expression of the C-type lectin receptor, CD161 (KLRB1) (2). Although the physiological function of CD161 is still not known, a recent study found that TCR-dependent MAIT cell activation could be blocked by anti-CD161 antibodies (22). Currently, the co-expression of CD161 and TRAV1-2 has been widely used to delineate MAIT cells *ex vivo* (2).

TRAV1-2 $^+$ CD161 $^+$ MAIT cells have been associated with additional cell surface markers, including the IL-18R, the dipeptidyl peptidase-4 or CD26, the ABCB1 drug resistance transporter (CD243), and the chemokine receptors CCR6, CXCR6, and CCR5, which are associated with trafficking to tissues including the intestine and liver (2, 8, 18, 23). Furthermore, the transcription factor PLZF (ZBTB16), which was previously associated with “innate” effector function in CD1d-restricted iNKT cells in mice, has been associated with MAIT cells in the thymus, blood, and tissues of humans as early as the second trimester of gestation (2, 21, 24).

Following the discovery that MAIT cells were activated by microbial ligands, MAIT cells have been identified in several ways including function. Functional MAIT cells have been defined as CD8 $^+$ TRAV1-2 $^+$ T cells that produce the pro-inflammatory cytokines, IFN- γ and TNF, in an MR1-dependent manner when co-incubated with infected cells (6–8, 25). To overcome the limitation that CD161 down-regulation can occur in activated MAIT cells, a simple phenotypic panel for defining those MAIT cells with the capacity to detect infected cells in the absence of *ex vivo* stimulation was identified for MAIT cells from healthy human blood. Sharma et al. demonstrated that all CD8 $^+$ TRAV1-2 $^+$ CD26 hi T cells could produce TNF in response to infected cells (25). MAIT cells have also been reported to produce IL-17 in response to TCR-independent stimulation (18). Therefore, while peripheral

blood MAIT cells with pro-inflammatory function can be defined phenotypically as CD8⁺ or double negative TRAV1-2⁺ CD26^{hi} CD161^{hi} T cells, the phenotype of tissue-derived MAIT cells, or potentially functionally distinct MAIT cell subsets, remains to be validated.

The elucidation of the crystal structure and identification of small molecule ligands for MR1 have resulted in the generation of a human MR1 tetramer (6, 19). The MR1 tetramer consists of four molecules of biotinylated MR1, each presenting one MAIT cell antigen (rRL-6-CH₂OH or 5-OP-RU), and bound to four molecules of fluorescently labeled streptavidin. In human blood and small intestine, this tetramer identifies MAIT cells, as it binds to virtually all TRAV1-2⁺ CD161^{hi} T cells in a TCR-dependent fashion (19). Using the human MR1 tetramer, a number of novel observations have been made (19, 26). For example, Rentrugoon et al. have shown that *TRAV1-2* can rearrange with *TRAJ12* and *TRAJ20* in addition to *TRAJ33*, suggesting MAIT cell TCRs are more diverse than originally thought (19). In addition, their data suggest that a subset of CD3⁺ T cells binding the MR1 tetramer have minimal to no expression of CD161. These data may indicate that CD161 is not sufficient for MAIT cell identification. Thus, the MR1 tetramer will allow for detection of MAIT cells regardless of their *ex vivo* cell surface molecule expression or anti-microbial function, facilitating an unbiased characterization of MAIT cells in human health and disease.

MAIT Cells in Mice

Mouse MAIT cells, defined by expression of the invariant TCR α chain *TRAV1* *TRAJ33* (V α 19J α 33), are present at very low frequencies in wild-type C57BL/6 and BALB/c laboratory mouse strains (1). Identification of MAIT cells in mice has been significantly hindered by the lack of a commercially available antibody for the murine TCR α chain, *TRAV1*. NK1.1 was shown to be expressed on mouse MAIT cells derived from transgenic C57BL/6 mice (27). However, NK1.1 is expressed on a limited number of laboratory mouse strains, and therefore its utility in defining MAIT cells is not clear. Due to the reagent limitations and the relatively low frequency of MAIT cells in mice, their identification has been restricted to the isolation of double negative T cells via fluorescence-activated cell sorting (FACS), followed by the quantification of *TRAV1* *TRAJ33* transcripts (1). Nonetheless, several *in vivo* mouse studies have contributed valuable insight suggesting a role for MAIT cells in the early immune response to respiratory pathogens (10, 11). These studies will be discussed in detail below.

MAIT Cells have Considerable TCR Diversity

While little is known about the TCR repertoire of MAIT cells in the thymus, their repertoire in peripheral blood has been the subject of several recent reports. These studies have shown that the MAIT cell TCR repertoire in the periphery is more heterogeneous than previously thought (19, 28, 29). Historically, MAIT cells were defined by their expression of the TCR α chain *TRAV1-2* adjoined to *TRAJ33* and paired with a limited set of TCR β chains (*TRBV6* and *TRBV20*) (13). This definition was further refined

based upon the finding of N-nucleotide additions within the V α and J α (CDR3 α) junctional region, lending to the modification of their name from “invariant” to “semi-invariant” (26, 30). This limited array of TCR α and β chains suggested MAIT cells detected a conserved antigen. However, more recently, several groups have identified substantial diversity in *TRAJ* gene usage, TCR β chain pairing, and the CDR3 α regions associated with *TRAV1-2* of MR1-restricted T cells (19, 28). Heterogeneity in TCR usage suggests the possibility that MAIT cells recognize a more diverse set of ligands.

MAIT Cells Recognize Microbe-Derived Riboflavin Metabolites

Recently, the laboratories of McCluskey and Rossjohn successfully identified small molecules derived from the folic acid (vitamin B₉) and riboflavin (vitamin B₂) metabolic pathways as the first known ligands for MR1(6). They determined that metabolites from the riboflavin pathway, but not the folic acid pathway, activated Jurkat T cells expressing the invariant TCR α chain *TRAV1-2* paired with *TRBV6.1*, *TRBV6.4*, or *TRBV20*. These data were intriguing as previously the only known antigens for T cells consisted of peptides, glycolipids, sphingolipids, and in mice short formylated peptides (31). Thus, these data identified small molecules as a new and unique class of T cell antigens.

Using culture supernatant from *Salmonella typhimurium*, a microbe previously shown to activate MAIT cells, the ribityllumazines, 6-hydroxymethyl-8-d-ribityllumazine (rRL-6-CH₂OH), 7-hydroxy-6-methyl-8-d-ribityllumazine (RL-6-Me-7-OH), and 6,7-dimethyl-8-d-ribityllumazine (RL-6,7-diMe) were found to bind MR1 and activate MAIT cells. Furthermore, the molecule rRL-6-CH₂OH proved to be the most efficient at activating human MAIT cells. These data suggest that there are multiple ligands for MR1 that may vary in their ability to activate the MAIT TCR. In addition to ribityllumazines, a photo degradation product of folic acid, 6-formyl pterin (6-FP), was identified, bound to MR1's ligand binding cavity in the first crystal structures (6, 32). However, unlike riboflavin metabolites, MR1-bound 6-FP was unable to activate MAIT cells. Given these data, it is possible that molecules derived from other metabolic pathways in addition to riboflavin and folic acid might provide additional sources of MR1 ligands. Interestingly, although both microbes and plants can produce folic acid and riboflavin, only ribityllumazines have been shown to activate MAIT cells. It remains to be determined if these exogenously produced molecules represent the complete repertoire of MR1 ligands.

Recent studies have characterized additional MR1 ligands, termed “neo-antigens” (33). Neo-antigens are small organic molecules, generated by the modification of unstable riboflavin metabolites by glyoxal/methylglyoxal, and are identified as 5-(2-oxoethylideneamino)-6-d-ribitylaminouracil(5-OE-RU) and 5-(2-oxopropylideneamino)-6-d-ribitylaminouracil(5-OP-RU). These neo-antigens can bind MR1 and activate MAIT cells, implying that in addition to the previously defined riboflavin intermediates, molecular bi-products created from the interaction between microbial- and potentially host-derived small organic molecules may represent new antigens for MAIT cells. It remains

possible that riboflavin metabolites and their physiological bi-products differ between microbial species, providing a plausible basis for MAIT cells to respond selectively to discrete microbes. In support of this hypothesis, Gold et al., have found selective microbe-associated TCR usage within an individual. In brief, functional MAIT cells were isolated from four human donors based on their response to *Mycobacterium smegmatis*, *S. typhimurium*, or *C. albicans* (28). Evaluation of the MAIT cell TCR repertoire of each individual demonstrated that there are distinct TCR usages in response to the three different microbes. These data suggest that MAIT cells could be selectively expanded in response to distinct microbial ligands. The possibility that TCR usage could be associated with selective ligand recognition was confirmed by the finding that individual MAIT cell clones had distinctive functional responses to two known ribityllumazine antigens. MAIT cells may nevertheless expand in the periphery due to ongoing exposure to exogenous antigen that may or may not be associated with microbial infection. In sum, these data suggest that individual MAIT TCRs can display ligand selectivity, implying that, like classically restricted T cells, an individual's MAIT TCR repertoire may reflect previous or ongoing microbial exposures.

While most microbes produce riboflavin, the process and molecules involved in the metabolic pathway are markedly different between bacterial and yeast species (34). Some microbes such as *M. smegmatis* and several *Candida* species are considered "overproducers" of riboflavin (34). Thus, the ability of microbes to produce riboflavin has been reported to fluctuate dramatically depending on the availability of chemical elements, including, but not limited to, iron, manganese, zinc, and magnesium (34). These data indicate that the physiological levels of these molecules may affect antigen production by the microbe. However, the mechanisms by which many microbes individually regulate riboflavin synthesis and how chemical elements may regulate these pathways remain unknown. As a result, it remains an open question as to whether or not MAIT cells with pathogen specificity can be defined. Thus, one of the top priorities in the field of MAIT cell immunobiology is the identification of these microbial antigens.

MR1 Structure

The structural elucidation of MR1 provided the first insight into the nature of ligands presented by this molecule (6, 32). The overall backbone structure of MR1 is most closely related to the classical class I MHC, HLA-A2; however, the antigen-presenting groove has a number of unique features. The MR1 cavity is smaller and has two pocket structures capable of binding small molecule antigens (Figure 1A). The A' pocket, named as such due to the similarity in location to the A' tunnels in CD1 molecules, is lined with aromatic and basic residues, creating a small, positively charged cavity that is almost entirely sequestered from external solvent. An additional pocket, termed F' (similar in location to the F' tunnel in CD1 molecules), is more shallow and can be variable in size due to flexibility noted in the structure of the $\alpha 2$ helix of human MR1(6). A comparison between the two molecules in the asymmetric unit of the human structure revealed an ~11 Å shift between the positioning of the N-terminal portion of the

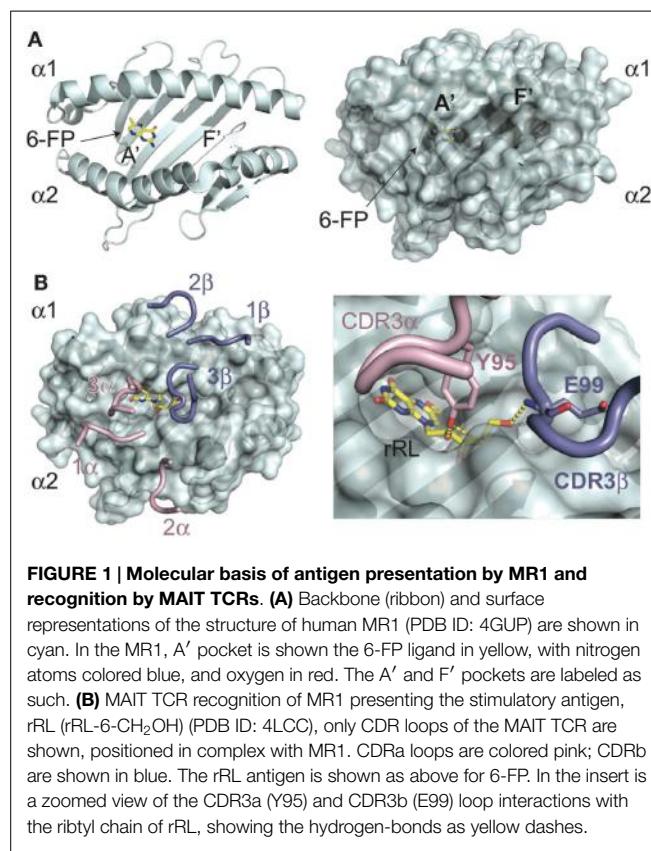


FIGURE 1 | Molecular basis of antigen presentation by MR1 and recognition by MAIT TCRs. (A) Backbone (ribbon) and surface representations of the structure of human MR1 (PDB ID: 4GUP) are shown in cyan. In the MR1, A' pocket is shown the 6-FP ligand in yellow, with nitrogen atoms colored blue, and oxygen in red. The A' and F' pockets are labeled as such. **(B)** MAIT TCR recognition of MR1 presenting the stimulatory antigen, rRL (rRL-6-CH₂OH) (PDB ID: 4LCC), only CDR loops of the MAIT TCR are shown, positioned in complex with MR1. CDR α loops are colored pink; CDR β are shown in blue. The rRL antigen is shown as above for 6-FP. In the insert is a zoomed view of the CDR3 α (Y95) and CDR3 β (E99) loop interactions with the ribityl chain of rRL, showing the hydrogen-bonds as yellow dashes.

$\alpha 2$ helix, with one molecule having a significantly inward-shifted helix resulting in a pseudo-collapsed F' pocket. The structure of the A' pocket was almost identical between the two structures, suggesting that this flexibility would not directly affect antigens presented in this pocket, although this phenomenon may shed light onto the molecular mechanisms of ligand loading. Curiously, this conformational flexibility was not apparent in the structure of bovine MR1 solved in complex with a MAIT TCR; either bovine MR1 has greater rigidity in this region or complexation with a TCR stabilizes this region of MR1(32).

In both crystal structures of MR1, electron density consistent with a bound ligand in the A' pocket was apparent. The protein used in producing these crystals came from two different sources; human MR1 was refolded in the presence of RPMI culture media, whereas bovine MR1 was secreted from insect cells. The ligand from human MR1 was characterized as 6-FP, a pterin-based compound discussed previously. The electron density in bovine MR1 was similar to that identified in human MR1, consistent with a pterin-like compound such as 6-FP. This compound is prevalent in cell culture media, explaining its availability for forming complexes with MR1. In both structures, the ligand electron density was continuous with a lysine residue (K43) deep within the A' pocket, supporting a covalent attachment of this compound via Schiff base. While it has yet to be demonstrated whether 6-FP and the covalent attachment are relevant in the normal functioning of MR1 presentation *in vivo*, exogenous addition of this compound leads to up-regulation of MR1 on the cell surface, yet does not lead to, in fact can be antagonistic toward MAIT activation (6),

suggesting it can stabilize MR1 but cannot provide a stimulatory signal to MAIT cells through their TCR.

The elucidation of lumazine-based compounds, previously described as MAIT cell antigens, is variants of ribityl-lumazine compounds, which contain a two-ring core structure with a ribityl extension. The ring structures are well-accommodated within the aromatic environment of the A' pocket and, depending on the lumazine derivative, also establish hydrogen-bonding networks with the basic residues located within this pocket. The ribityl group extends from the A' pocket and is engaged to differing degrees depending on the orientation of the lumazine group, by the MAIT TCR (30, 35). It is these additional ligand-mediated contacts between the CDR3 α and, depending on the sequence of the MAIT TCR, CDR3 β loops that enhance MAIT TCR binding leading to MAIT cell activation (Figure 1B). Other derivatives of intermediaries of the riboflavin pathway that are highly stimulatory to MAIT cells, including the “neo-antigens” 5-OE-RU and 5-OP-RU, are presented by MR1 via a covalent Schiff base attachment (33). All characterized ligands for MR1 have been shown to be presented through interactions, either covalent or non-covalent, with residues lining the A' pocket. Whether there are other ligands for MR1, potentially being presented also in the F' pocket, is unclear at this time, although considering the universe of ligand possibilities for this protein it is likely that there are at least some non-B vitamin small molecules that derive from endogenous or exogenous sources that can be presented by MR1. Indeed, it is likely the B vitamin ligands identified thus far are only a subset of the ligand repertoire presented by this intriguing MHC-like protein and that there are other small molecule candidates for activating MAIT cells during microbial surveillance and, potentially, other non-microbial related surveillance activities.

MR1-Dependent Antigen Processing and Presentation

MR1 transcripts and protein are expressed ubiquitously in all mammalian cells tested (36–38). However, unlike classical MHC molecules, the MR1 protein resides primarily in intracellular endocytic compartments, while cell surface expression is often undetectable (39). One hypothesis is that MR1 is under tight regulation as to avoid indiscriminate activation of MAIT cells. To this extent, cells infected with Mtb, a pathogen known to elicit MAIT cell responses, or cells treated with the newly identified MR1 ligand, 6-FP, increased and stabilized expression of MR1 on the cell surface (5, 40). These data suggest that the availability MR1 ligand(s) is requisite for MR1 surface expression and therefore MAIT cell activation.

A recent study implies that in humans with dysregulated immune systems, MAIT cell prevalence and activation were associated with pathology (41). For example, MAIT cells were enriched and activated, as determined by expression of Ki67, NKG2D, and BTLA, in ileum tissue sections taken from humans with inflammatory bowel disease compared to healthy control subjects (41). Thus, careful regulation of MR1 expression may be necessary in tissues such as the gut where exposure to microbes is ubiquitous. Regulation of surface expression of MR1 appears to be a limiting factor for MAIT cell activation, as stabilizing

endogenous MR1 cell surface expression *ex vivo* has the potential to activate MAIT cells (42). At present, the mechanisms by which antigens are processed and presented in the context of MR1 are largely unknown.

MAIT Cells Play a Protective Role in Antimicrobial Immunity

Le Bourhis et al. provided the first *in vivo* evidence suggesting that MAIT cells were protective against bacterial pathogens (8). In this study, *TRAV1* (iV α 19) and *TRBV6* (V β 6)-transgenic mice, on an MR1-sufficient (MR1 $^{+/+}$) or an MR1-deficient (MR1 $^{-/-}$) background, the latter of which lack MAIT cells, were injected intraperitoneally with *E. coli* or *Mycobacterium abscessus*. In both cases, MR1 $^{-/-}$ mice had increased bacterial burden implicating a role for MAIT cells in the protection against two different microbes. In this same study, control C57BL/6 mice expressing wild type levels of MR1 had no significant change in mycobacterial burden in comparison to MR1 $^{-/-}$ mice. Notably, *TRAV1*- and *TRBV6*-transgenic mice have increased numbers of MAIT cells in comparison to wild type mice, indicating that the augmented frequencies of MAIT cells in the transgenic mice resulted in protection (2). Following this study, several groups have found that MAIT cells were associated with early protection against bacterial pathogens in mouse models of infection, the details of which will be discussed below.

Mycobacteria

In an effort to determine the role of MAIT cells in protection against mycobacterial infections *in vivo*, Chua et al. challenged MR1 $^{-/-}$ mice with a low dose aerosol infection of *Mycobacterium bovis* BCG (10). MR1 $^{-/-}$ mice had significantly increased bacterial burden at day 10 following infection in comparison to MR1 $^{+/+}$ mice. However, at day 30 post-infection, the bacterial burden of MR1 $^{+/+}$ and MR1 $^{-/-}$ mice were not significantly different. These data suggest that MAIT cells enhance early containment of mycobacterial infection in the lungs. Further studies are needed to determine if MR1 $^{-/-}$ mice infected with aerosolized Mtb will recapitulate this phenotype.

In the same study, MAIT cells contributed to enhanced bacterial containment within macrophages in an MR1-independent manner. Survival of BCG in macrophages was dramatically reduced during an *in vitro* co-culture of enriched MAIT cells, derived from the double negative, CD8 $^{+}$, and CD4 $^{+}$ T cell subsets of *TRAV1* transgenic mice. However, when a similar fraction of cells from wild type mice (which contain very few MAIT cells) were co-incubated with infected macrophages, no effect on bacterial containment was noted. To determine if the anti-mycobacterial capacity of MAIT cells was dependent on activation through MR1, MAIT cells were co-incubated with MR1 deficient macrophages that were infected with BCG. Surprisingly, absence of MR1 did not preclude MAIT cells from reducing intracellular survival of BCG. To determine if IL-12 secretion by infected macrophages was responsible for MAIT cell activity, a blocking antibody to IL-12 was included in a co-incubation of MAIT cells and infected macrophages. This experiment showed that in

the absence of IL-12, MAIT cells lost their bactericidal capacity. The authors suggest that MAIT cell activation and antibacterial capacity were dependent on IL-12 and not MR1. However, the contribution of IL-12 *in vivo* and the mechanism by which it enhanced mycobacterial control were not directly demonstrated.

The possibility that MAIT cells can be activated in an MR1-independent manner has been supported by the recent observation that IL-12 and IL-18 production by macrophages infected with *E. coli* is sufficient to induce IFN- γ production by human liver-derived MAIT cells following an overnight co-culture (20). Given the ability of IFN- γ to augment IL-12 production, it is possible that IL-12 secretion could stimulate a positive feedback cycle in which MAIT cell production of IFN- γ further amplifies the production of IL-12, which in turn promotes further MAIT cell activation. By contrast, the ability of human blood-derived MAIT cells to produce IFN- γ in response to mycobacteria-infected APCs has been shown to be MR1-dependent (7, 8).

Klebsiella

Georgel et al. evaluated the role of MAIT cells in MR1 $^{-/-}$ mice given a high dose (2×10^8 /mouse) intraperitoneal infection with the Gram-negative bacterium, *K. pneumonia* (9). In wild type mice, *K. pneumonia* was cleared within the first 72 h of infection, whereas in MR1 $^{-/-}$ mice, bacteria had spread from the site of injection to several tissues including the lungs. Overall, the MR1 $^{-/-}$ mice had significantly increased mortality. These data are in agreement with that obtained in the BCG challenge model, in that mice lacking MAIT cells had delayed bacterial clearance, suggesting MAIT cells facilitate the early recognition and clearance of bacterial infection.

In the same model, MR1 $^{-/-}$ mice showed equivalent protection to wild type mice when infected with other Gram-negative species, including *E. coli*, *Shigella dysenteriae*, and *Yersinia enterocolitica*. These data would suggest that MAIT cells are more important for clearance of some bacterial pathogens but not others. In this regard, it is important to note in this study each of the bacterial strains were administered by intraperitoneal injection, and therefore it remains possible that MAIT cells could facilitate bacterial containment of the same strains following mucosal routes of infection. Here, we postulate that MAIT cells may have anatomic localization that might favor their role in the respiratory tract.

Francisella

In support of the hypothesis, Meierovics et al. evaluated the role of MAIT cells in the host response to a low-dose intranasal infection with the Gram-negative bacterium, live vaccine strain of *Francisella tularensis* (LVS). In wild type mice, MAIT cells were enriched in the lungs as early as 8 days and up until the latest time point tested (18 days) post-infection. Furthermore, in wild type mice, the frequency of MAIT cells increased in the lung as the bacterial burden decreased, indicating an inverse correlation between the frequency of MAIT cells and the bacterial burden. Alternatively, at days 10–15 post infection, MR1 $^{-/-}$ mice had significantly higher bacterial burden, 1×10^6 CFU versus $1-5 \times 10^5$ CFU in wild type mice. Whereas it took wild type

mice ~17 days to clear the infection, it took MR1 $^{-/-}$ mice five additional days (~22 days). These findings support the hypothesis that MAIT cells contribute to early control of LVS in mice.

Mucosal associated invariant cells in the lungs of mice infected with LVS were pro-inflammatory as they produced IFN- γ , TNF, and IL17A during both early and late infection. As described for the intracellular containment of BCG, co-incubation of purified MAIT cells with LVS-infected macrophages resulted in bacterial growth inhibition in an IL-12-dependent manner. Together, these data suggest that the increase in MAIT cell frequencies in response to pulmonary LVS infections and early control of the bacteria within the lungs is MR1-dependent, but that IL-12 plays a direct role in the containment of intracellular infection.

A Role for MAIT Cells in Human Antibacterial Immunity

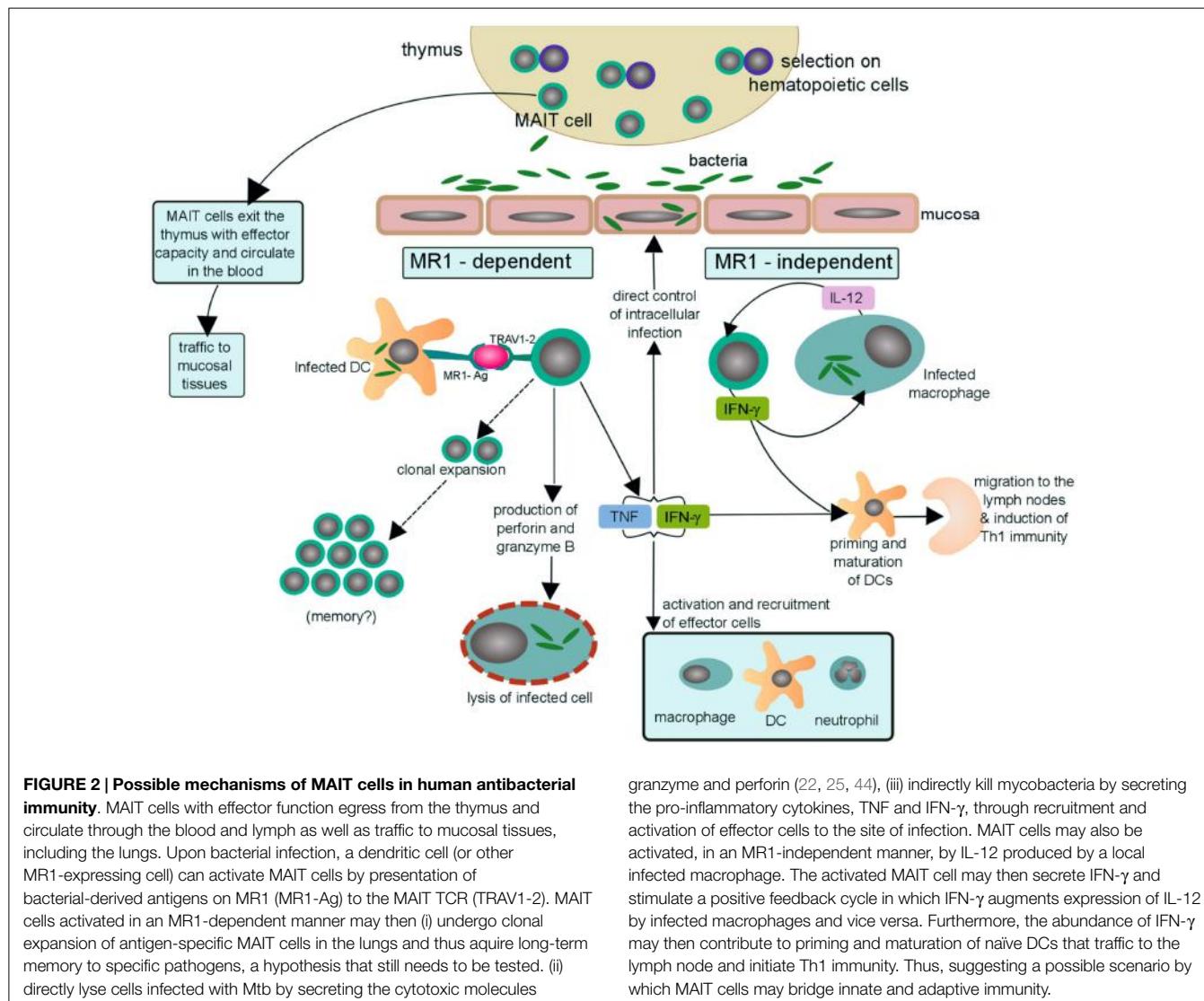
Several studies have implicated MAIT cells in the recognition and possible control of intracellular infection in humans. MAIT cells are present in equal or greater numbers in the lungs of healthy humans, compared to peripheral blood and lymph nodes (7, 8). Thus, MAIT cells are physically positioned to serve as sentinels in the detection of respiratory pathogens, such as Mtb, in the lung. The plausibility of MAIT cells serving as sensors of pulmonary infection with Mtb is supported the observation that subjects with pulmonary TB have diminished MAIT cell frequencies in the peripheral circulation (7, 8). These findings could reflect a dynamic relationship where the number of MAIT cells in peripheral blood is inversely correlated with the presence of Mtb in the lung. Here, we postulate that the presence of mycobacterial antigen in the lungs results in trafficking to the site of infection and possible expansion of antigen-selective MAIT cells. The hypothesis that bacterial infection could result in the egress of MAIT cells from the peripheral circulation is supported by the observation that MAIT cell frequencies are diminished in the blood of individuals with bacterial sepsis (43).

The observations made thus far in humans and mice can be used to develop a model by which MAIT cells contribute to the control of intracellular infection. This model is presented in **Figure 2** and possesses the following: (1) thymically derived MAIT cells have inherent effector capacity, and by virtue of their preferential residence in mucosal sites can serve as early sensors of bacterial or fungal infection; (2) early recognition of an infected cell could result in the direct control of intracellular infection through the release of pro-inflammatory cytokines and/or cytotoxic molecules; (3) early recognition of an infected cell could promote the acquisition of Th1 immunity through the release of IFN- γ which in turn promotes the production of IL-12 from tissue-resident DCs; (4) IL-12 may play a special role both in the promotion of Th1 immunity and augmentation of the MAIT cell response; and (5) microbial exposure results in the expansion and maintenance of MAIT cells that are selective for these pathogens.

Outstanding Questions

MAIT Cells in Autoimmunity

Mucosal associated invariant cells have been implicated in autoimmunity and inflammatory disorders, including chronic



inflammatory demyelinating polyneuropathy, experimental autoimmune encephalomyelitis, multiple sclerosis, arthritis, celiac disease, and as mentioned previously IBD (41, 45–50). One hypothesis is that these autoimmune conditions could be instigated or exacerbated by prior or ongoing microbial infections. Here, we postulate that bacterially-derived MR1 ligands could serve as “molecular mimics” of host-derived compounds allowing for MAIT cell autoreactivity. In this context, the MAIT cell TCR may recognize host-derived pterins or pterin-like small molecules that may serve as ligands for MR1. Additionally, host-derived molecules may bind MR1 and activate MAIT cells, or like 6FP, serve to stabilize MR1.

It is also possible that MAIT cells may be activated in an MR1-independent manner by pro-inflammatory cytokines as a result of ongoing tissue inflammation. These activated MAIT cells may then secrete pro-inflammatory cytokines or exert their cytolytic potential in an indiscriminate manner. While MAIT cells have been associated with autoimmunity, it remains unknown if they serve a pathological role in these diseases. It is also possible that

they play a protective or regulatory role. With the advent of the MR1 tetramer and the development of comprehensive *ex vivo* functional assessment, the physiological function of these cells in autoimmunity can be further elucidated.

The Role of MAIT Cells in Vaccination

Vaccines serve to initiate long-term protection through induction of adaptive immunity. Currently available vaccines are often administered systemically, and hence may be less effective in the initiation and maintenance of mucosal immunity. The desirability of mucosal vaccination might be particularly evident in infections that are spread via aerosol (51). In this regard, the role that MAIT cells might play in a mucosal vaccine is largely unexplored. One possibility is that MAIT cell expansions in the lung do not reflect long-term memory but depend on the ongoing presence of microbial ligand. In this regard, it might be envisioned that MAIT cells could be harnessed as “adjuvants” in mucosally delivered vaccines. Alternately, if MAIT cells have memory, then vaccination with selective ligands could result in the stable expansion of MAIT cells.

Could MAIT Cells Be Used as Mucosal Adjuvants?

Given that conventional systemic vaccines rely on the presence of an adjuvant to elicit sustained B cell and T cell responses, we postulate that the activation of MAIT cells in the lung could serve to facilitate the acquisition of adaptive immunity. At present, FDA-approved adjuvants are TLR agonists. While these are traditionally thought to result in the direct activation of APCs such as DCs, we postulate that the activation of lung-resident MAIT cells could promote the acquisition of Th1 immunity, and hence could serve as an adjuvant. With the recent discovery of vitamin B metabolites serving as MAIT cell antigens, one could envision ribityllumazines as a class of “Vita-PAMPS,” that if safely delivered to the mucosal surface where MAIT cells are present, could serve as a mucosal adjuvant (52).

Could MAIT Cells Be Targeted for a Mucosal Vaccine?

Alternately, we speculate that the expansion and maintenance of microbial selective MAIT cells could be used as a mucosal vaccination strategy. These approaches will depend on the identification of microbial-selective ligands, their stable formulation, and their delivery either systemically or via aerosol. While either the adjuvant or vaccination strategies are attractive, significant challenges remain. First, antigens that are selective for discrete pathogens, such as *Mtb*, remain to be identified. Second, many of the ligands identified to date are unstable, making them unsuitable for vaccination studies. Third, animal models that reflect a human MAIT cell immunobiology have not been developed. In this regard, a mouse model would be ideal, and will likely require the identification of mouse strains that have MAIT cell frequencies and reactivity that are similar to humans. Alternately, it is possible that other animal species, such as the rabbit, guinea pig, or non-human primates, may be needed.

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Concluding Remarks

While existing data strongly support a role for MAITs in the recognition and control of microbial infection, particularly at the mucosal surface, there remains much that is not known. For humans, a direct demonstration of the role of MAIT cells in the host response to microbial infection will either require the identification of MR1 variants that alter MAIT cell function, and potentially increase the host-vulnerability to infection, or the identification of altered developmental pathways that alter MAIT cell frequency and function. In this regard, it might be expected that these deficiencies affect other cell types, such as iNKT cells. Alternately, vaccination strategies that harness MAIT cells would also allow for more direct demonstration of the protective capacity of MAIT cells. Finally, given the tight regulation of MR1 surface expression and MAIT cell activation, it is possible that microbial activation of MAIT cells could have the unintended consequence of promoting autoimmunity.

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CD1d- and MR1-restricted T cells in sepsis

Peter A. Szabo^{1†}, Ram V. Anantha^{1,2†}, Christopher R. Shaler¹, John K. McCormick^{1,3,4} and S.M. Mansour Haeryfar^{1,3,4,5*}

¹Department of Microbiology and Immunology, Western University, London, ON, Canada, ²Division of General Surgery, Department of Medicine, Western University, London, ON, Canada, ³Centre for Human Immunology, Western University, London, ON, Canada, ⁴Lawson Health Research Institute, London, ON, Canada, ⁵Division of Clinical Immunology and Allergy, Department of Medicine, Western University, London, ON, Canada

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Olivier Lantz,
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University of Southern California, USA

*Correspondence:

S. M. Mansour Haeryfar,
Department of Microbiology and
Immunology, Schulich School of
Medicine and Dentistry, Western
University, 1151 Richmond Street,
London, ON N6A 5C1, Canada
mansour.haeryfar@schulich.uwo.ca

[†]Peter A. Szabo and Ram V. Anantha
have contributed equally to this work.

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Dysregulated immune responses to infection, such as those encountered in sepsis, can be catastrophic. Sepsis is typically triggered by an overwhelming systemic response to an infectious agent(s) and is associated with high morbidity and mortality even under optimal critical care. Recent studies have implicated unconventional, innate-like T lymphocytes, including CD1d- and MR1-restricted T cells as effectors and/or regulators of inflammatory responses during sepsis. These cell types are typified by invariant natural killer T (iNKT) cells, variant NKT (vNKT) cells, and mucosa-associated invariant T (MAIT) cells. iNKT and vNKT cells are CD1d-restricted, lipid-reactive cells with remarkable immunoregulatory properties. MAIT cells participate in antimicrobial defense, and are restricted by major histocompatibility complex-related protein 1 (MR1), which displays microbe-derived vitamin B metabolites. Importantly, NKT and MAIT cells are rapid and potent producers of immunomodulatory cytokines. Therefore, they may be considered attractive targets during the early hyperinflammatory phase of sepsis when immediate interventions are urgently needed, and also in later phases when adjuvant immunotherapies could potentially reverse the dangerous state of immunosuppression. We will highlight recent findings that point to the significance or the therapeutic potentials of NKT and MAIT cells in sepsis and will also discuss what lies ahead in research in this area.

Keywords: CD1d, MR1, NKT cell, MAIT cell, LPS, α -galactosylceramide, infection, sepsis

Preamble

Sepsis is a life-threatening syndrome typically associated with early hyperinflammation, immunosuppression in its protracted phase, and a continuum of organ dysfunction abnormalities. It is a significant cause of death across all age groups and in both developed and developing countries. It also negatively affects the quality of life among survivors. Sepsis is usually a consequence of infection although sterile tissue damage inflicted by non-infectious causes or conditions, such as pancreatitis, ischemia-reperfusion injury, and cancer may also lead to sepsis (1). In this article, we will only focus on the syndrome caused by disproportionate, excessive, or sometimes defective host responses to infection. We will provide a general overview of sepsis, its epidemiology, prognosis, management, and immunopathogenesis. We will briefly discuss experimental immunotherapeutic strategies tested in animal models of sepsis or used in clinical trials. Many such strategies have targeted antigen-presenting cells (APCs) and conventional T cells or their products, such as inflammatory cytokines, albeit with little success. Recent progress in our understanding of natural killer T (NKT)

cell and mucosa-associated invariant T (MAIT) cell responses to infection and their regulatory functions may open a new front in our fight against sepsis. These unconventional T cells respond rapidly to infection by secreting large quantities of pro- and/or anti-inflammatory cytokines, thereby controlling the effector functions of numerous other cell types belonging to both innate and adaptive arms of immunity. Also importantly, NKT cells can be easily manipulated by “disease-tailored” synthetic glycolipids. Therefore, the quick and wide-ranging actions of NKT cells, and potentially of MAIT cells, may be exploited to the host’s benefit in different forms or stages of sepsis. We will review NKT and MAIT cell functions in antimicrobial immunity and highlight recent findings on these cell types in the context of sepsis.

NKT Cells: A Brief Overview

Natural killer T cells are innate-like T lymphocytes with impressive immunomodulatory properties. They express glycolipid-reactive $\alpha\beta$ T cell receptors (TCRs) along with several characteristic markers of NK cells (e.g., mouse NK1.1 and human CD161) (2, 3). NKT cells develop in the thymus where they are positively selected by CD1d $^+$ CD4 $^+$ CD8 $^+$ thymocytes and consequently become “CD1d-restricted” (4). As such, CD1d-deficient mice are devoid of NKT cells (5). CD1d is a monomorphic major histocompatibility complex (MHC) class I-like glycoprotein that is highly conserved across mammalian species (6). It is a member of the CD1 family of lipid antigen (Ag)-presenting molecules (7, 8). The CD1 family in human has five members, namely CD1a–e, while rodents only express CD1d. Murine and human CD1d can present normal self- and tumor-derived lipids as well as microbial glycolipids to NKT cells. The discovery of CD1d restriction led to the invention of glycolipid-loaded CD1d tetramer reagents enabling accurate tracking, enumeration, and phenotypic and functional analysis of NKT cells (9–11).

The major subset of NKT cells is defined by the expression of a canonical or invariant TCR (*i*TCR) with a unique α chain rearrangement (V α 14–J α 18 and V α 24–J α 18 in mice and humans, respectively), which is paired with one of only a limited choices of β chains (V β 8.2, V β 2 or V β 7 in mice and V β 11 in humans). These cells are called type I or invariant NKT (*i*NKT) cells (2, 3). Two phenotypically distinct subpopulations of *i*NKT cells have been identified in mice, the CD4 $^+$ CD8 $^-$ subset and the double-negative (CD4 $^-$ CD8 $^-$) subset (12). An additional CD8 α^+ subset exists in humans (13). *i*NKT cells constitutively express CD69, CD25, and CD44 on their surface, which is consistent with their “partially-activated” or “memory-like” status even in germ-free mice (14) and in human cord blood (15).

*i*NKT cells are present at low frequencies in the circulation and in various tissues including bone marrow, thymus, spleen, and lymph nodes. However, they are abundant in the mouse liver and in the human omentum (16). The prevalence of *i*NKT cells varies considerably among different individuals for reasons that are currently unknown. Also importantly, *i*NKT cell subsets found in different anatomical locations exhibit functional or even transcriptional heterogeneity. For instance, interleukin (IL)-4- and IL-13-producing human peripheral blood *i*NKT cells fall exclusively within the CD4 $^+$ subset, whereas *i*NKT cells that

synthesize tumor necrosis factor (TNF)- α and interferon (IFN)- γ upon *ex vivo* stimulation can be either CD4 $^+$ or double negative (17). Another example is the case of adipose tissue *i*NKT cells that secrete IL-10, impart an anti-inflammatory phenotype to macrophages, and control the expansion and suppressor function of regulatory T (Treg) cells (18). Moreover, adipose tissue *i*NKT cells lack promyelocytic leukemia zinc finger (PLZF), a transcription factor otherwise regarded as a “master regulator” of *i*NKT cell effector functions (19).

*i*NKT cells are armed with cytotoxic effector molecules such as perforin, granzymes, TNF- α , Fas ligand, and TNF-related apoptosis-inducing ligand (TRAIL), and may be able to lyse neoplastic or infected cells directly (20–22). However, they are best known for their immunomodulatory functions mediated by the early production of pro- and/or anti-inflammatory cytokines. *i*NKT cells can thus transactivate numerous downstream effector cell types including natural killer (NK) cells, macrophages, dendritic cells (DCs), conventional CD4 $^+$ and CD8 $^+$ T cells, and B cells. They are rapid producers of enormous quantities of T helper (Th)1-, Th2-, and Th17-type cytokines, although Th9- and Th10-like *i*NKT cells have also been described (23, 24). The constitutive presence of pre-formed messenger RNA (mRNA) encoding at least some of such cytokines in *i*NKT cells explains the rapidity with which they are released (25).

The identity of endogenous CD1d ligand(s) that participate in positive selection and also perhaps in peripheral maintenance of *i*NKT cells remains ill-defined and controversial. *i*NKT cells can recognize and respond to certain glycolipids present in various microbes, including but not limited to *Novosphingobium* spp., *Ehrlichia* spp., *Borrelia burgdorferi*, *Streptococcus pneumoniae*, and *Streptococcus agalactiae* (26–28). Of note, the latter pathogen, which is often referred to as group B streptococcus, is a common cause of neonatal sepsis.

Of all exogenous glycolipid agonists of *i*NKT cells, α -galactosylceramide (α -GalCer) has been used most extensively, not only as a research tool but also in clinical trials for cancer and viral diseases (29). α -GalCer was initially isolated from an extract of a marine sponge called *Agelas mauritanicus* (30), and is believed to have originated from microbes co-existing in a symbiotic relationship with this sponge. Until recently, α -GalCer was considered to be a merely exogenous and unnatural glycolipid given the presence of only one glucosylceramide synthase and one galactosylceramide synthase in mammalian species, both of which are β -transferases. However, a recent report has demonstrated the presence of endogenous α -anomeric glycolipids including α -GalCer in mammals, due perhaps to the operation of an “unfaithful” enzyme or a novel, as-yet-unidentified pathway (31).

α -GalCer and its analogs possess a lipid tail that can be buried deep inside the hydrophobic pocket of CD1d, while their galactose head protrudes out of CD1d to be contacted by the *i*TCR α chain (32). The length and composition of acyl and phytosphingosine chains of synthetic α -GalCer analogs impact the binding affinity of α -GalCer:CD1d:*i*TCR interactions (33), which partially determines the type of cytokines that an activated *i*NKT cell will secrete. For example, OCH is a sphingosine-truncated derivative of α -GalCer with Th2-skewing

characteristics (34). We have successfully used this glycolipid to delay Th1-mediated cardiac allograft rejection (35), to prevent or cure citrulline-induced autoimmune arthritis (36), and to reduce the severity of intra-abdominal, polymicrobial sepsis (37) in mouse models. Another Th2-favoring agonist of *i*NKT cells is C20:2, an α -GalCer analog with a short fatty acyl chain containing two unsaturation sites at carbon-11 and -14 positions (38). C20:2 is reportedly superior to OCH in polarizing human *i*NKT cells. Th1-biasing ligands of *i*NKT cells can be exemplified by a C-glycoside analog of α -GalCer, also known as α -C-GalCer, which potentiates IL-12 and IFN- γ production in mice (39). Therefore, α -C-GalCer may be useful in adjuvant glycolipid immunotherapy of cancer and infectious diseases.

Cell membrane location of glycolipid loading onto CD1d and its presentation within or outside lipid rafts (40), the type of CD1d $^+$ APCs involved (41), the presence and intensity of costimulatory and danger signals transmitted or exchanged (29), and the cytokine milieu in which *f*NKT cell priming occurs are among other important factors that shape the cytokine profiles of *i*NKT cells. Remarkably, mouse *i*NKT cells can recognize human CD1d and *vice versa* (6), and *i*NKT cells from either species are responsive to α -GalCer. Therefore, at least some of the findings obtained in mouse models of CD1d-mediated *i*NKT cell activation are likely to be translatable to the clinic.

*i*NKT cells can also be activated in the absence of exogenous glycolipids. During infection, microbial components may engage pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) on APCs, thus resulting in secretion of IL-12 and IL-18. Together, these cytokines trigger indirect activation of *i*NKT cells (42, 43), which is often dependent upon the presence of CD1d. This indicates an intriguing but poorly understood role for endogenous lipids in the context of antimicrobial immunity. A combination of IL-12 and IL-18 can also reportedly induce *i*NKT cell responses in a truly *i*TCR-independent fashion (44). We recently reported that group II bacterial superantigens (SAGs) can directly activate *i*NKT cells in a CD1d-independent manner (45). Therefore, *i*NKT cells may serve as effectors and/or regulators of early cytokine responses to bacterial SAGs.

Type II or variant NKT (ν NKT) cells are CD1d-restricted cells with a relatively diverse $\alpha\beta$ TCR repertoire (3, 11). They exhibit reactivity with certain self lipids, but not with α -GalCer (46). Compared with *i*NKT cells, ν NKT cells are less frequent in mice but more prevalent in humans (47). A major fraction of ν NKT cells can recognize sulfatide, a self glycolipid that is highly enriched in the central nervous system, kidney and liver (48). Several other endogenous lipids, including but not limited to β -D-glucopyranosylceramide (β -GlcCer), have also been recently discovered to activate ν NKT cells (49). Given the relative promiscuity of ν NKT cell Ag receptors, it is not too far-fetched to envisage scenarios where ν NKT cells recognize microbial lipids cross-reactive to self components. In addition, infection may lead to the release of self lipids in sufficient quantities to induce ν NKT cell activation.

Our overall understanding of ν NKT cell responses in health and disease is limited. This is in large part due to a lack of firm molecular markers, stable reagents and direct methods to detect

and characterize ν NKT cells. Sulfatide-loaded CD1d-tetramer reagents have been generated (46). However, they are not popular due to their low stability and high background staining. In addition, there is no mouse model of pure ν NKT cell deficiency. CD1d $^{-/-}$ mice are devoid of both *i*NKT and ν NKT cells since CD1d is required for the positive selection of both cell types in the thymus (50). Experimental evidence indicates that ν NKT cells have an activated phenotype and depend on PLZF for their development (49), and that ν NKT and *i*NKT cells may exert opposing functions with broad implications for antitumor responses (51) and antimicrobial immunity (52, 53).

MAIT Cells and Their Roles in Microbial Immunity

MAIT cells are another evolutionarily conserved subset of innate T lymphocytes that have captured the attention of the immunological community in the past few years (54, 55). MAIT cells develop in the thymus where they rearrange their semi-invariant TCR with a characteristic V α 19–J α 33 and V α 7.2–J α 33 TCR α chain in mice and humans, respectively (56, 57).

Similar to NKT cells, MAIT cells are positively selected by CD4 $^+$ CD8 $^+$ thymocytes (58). However, their selection requires the expression of MHC-related protein 1 (MR1), as opposed to CD1d, on thymocytes. Accordingly, MR1-deficient mice lack MAIT cells in their T cell repertoire (59). MR1 is a monomorphic, non-classical MHC I molecule that is markedly conserved among various mammals (60–62). There is 90% sequence homology between mouse and human MR1 ligand-binding domains and a high degree of functional cross-reactivity, which is highly reminiscent of cross-species CD1d conservation.

MAIT cells are infrequent and immature in the human fetal thymus (63). Their maturation is accompanied by a gradual, post-thymic acquisition of PLZF expression and the ability to secrete IFN- γ and IL-22 upon exposure to microbes in mucosal layers. A PLZF-expressing CD161 high CD8 $^+$ population is detectable in human cord blood, from which V α 7.2 $^+$ MAIT cells emerge in adults (64).

MAIT cells are severely depleted in B cell-deficient patients and mice, and are also entirely absent in the peripheral tissues of germ-free mice (59), indicating that B cells and commensal microflora are essential for MAIT cell peripheral maintenance/expansion. Therefore, it is not surprising that MAIT cells preferentially accumulate in the mucosal compartments, such as the gut lamina propria, hence their denomination. MAIT cells are also present in other tissues. In human, they are particularly abundant in peripheral blood and can comprise up to ~50% of all T cells in the liver (65, 66). There are far fewer MAIT cells in mice than in humans. This, together with other differences between the two species (67), indicates that caution needs to be exercised in extrapolating experimental data from mice to human conditions.

Until recently, there was no single reagent to directly detect mouse MAIT cells. In addition, human MAIT cells have been commonly defined as CD3 $^+$ V α 7.2 $^+$ CD161 $^+$. However, recent identification of a MAIT cell Ag, namely reduced 6-hydroxymethyl-8-D-ribityllumazine (rRL-6-CH₂OH), led to the development of MR1 tetramer reagents loaded with this compound to

accurately identify mouse and human MAIT cells (68). Once widely available, these reagents will undoubtedly boost research in the area of MAIT cell biology. Human peripheral blood MAIT cells are CD45RA⁻CD45RO⁺CD62L^{low}CD95^{high}, which is consistent with an effector memory phenotype (65). They also express the receptors for IL-12, -18, and -23. Human hepatic MAIT cells have a more activated phenotype and express elevated levels of CD69 in comparison with their blood counterparts (66). They are also human leukocyte Ag (HLA)-DR⁺ and CD38⁺. This may be due to continuous exposure to microbial Ags accessing the liver from the gut through the portal system.

MAIT cells bridge innate and adaptive arms of immunity to microbial intruders. They quickly amass in sites of infection where they can keep pathogens in check. For instance, in a mouse model of pulmonary infection with *Francisella tularensis*, MAIT cells reduce bacterial burden in the lungs and prevent mortality from infection even in the absence of conventional T cells (69). They can produce inflammatory cytokines such as IFN- γ , IL-17, and TNF- α readily, amply and promptly after TCR stimulation (54, 55). Human MAIT cells express granzymes A and K, and are able to kill infected cells (70). They were shown to lyse, in an MR1-dependent fashion, epithelial cells infected by the intestinal pathogen *Shigella flexneri* (71), and THP1 monocytic cells infected by *Escherichia coli* (*E. coli*) (70). MAIT cells are responsive to a variety of bacteria and yeasts including *Lactobacillus acidophilus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* (*S. aureus*), *Staphylococcus epidermidis*, *Candida albicans*, *Candida galbrata*, and *Saccharomyces cerevisiae*. A limited number of studies have utilized MR1-deficient mice to explore the antimicrobial potentials of MAIT cells *in vivo*. The ability to control infection with *Klebsiella pneumoniae*, *Mycobacterium bovis* bacillus Calmette–Guérin (BCG), or *Francisella tularensis* was found to be impaired in MR1-deficient mice (69, 72, 73).

McCluskey's and Rossjohn's research teams discovered that vitamin B metabolites represent a class of MR1-restricted Ags (74). A folic acid (vitamin B9) metabolite called 6-formyl pterin (6-FP) was found to bind MR1 without stimulating MAIT cells. In contrast, MR1 ligands derived from the riboflavin (vitamin B2) biosynthesis pathway could activate MAIT cells. Of note, this pathway is operational in all of the microorganisms that activate MAIT cells, but not in those that reportedly fail to do so.

To confirm that the riboflavin pathway supplies human MAIT cell ligands, Corbett et al. mutated various enzymes of the riboflavin operon in the Gram-positive bacterium *Lactococcus lactis* followed by testing the MAIT cell-activating capacity of the mutants (75). This approach led to the identification of 5-amino-6-D-ribitylaminouracil (5-A-RU), an early intermediate of the riboflavin pathway, as a key compound in generating MAIT cell "neo-antigens." Through non-enzymatic interactions, 5-A-RU forms simple adducts with small molecules arising from other metabolic pathways (e.g., glycolysis), such as glyoxal and methylglyoxal, thus giving rise to 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU) and 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU), respectively. MR1 in turn captures, stabilizes, and presents these neo-antigens to MAIT cells. Recent work from Olivier Lantz's laboratory demonstrated that most, if not all, mouse MAIT cell ligands harbored by the Gram-negative

bacterium *E. coli* are also related to the riboflavin pathway (76). MR1-mediated activation of mouse MAIT cells was most robust upon stimulation with a mixture of 5-A-RU and methylglyoxal, and also detectable when a combination of 5-A-RU and glyoxal was used. This study also reported the synthesis of a new 6-FP variant in which the amine and the formyl group are blocked. This compound could efficiently inhibit the activation of MAIT cells by semipurified soluble bacteria (SPB) or by 5-A-RU plus methylglyoxal, and may therefore represent a new class of inhibitors of MAIT cell activation. Finally and importantly, *in vivo* activation of MAIT cells was demonstrated for the first time when iV α 19 transgenic mice on a C $\alpha^{-/-}$ background, which harbor many MAIT cells, were directly injected with the SPB fraction from riboflavin-sufficient *E. coli* or with a mixture of 5-A-RU and methylglyoxal. Interestingly, administration of 5-A-RU alone failed to activate MAIT cells, which may be probably due to its instability and/or low bioavailability for interaction with small metabolites and loading onto MR1 (76).

Mammals do not synthesize riboflavin, but host-derived metabolites could potentially generate adducts with 5-A-RU of bacterial origin (75). MR1-restricted recognition of the formed neo-antigens may be considered a new mechanism of self–non-self discrimination, especially in mucosa-associated lymphoid tissues. MR1 ligands are ubiquitous and present in many bacteria, including commensals. In addition, they can readily diffuse across epithelial barriers (55). Therefore, how MAIT cell activation is controlled *in vivo* remains enigmatic at this point.

MR1-independent responses can also be mounted by MAIT cells. The *in vitro* response of MAIT cells to BCG-infected cells is an example (73). Moreover, MAIT cells can produce IFN- γ when cultured with a combination of IL-12 and IL-18 in the absence of TCR triggering (77). Therefore, bystander activation of MAIT cells may occur during infection with viral pathogens or other germs that do not harbor MR1 ligands.

Sepsis

Definitions and Epidemiology

Although sepsis is often discussed in the context of intensive care in modern settings, the syndrome is almost as old as medicine itself. Derived from the Greek *sipsi* meaning "make rotten," the term sepsis was first coined by Hippocrates (460–370 BC) to describe the unpleasant process of organic matter putrefaction (78). Avicenna (980–1037 AD), the great Persian physician/scientist/philosopher, noted the frequent coincidence of blood putrefaction, what is known today as septicemia, and fever in the aftermath of surgery (79). The centuries that followed witnessed important discoveries linking germs to a wide array of disorders including sepsis. However, the germ theory of disease failed to fully explain the pathogenesis of sepsis since many patients succumbed to it despite successful eradication of the microbial intruder(s). Therefore, the host response to the germ, and not the germ *per se*, was proposed to drive the pathogenesis of sepsis (80).

The modern terminology for sepsis and its sequelae was standardized during an American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference in 1991 (81).

Accordingly, sepsis is defined as documented or suspected infection accompanied by at least two of the following abnormalities: (i) a body temperature of $>38^{\circ}\text{C}$ or $<36^{\circ}\text{C}$; (ii) a heart rate of >90 beats/min; (iii) a respiratory rate of >20 breaths/min or PaCO_2 of <32 mm Hg; (iv) a blood leukocyte count of $>12,000/\text{mm}^3$ or $<4,000/\text{mm}^3$, or detection of $>10\%$ immature neutrophils (*aka.* band cells) in the leukocyte differential count. The panel of experts recommended the application of the term “severe sepsis” when sepsis is further complicated by organ dysfunction, perfusion abnormalities (e.g., lactic acidosis, oliguria, acute alteration in mental status), or hypotension (a systolic blood pressure of <90 mm Hg or a reduction of ≥ 40 mm Hg from the baseline in the absence of other causes of hypotension). It needs to be noted that the terms “sepsis” and “severe sepsis” have often been used interchangeably. Finally, a severely septic patient should be classified as having “septic shock” when her/his hypotensive state is refractory to fluid resuscitation.

Sepsis is a leading cause of death following hospitalization and represents a major challenge in the management of critically ill patients in non-coronary intensive care units (ICUs) (82). It is estimated that 25% of patients who develop severe sepsis die during hospitalization, and septic shock is associated with mortality rates approaching 50% (83). Alarmingly, the incidence of severe sepsis is on the rise (84). Of equal importance, sepsis worsens the quality of life among survivors and increases their risk of morbidity and early death. In fact, the 5-year mortality rate in the sepsis survivor pool can be as high as 75% (84).

Risk Factors and Prognosis

In general, the prognosis of sepsis is dependent upon demographic, socioeconomic, and iatrogenic factors in addition to the patient’s medical history, immunological, nutritional and overall health status, and the type of microorganism(s) involved in triggering or perpetuation of sepsis (79, 85). For instance, being over 65 years of age, being a male, being a nursing home resident, being in a poor nutritional state, having low household income, or receiving treatment in a non-teaching hospital predisposes to sepsis and to its elevated severity. Several studies have found that age is an independent predictor of mortality from sepsis (86–88). However, the elderly are vulnerable to sepsis also due to a higher likelihood of pre- or co-existing morbidities (e.g., diabetes and cardiovascular problems) requiring medication, malnutrition, repeated and/or prolonged hospitalizations, decline in immunity, and functional restrictions (89). Some of the above factors are taken into account in calculation of Mortality in Emergency Department Sepsis (MEDS) score to predict 1-year mortality (85).

Adverse iatrogenic factors include steroid therapy and immunosuppression prior to surgery and a need for multiple operations (79). Invasive devices such as urinary and intravenous catheters and breathing tubes also increase the risk of sepsis. The main predisposing factor for urinary tract infections, which are the most frequent nosocomial infections in surgical patients, is the usage of an indwelling urinary catheter. Vascular catheters, especially central venous catheters, are also common vehicles for nosocomial infections caused by Gram-positive skin commensals.

It cannot be overstated that the prognosis of sepsis is also determined by the speed with which the diagnosis is established and proper management strategies implemented. The earlier the treatment is started, the more favorable the outcome will be.

Clinical Management

Despite advances in our understanding of sepsis at organismal, cellular and molecular levels, not even a single drug is approved as a mechanism-based treatment option for sepsis. The clinical guidelines established by the Surviving Sepsis Campaign (SSC), an international consortium of professional societies committed to reducing mortality from severe sepsis and septic shock, are organized into two “bundles,” each comprising a select but non-specific set of care elements distilled from evidence-based practice (90). The initial “resuscitation bundle” should be applied within 6 h after the patient’s presentation to prevent or resolve cardiorespiratory insufficiency and to combat the immediate threats posed by uncontrolled infection(s). Hemodynamic resuscitation is achieved by administration of intravenous fluids and vasopressors while oxygen therapy and mechanical ventilation can also be supplied as needed. The timely management of infection requires obtaining blood cultures before broad-spectrum antibiotic therapy is launched as well as source control (e.g., drainage of pus). The subsequent “management bundle” is typically accomplished in the ICU where the attention is shifted toward monitoring and supporting vital organ functions and avoiding complications. In addition, the efficacy of antibiotic therapy is evaluated for potential de-escalation to prevent the emergence of microbial resistance and to lower the risk of drug toxicity (90, 91).

A recent meta-analysis of 13 randomized controlled trials has demonstrated that early goal-directed therapy, which is perhaps best exemplified by the SSC-recommended resuscitation bundle, reduces overall mortality from sepsis when initiated within the first 6 h (92). This should reinforce the notion that there usually exists a short window of opportunity in which current management strategies or novel future therapies are expected to be most effective.

Immunopathogenesis and Immunosuppression in Sepsis

Disproportionate or dysregulated immune responses to infection constitute a major culprit in sepsis-related death. Sepsis is no longer considered a merely or even mainly hyperinflammatory syndrome. Rather, in “sepsis-prone” individuals and conditions, infection triggers a highly complex response that is variable in proportion or in pro- versus anti-inflammatory nature depending upon the pathogen load and virulence, genetic and other host factors including age and co-morbidities, and the time point at which the response is evaluated (91). Pro-inflammatory responses mounted in septic patients help eradicate the inciting microbe(s) but may cause collateral organ damage. On the other hand, anti-inflammatory and immunosuppressive mechanisms contribute to tissue recovery but also make the patient susceptible to secondary infections and opportunistic pathogens, especially during protracted sepsis (93).

The pioneering studies of Tracey et al. in the mid-1980s revealed that many deleterious features of endotoxin administration to

rats could be simulated by human cachectin (*aka.* TNF) (94) and that cachectin-neutralizing antibody F(ab')₂ fragments could prevent acute and otherwise lethal septic shock in *E. coli*-infected baboons (95). We now know that acute septic shock, which occurs in a relatively small fraction of patients with sepsis, is indeed a dangerous immunopathology mediated by an overly exuberant TNF response (91). TNF- α and other pro-inflammatory cytokines including IL-1 β and IL-6 and chemokines like IL-8 are released from activated macrophages and other APCs after they sense the presence of invading microbes by PRRs (e.g., TLRs) and phagocytose them. This in turn leads to neutrophil mobilization, lymphocyte activation, and more pro-inflammatory cytokine (e.g., IFN- γ) secretion. These cytokines limit microbial infections, but their elevated levels are associated with a poor outcome in sepsis (96, 97). The pleotropic cytokine IL-3 was recently found to be an upstream orchestrator of inflammation in the early phase of polymicrobial sepsis modeled by the cecal ligation and puncture (CLP) procedure in mice (98). In addition, retrospective and prospective analyses of plasma IL-3 in septic patients linked heightened levels of this cytokine to a poor outcome.

Both pro- and anti-inflammatory processes get underway promptly after the initiation of sepsis. A hyperinflammatory “cytokine storm” dominates the initial phase in many patients and accounts for death within the first 3 days from septic shock and multiple organ failure in a substantial fraction of patients (99). However, more than 70% of deaths due to sepsis occur after the first 3 days, with many occurring weeks later. One needs to keep in mind that many, if not most, epidemiological studies on sepsis have been conducted in developed countries with an aging population and advanced ICU facilities. Therefore, the reported decline in mortality rates of early sepsis is likely owed to better management protocols and also perhaps a reflection of immunosenescence in the elderly.

Death during protracted sepsis is sometimes the result of the family's decision to withdraw aggressive support measures to switch to palliative care for patients with severe co-morbidities and a slim chance of recovery. However, the fact remains that with or without such decisions, many patients in this phase succumb to stubborn infections that are difficult to resolve even with broad-spectrum antimicrobial therapy and infection source control (100). In a retrospective review of macroscopic autopsy findings, approximately 77% of surgical ICU patients who had died from sepsis or septic shock were found to have continuous septic foci (101), suggesting a failure to clear the inciting pathogen and/or to eradicate nosocomial infections. This is thought to be a consequence of immunosuppression (99, 100), especially in patients who survive the early hyperinflammatory phase. The reported inability of many septic patients to elicit normal delayed-type hypersensitivity (DTH) skin reactions to standard recall Ags (102) and the frequent reactivation of latent viruses (e.g., cytomegalovirus, Epstein–Barr virus, herpes simplex virus, human herpesvirus-6), sometimes involving multiple viruses at the same time in prolonged sepsis (103), also point to a profound state of immunosuppression.

Multiple other findings lend support to the notion of sepsis-induced immunosuppression. In an earlier study, van Dissel et al. demonstrated that a high ratio of plasma IL-10:TNF- α correlates

with increased mortality in febrile patients with community-acquired infection and cautioned against the application of pro-inflammatory cytokine inhibition in sepsis (104). In a separate study, circulatory levels of IL-10 paralleled the sepsis score, and its sustained overproduction was deemed a predictor of severity and fatal outcome (105).

A global cytokine depression has been noted in numerous other studies. After stimulation with lipopolysaccharide (LPS), whole blood samples from septic patients contained less IL-1 β , TNF- α , and IL-6 in comparison with samples obtained from non-septic patients admitted for hernia repair or cholecystectomy (106). Munoz et al. reported a profound decrease in the ability of freshly isolated monocytes from ICU patients with sepsis to produce IL-1 β , TNF- α , and IL-6 following *ex vivo* exposure to LPS (107). An important finding of this investigation was that monocytes from the survivor subpopulation, but not from those who eventually died from sepsis, regained their cytokine production capacity. Also interestingly, the blunted pro-inflammatory cytokine response was most pronounced in patients with Gram-negative infections. This may be a manifestation of the long-known phenomenon of “endotoxin tolerance,” according to which LPS-exposed cells become refractory to subsequent LPS challenges (108). Endotoxin tolerance arguably serves to protect against uncontrolled inflammation in sepsis, but is also correlated with a high risk of secondary infection and mortality. In septic patients, monocytes are also hyporesponsive to CD40 ligation, which would otherwise result in the upregulation of classic costimulatory molecules B7-1 (CD80) and B7-2 (CD86) and enhanced ability of monocytes to activate T lymphocytes (109). The CD40–CD40L cross-talk does not directly involve the CD14/TLR-4 pathway governing cellular responses to LPS. Therefore, endotoxin tolerance may only partially explain monocyte hyporesponsiveness in sepsis.

Sepsis-induced immunological shortcomings are not limited to leukocytes traveling in the bloodstream. Boomer et al. found that post-mortem splenocytes from septic patients secreted significantly less TNF- α , IFN- γ , IL-6, and IL-10 in response to LPS, CD3/CD28 co-ligation, or stimulation with phorbol 12-myristate 13-acetate (PMA) plus ionomycin when compared with splenocytes from patients who were declared brain dead or those who underwent emergency splenectomy due to trauma (110). Moreover, cytofluorimetric analyses of splenic cell populations revealed signs of T cell exhaustion or anergy. For instance, the frequency of CD4 $^{+}$ T cells displaying the anergy/exhaustion marker programmed cell death 1 (PD-1) and that of CD8 $^{+}$ T cells expressing the prototype co-inhibitory molecule cytotoxic T-lymphocyte antigen-4 (CTLA-4) were higher in septic than in control patients. Both subsets also expressed low levels of IL-7 receptor α chain (CD127) that promotes cell survival. Consistent with these observations, splenic APCs from septic patients exhibited decreased B7-2 and HLA-DR and increased PD-ligand 1 (PD-L1) levels. It is noteworthy that weak expression of HLA-DR is a common abnormality in sepsis. In fact, measuring monocytic HLA-DR levels has been used to identify an immunosuppressed state in patients with sepsis and septic shock and to monitor the efficacy of sepsis immunotherapy (111).

Boomer et al. also demonstrated that within the post-mortem lung tissues of septic patients, PD-1 expression on CD4⁺ cells and PD-L1 expression on plasmacytoid dendritic cells (pDCs) were augmented in comparison with control lung tissues obtained from transplant donors or cancer resections (110). Finally, this comprehensive study reported two- and three-fold increases in the frequencies of splenic Treg cells and lung myeloid-derived suppressor cells (MDSCs), respectively, in sepsis. Treg cells are relatively resistant to sepsis-induced apoptosis, and their percentage increases also in the circulation of patients with sepsis (99, 112). Using the CLP mouse model, Delano et al. found that GR-1⁺CD11b⁺ MDSCs that produce IL-10 among other cytokines and skew T cell responses toward a Th2 phenotype increase numerically and remain elevated within the spleen, lymph nodes, and bone marrow (113). Therefore, suppressor cell function appears to be a significant component of immunosuppression in sepsis.

Apoptotic death of naïve and adaptive cells of the immune system also contributes to immunosuppression. We detected widespread apoptosis in the spleen of mice with feces-induced peritonitis (FIP), which we used as a model of intra-abdominal sepsis (114). This was due to a profound apoptotic loss of splenic T cells, B cells, NK cells, and macrophages (37). Hotchkiss et al. performed rapid tissue harvesting at the bedside of patients dying from sepsis and demonstrated a marked loss of splenic CD4⁺ T cells, B cells and DCs (115, 116). Felmet and coworkers reported similar depletions, prolonged lymphopenia, and hypocellularity accompanied by apoptosis in the thymus, spleen and lymph node autopsies of pediatric ICU patients with nosocomial sepsis and multiple organ failure (117). Toti et al. found a dramatic depletion of B and T cells in the spleen of preterm and full-term neonates who died of early-onset sepsis due, likely, to *in utero* infection with Gram-positive or -negative microbes (*aka.* chorioamnionitis) (118). These findings indicate that immune effector cell loss during sepsis is a universal phenomenon across all age groups.

Apoptosis causes immunosuppression through multiple mechanisms. First, severe depletion of B and T cells creates “holes in the repertoire” of adaptive lymphocytes. This jeopardizes the ability of the immune system to launch highly specific responses to pathogens. Furthermore, immunological memory cannot be built to protect the survivors at later time points. Apoptosis-mediated shrinkage of the DC compartment not only weakens innate immunity but also contributes to functional T cell inadequacies since naïve T cells can only be primed by DCs. Apoptotic cells are immunosuppressive by nature and their uptake by phagocytic cells can stimulate the release of anti-inflammatory cytokines such as IL-10 (119). In addition, after ingesting apoptotic bodies, DCs may induce death in T cells with which they interact or render them anergic (120). The importance of immune cell apoptosis in the pathogenesis of sepsis can be underscored by the observations that Bcl-2 overexpression or treatment with z-VAD-fmk, a pan-caspase inhibitor, improves survival in mouse models of sepsis (121, 122).

Immunotherapy for Sepsis

Advances in our understanding of the pathogenesis of sepsis have prompted more than 40 clinical trials of immunotherapeutic

agents to date. However, the results have been by and large disheartening, with many trials yielding no benefits while a few even aggravated the syndrome, thus leading to their premature termination.

Most previous trials have employed agents that neutralize pathogens or their products [e.g., intravenous immunoglobulin (123) and the anti-endotoxin antibody nebacumab (124)], interfere with pathogen recognition by the host [e.g., the TLR4 antagonist eritoran (125)], or target pro-inflammatory cytokines/mediators [e.g., the anti-TNF- α antibody afelimomab (126) and the recombinant TNF receptor p55–IgG1 Fc fusion protein lenercept (127)] or their receptors [e.g., the IL-1 receptor antagonist anakinra (128) and the platelet-activating factor receptor antagonist lexipafant (129)]. Pro-inflammatory cytokines sometimes exert redundant functions. Therefore, therapeutic approaches targeting individual cytokines are often ineffective. Non-specific corticosteroid therapy has also been used in sepsis, albeit to little avail (130).

Dampening hyperinflammatory responses may benefit some patients in the early phase of sepsis. However, it is now recognized that many others have a global cytokine depression or even a predominance of anti-inflammatory cytokines. Equally important is the fact that most patients rapidly progress to an immunosuppressed state associated with a higher susceptibility to secondary and opportunistic infections, in which case weakening the immune system may be counterintuitive. This may explain, at least partially, the failure of the vast majority of previous trials designed to block inflammatory mediators in sepsis. In fact, apart from prophylactic measures and antibiotic administration, adjuvant therapy to restore immune competence in immunosuppressed septic patients may prove beneficial or even lifesaving (99). In an earlier application of such approaches, Döcke et al. administered IFN- γ to a small cohort of septic patients whose monocytes had reduced HLA-DR expression and whose whole blood cells produced only minute amounts of TNF- α in response to LPS stimulation (131). Treatment with IFN- γ reversed these deficits and also importantly resulted in resolution of sepsis in most cases. In a more recent case report by Nalos and coworkers, successful IFN- γ therapy in a male patient with type-2 diabetes and prolonged, disseminated *S. aureus* sepsis was documented (132).

Granulocyte-macrophage colony-stimulating factor (GM-CSF), a hematopoietic growth factor that stimulates the production of neutrophils and monocytes from bone marrow stem cells, has also been used and shown promise in immunosuppressed septic patients. In a relatively small-scale clinical trial, GM-CSF administration was safe and normalized the expression of monocytic HLA-DR and shortened the duration of mechanical ventilation and hospital/ICU stay due to sepsis (111). In a subsequent study, GM-CSF restored the *ex vivo* TNF- α production capacity of whole blood cells and prevented nosocomial infections in pediatric patients with multiple organ dysfunction syndrome (133).

IL-7 and IL-15 are two other immune-enhancing cytokines with enormous therapeutic potentials. Dubbed as the “maestro of the immune system” (134), IL-7 is a pleiotropic cytokine with diverse biological properties, some of which may correct

immunological abnormalities linked to sepsis. Clinical trials of IL-7 in other conditions (e.g., metastatic cancer, HIV-1 infection, and progressive multifocal leukoencephalopathy) have demonstrated that its systemic administration is safe and well tolerated (135–137). Furthermore, it seldom causes fever or significant pro-inflammatory cytokine production. IL-7 induces naïve and memory T cell proliferation without a predilection for Treg cell expansion (138). Therefore, its administration could potentially replenish the T cell pool following drastic lymphocyte depletion in sepsis. IL-7 is known to upregulate the expression of the anti-apoptotic molecule Bcl-2 in T cells, thus promoting their survival (139) and that of cell adhesion molecules (140), thus potentiating leukocyte trafficking into the site(s) of infection. In addition, treatment with IL-7 increases the diversity of the TCR repertoire (138, 139), which in turn improves the breadth of pathogen-specific T cell responses. Together, these activities can immensely help combat pathogens during sepsis. The therapeutic benefit of IL-7 has been validated in CLP. Using this animal model, Unsinger et al. found that recombinant human IL-7 (rhIL-7) can normalize the DTH reaction, block T cell apoptosis, restore IFN- γ production, and improve host survival (140). Similar results were obtained in a “two-hit” model of fungal sepsis in which mice underwent CLP to induce peritonitis followed by an intravenous injection of *Candida albicans* (141) to mimic delayed secondary infections in ICU patients. Venet et al. reported that IL-7 plasma levels and CD127 expression by T lymphocytes remain unaltered in septic shock (142). More importantly, T cells from septic patients and healthy volunteers exhibited comparable signal transducer and activator of transcription 5 (STAT5) phosphorylation and Bcl-2 upregulation when exposed to rh-IL-7. In addition, rh-IL-7 augmented T cell proliferation and IFN- γ production by CD8 $^{+}$ T cells in response to anti-CD2/CD3/CD28-coated beads that were used *ex vivo* as artificial APCs. Therefore, the IL-7:IL-7 receptor machinery appears to be fully operative in septic patients and may thus be utilized to reverse their immunological impairments.

IL-15 is another pleotropic cytokine involved in the development, maintenance, and proliferative responses of multiple lymphocyte lineages. It optimizes effector and memory CD8 $^{+}$ T cell functions under normal conditions and also reportedly controls the homeostatic recovery of naïve CD8 $^{+}$ T cells after CLP-induced sepsis (143). Unlike IL-7, IL-15 is a potent promoter of NK cell and DC functions, which can be defective in sepsis. In fact, IL-15 therapy was demonstrated to block NK cell, DC, and CD8 $^{+}$ T cell apoptosis, to increase IFN- γ levels in the circulation, and to improve survival of mice rendered septic by the CLP procedure or *Pseudomonas aeruginosa* pneumonia (144). In a recent study, septic patients with severe lymphopenia had low expression of Bcl-2 mRNA in their peripheral blood mononuclear cells despite moderately increased plasma IL-15 concentrations (145). Whether such IL-15 quantities are still insufficient and whether treatment with exogenous IL-15 may help correct immunological incompetence in sepsis warrant further investigation.

Several other studies have focused on blockade of co-inhibitory receptors (e.g., PD-1) to alleviate sepsis-induced immunosuppression. Since the induced expression of PD-1 on T cells was first linked to their exhaustion in the context of chronic viral infection (146), interfering with PD-1:PD-L1 interactions has been viewed

as a tempting therapeutic approach to rejuvenating T cells in various conditions including sepsis. Administration of an antagonistic monoclonal antibody (mAb) to PD-1 after the CLP procedure rescued the DTH response and prevented the expression loss of the pro-survival protein Bcl-xL in splenic T cells (147). This was accompanied by a reduction in depletion of lymphocytes and DCs and mortality. In a separate study, treatment with an anti-PD-L1 Ab either before or after CLP led to improved survival of septic mice (148). In addition, PD-L1 blockade prevented the loss of B and T cells, increased blood levels of IL-6 and TNF- α while decreasing IL-10, and lowered bacterial burden in the circulation and within the peritoneal cavity. Therefore, the PD-1:PD-L1 axis is an attractive target for sepsis immunotherapy.

Tailoring immune intervention strategies to patients' factors and conditions (e.g., age, cytokine profiles, immune competence, co-morbidities) and to the phase of sepsis (i.e., early versus protracted) will improve the likelihood of success (99). Biomarker-guided, personalized therapies that are carefully timed and sufficiently monitored using laboratory and/or clinical measures should prevent short- and long-term, adverse consequences of sepsis. Agents that block inflammatory cytokines need to be short-acting, used in early sepsis, and reserved for a group of patients with drastically elevated pro-inflammatory cytokine levels. On the contrary, adjuvant immunotherapy will benefit septic patients who are in an immunosuppressed state. Failure of leukocytes to produce TNF- α in response to LPS stimulation *ex vivo*, subnormal expression of monocytic HLA-DR, upregulated expression of PD-1 or PD-L1 on circulating leukocytes, infections caused by opportunistic pathogens (e.g., *Candida* spp.) and reactivation of otherwise latent viruses, such as cytomegalovirus and herpes simplex virus, can help identify such patients.

Animal Models of Sepsis

Using preclinical models that reliably replicate human sepsis is essential for the development of novel diagnostic biomarkers, prognostic indicators and therapeutic modalities that can be truly translatable from the benchtop to the bedside. Common animal models of sepsis, which are summarized in **Table 1**, utilize a variety of septic triggers or insults including LPS injection, systemic administration of microbes, surgical disruption of the intestinal barrier integrity, and direct introduction of feces into the peritoneal cavity.

Clinical and paraclinical (e.g., biochemical) features of sepsis serve as guiding principles for the development of *bona fide* animal models and for validation of their relevance to the human syndrome. Such models should take into consideration both the early hyperinflammatory state, which is characterized by massive pro-inflammatory cytokine production and its consequences (e.g., fever), and the concurrent or subsequent anti-inflammatory responses that contribute to anergy, immunosuppression and susceptibility to secondary and opportunistic infections. Hemodynamic changes sometimes requiring fluid resuscitation, organ damage, apoptotic death of immunocytes, and mortality from sepsis also need to be simulated. Animal models should also ideally permit therapeutic intervention at defined stages of sepsis and efficacy testing of such treatments. Accordingly, gross outcome measurements, such as weight loss and death

TABLE 1 | Common Animal Models of Sepsis.

Species	Model	Immunopathology and reported manifestations	Advantages	Disadvantages
Mouse	Endotoxicosis	Rapid but transient inflammatory cytokine response, hypotension (149); leukopenia (150) hypodynamic cardiovascular changes (151); multi-organ injury, mortality within days	Simple and reproducible	Lack of infectious focus; cytokine response magnitude may not represent human sepsis (149); poor reflection of complex physiological/immunological changes of human sepsis
	Systemic bacterial administration	Rapid but transient inflammatory cytokine response when given i.v., slow and sustained inflammatory cytokine response when given i.p. (180); bacteremia, hypotension, hypodynamic cardiovascular changes with infected fibrin clot (181); multi-organ injury, mortality within hours to days	Simple and reproducible	Variability introduced by the choice of bacterial strain and administration route; large bolus of bacteria may not reproduce changes of human sepsis; may reflect endotoxicosis in the case of Gram-negative bacteria
	Host barrier disruption (CLP/CASP)	Rapid pro/anti-inflammatory cytokine response (187) that is more severe in CASP vs CLP (201); polymicrobial bacteremia, hypotension, hyperdynamic cardiovascular changes (188); T and B cell apoptosis, immunosuppression (189); multi-organ injury, mortality within hours to days	Polymicrobial, severity controlled by size of puncture/stent diameter; CLP reproduces immunosuppressive phase	Requires surgical techniques; high experimental variability; abscess formation may prevent disease development (201)
	Feces-induced peritonitis (FIP)	Rapid pro/anti-inflammatory cytokine response, systemic bacterial dissemination, splenocyte apoptosis (114); hypothermia, impaired metabolism, hypodynamic cardiovascular changes (203); mortality within days	Simple, controlled inoculum; reflects polymicrobial peritonitis	Microbial dose and composition of the fecal inoculum often unknown; cytokine response magnitude more severe vs. CLP (206)
Rat	Endotoxicosis	Rapid pro/anti-inflammatory cytokine response (152); hypermetabolism, hypotension, hypodynamic cardiovascular changes with lethal dose i.v. (153); hyperdynamic cardiovascular changes with non-lethal dose i.p. (165); multi-organ injury, mortality within hours to days	Simple and reproducible	Lack of infectious focus, poor reflection of complex physiological/immunological changes of human sepsis
	Systemic bacterial administration	Rapid pro/anti-inflammatory cytokine response (170); hypotension, bacteremia, hypodynamic cardiovascular changes with high dose (171); hyperdynamic cardiovascular changes with low dose (177) and infected fibrin clot (185); mortality within hours to days	Simple and reproducible, can reproduce hyperdynamic changes in human sepsis	Large bolus of bacteria may not reproduce changes of human sepsis; may reflect endotoxicosis in the case of Gram-negative bacteria
	Host barrier disruption (CLP/CASP)	Rapid pro/anti-inflammatory cytokine response (190), hyperdynamic cardiovascular changes (191); polymicrobial bacteremia, leukopenia, thrombocytopenia (192); multi-organ injury, mortality within hours to days	Polymicrobial, severity controlled by size of puncture/stent diameter	Requires surgical techniques; high experimental variability
Rabbit	Endotoxicosis	Rapid inflammatory cytokine response, hypotension, hypodynamic cardiovascular changes with high dose (154); hyperdynamic cardiovascular changes with low dose (164); hypothermia, leukopenia (155); multi-organ injury, mortality within hours to days	Simple and reproducible, increased sensitivity to LPS compared to rodents	More expensive than rodent models; lack of infectious focus, poor reflection of complex physiological/immunological changes of human sepsis
	Systemic bacterial administration	Rapid inflammatory cytokine response, hypotension, leukopenia, thrombocytopenia (172); bacteremia, hypothermia, neutrophil apoptosis (182); multi-organ injury, mortality within hours	Simple and reproducible	More expensive than rodent models; less well-characterized; may reflect endotoxicosis in the case of Gram-negative bacteria
Pig	Endotoxicosis	Rapid pro/anti-inflammatory cytokine response, neutropenia, lymphopenia (156); hypotension, DIC, hypodynamic cardiovascular changes (157); hyperdynamic cardiovascular changes with fluid resuscitation (160); mortality within hours	Simple, reproducible, porcine physiology and LPS sensitivity similar to humans	Expensive housing and care costs; lack of infectious focus; poor reflection of complex physiological/immunological changes of human sepsis
	Systemic bacterial administration	Rapid pro/anti-inflammatory cytokine response, bacteremia, DIC (173); hypotension, hypodynamic cardiovascular changes (174); multi-organ injury, mortality within hours to days	Simple, reproducible, porcine physiology similar to humans	Expensive housing and care costs; large bolus of bacteria may not reproduce changes of human sepsis; may reflect endotoxicosis in the case of Gram-negative bacteria
	Feces-induced peritonitis (FIP)	Inflammatory cytokine response, hypotension, hyperdynamic cardiovascular changes with fluid resuscitation (204, 205); leukocytosis, endotoxemia (174); multi-organ injury, mortality	Porcine physiology similar to humans; reflects polymicrobial peritonitis	Expensive housing and care costs; microbial dose and composition of the fecal inoculum often unknown
Non-Human Primate	Endotoxicosis	Rapid but transient pro-inflammatory cytokine response, hypotension, hypodynamic cardiovascular changes (158); thrombocytopenia, leukopenia (159)	Cross-reactivity with human therapeutics and diagnostic tools, most comparable to human physiology	Most expensive housing and care costs; ethical concerns; more accurately reflects human endotoxicosis rather than sepsis
	Systemic bacterial administration	Rapid pro/anti-inflammatory cytokine response, hypotension, leukopenia, thrombocytopenia, DIC (175); hypodynamic cardiovascular changes that become hyperdynamic with fluid resuscitation (176); multi-organ injury, mortality within hours to days	Cross-reactivity with human therapeutics and diagnostic tools, most comparable to human physiology	Most expensive housing and care costs; ethical concerns; may reflect endotoxicosis in the case of Gram-negative bacteria

CASP, *colon ascendens* stent peritonitis; CLP, cecal ligation and puncture; DIC, disseminated intravascular coagulation; FIP, feces-induced peritonitis; i.p., intraperitoneal; i.v., intravenous; LPS, lipopolysaccharide.

rates, should be complemented with laboratory assessments of immune competence or incompetence (e.g., cytokine production and anergy/exhaustion marker expression).

Findings from models in which young, adult and otherwise healthy animals are utilized may not accurately represent the “real-life” features of sepsis in the rising elderly populations. This is a major limitation of animal models that place a disproportionate emphasis on sepsis-induced hyperinflammation, which no longer accounts for most deaths due to sepsis at least when advanced ICU facilities and robust practice of critical care are in place. Therefore, experimentation with older animals and those with co-morbidities may provide a more realistic picture of sepsis in vulnerable populations.

One of the most routinely utilized agents to induce sepsis in small and large animals is LPS (149–160), a glycolipid found abundantly in the outer membrane of Gram-negative bacteria. Following intravenous (i.v.) or intraperitoneal (i.p.) injection, LPS binds to the glycosylphosphatidylinositol (GPI)-anchored protein CD14 and signals through TLR-4 to provoke a systemic inflammatory response often referred to as “endotoxicosis” (161, 162). This response is characterized by pro-inflammatory cytokine production, multiple organ injury and hypotensive shock, which are hallmarks of early sepsis. LPS administration is simple and does not require advanced surgical techniques. In addition, its dosage can be easily controlled. However, one should keep in mind that exposure to large amounts of LPS may result in an immediate hypodynamic cardiovascular state that does not represent human sepsis (163). Several groups have overcome this problem by developing models that use sublethal doses of LPS (164, 165) or aggressive fluid resuscitation (166). Also importantly, bolus injection of LPS into laboratory animals triggers a severe inflammatory cytokine response that differs in magnitude and sustenance from what is observed in clinical sepsis (163, 167).

Lethal shock and disseminated intravascular coagulation (DIC) can be induced in mice by two consecutive injections of LPS separated by a 24-h interval (168). In this model, which is known as “generalized Shwartzman reaction,” a “super low” dose of LPS is injected followed by a larger systemic dose that elicits rapid pro- and anti-inflammatory responses, coagulopathy and multi-organ damage. It is noteworthy that the initial priming dose in Shwartzman reaction is smaller than that causing endotoxin tolerance (169). It is believed that tolerizing doses of LPS activate the canonical nuclear factor- κ B (NF- κ B) pathway leading to robust expression of pro-inflammatory mediators as well as a myriad of suppressive elements designed to prevent progressive inflammation (169). In contrast, super low doses of LPS, such as those used in the priming phase of Shwartzman reaction, fail to activate the NF- κ B pathway. Instead, they trigger the activation of CCAAT/enhancer binding protein δ (C/EBP δ) in an IL-1 receptor-associated kinase 1 (IRAK1)-dependent manner resulting in mild but persistent expression of inflammatory mediators (169).

All animal models of endotoxicosis lack an infectious focus. In addition, since LPS is only present in Gram-negative bacteria, these models do not represent polymicrobial sepsis caused by mixed Gram-positive and Gram-negative microbes.

Systemic administration of a large number of bacteria, typically *E. coli*, instigates a massive inflammatory cytokine response, cardiovascular collapse and rapid mortality (170–175). Fluid resuscitation or sublethal dosages of bacteria can be used to better mimic the septic response and its hemodynamic manifestations in humans (176–178). These models allow for bacterial strains and numbers to be carefully chosen and for host responses to develop against intact microbial pathogens. However, they are more similar to models of endotoxicosis than full-blown infections when Gram-negative bacteria are used. Many bacterial strains are complement-sensitive and lysed shortly after they enter the circulation, thus releasing their endotoxin content (179). Moreover, systemic bacterial infusion gives rise to serum TNF- α concentrations that are orders of magnitude larger than those found in septic patients or in peritonitis models (179). Lastly, the route of administration can impact the vigor of the septic response. For instance, a robust but transient TNF- α response is elicited following an i.v. challenge of mice with live *E. coli* O111, whereas an i.p. challenge leads to much lower but more sustained blood levels of TNF- α (180).

Surgical implantation of bacteria (e.g., *E. coli*)-laden fibrin clots into the peritoneum has also been used to induce sepsis in several species (181–185). Some of these models more accurately reproduce the hyperdynamic state and slow, sustained release of cytokines associated with human sepsis.

Cecal ligation and puncture (CLP) is considered by many as the “gold standard” of intra-abdominal sepsis models. This relatively simple surgical procedure involves a laparotomy and ligation of the cecum in a non-obstructing manner followed by puncturing the ligated portion to allow fecal content to leak into the otherwise sterile peritoneal cavity (186). Therefore, a source of necrotic tissue combined with an infectious focus that persistently challenges the host with enteric microbes causes polymicrobial sepsis. CLP-inflicted sepsis resembles the clinical syndrome since it can set in motion a systemic pro-inflammatory cytokine response as well as a compensatory anti-inflammatory response and a hyperdynamic cardiovascular state (162, 187–192). Furthermore, CLP is particularly useful for studying the delayed phase of sepsis in which immune responses are impaired. This is possible by the “two-hit” versions of the model, in which mice undergo CLP and are subsequently challenged with a secondary/opportunistic pathogen, such as *Streptococcus pneumoniae* (193), *Pseudomonas aeruginosa* (193–195), *Candida albicans* (141) or *Aspergillus fumigatus* (196). Logistically, the CLP procedure is quick to perform by an experienced experimentalist. It can also be readily modified to investigate varying degrees of inflammation and different survival intervals. The length of ligated cecum (197), the size of the needle used for the perforation (198), and the number of punctures made (199) can all determine the severity of sepsis and the speed with which death occurs. It needs to be noted that the CLP outcome may vary considerably among different laboratories and animals depending upon the experimentalist’s surgical expertise and the animals’ sex, age, strain, housing conditions, cecal content, and even cecal fullness when CLP is performed (167). Another disadvantage of the CLP model lies in the host’s natural ability to form an abscess in order to contain infection (200, 201). Therefore, treatments that promote abscess

formation may improve survival in CLP, which may introduce bias by adding a confounding variable in the experiment.

Another model of host barrier disruption leading to sepsis is colon ascendens stent peritonitis (CASP), in which a stent is inserted into the ascending colon to allow for leakage of fecal matter into the peritoneal cavity (201, 202). Although similar to CLP in principle, CASP represents persistent peritoneal infection rather than abscess formation and causes a more robust cytokine response and higher bacterial loads within several organs. The severity of and mortality from CASP are influenced by the diameter of the stent and also by its removal at defined time points. This mimics surgical interventions to eliminate infectious foci in humans.

Host barrier disruption models are heavily reliant on surgical techniques and relatively difficult to standardize. An alternative approach is to simply inject animals with a given amount of fecal solution i.p. (114, 174, 203–205). This is called the feces-induced peritonitis (FIP) model of polymicrobial sepsis, for which we recently developed a robust scoring system (114). Early inflammatory cytokine production in FIP is typically much more intense than that caused by CLP (206). The amount of feces to be injected i.p. can be adjusted to alter the severity and outcome of sepsis. An additional advantage of FIP is that fecal solutions with identical microbial loads and composition can be injected into multiple recipient cohorts. This is in contrast with barrier disruption models requiring the leakage of each animal's intestinal content into the peritoneal cavity, which is an inevitable source of variation. A limitation of the FIP model is that the dosage and species of bacteria introduced into the recipients are usually unknown given that intestinal flora vary according to the animal strain, commercial source and housing conditions. Finally, the state of immunosuppression that follows the hyperinflammatory phase of sepsis has not been fully characterized in FIP.

Despite the abundance of animal models for sepsis, there is currently no one truly clinically relevant model that fully recapitulates all the complex immunological, hemodynamic, and pathophysiological responses seen in human sepsis. The reason for outcome discrepancies between animal models and clinical sepsis is multifactorial, but partially stems from the heterogeneity of patient populations. Nevertheless, we continue to rely on current animal models and strive to come up with improved models in order to better understand the pathogenesis of sepsis and to design and test novel treatments for this fatal syndrome.

iNKT Cells and Sepsis

Several groups including ours have explored the effector or regulatory capacities of iNKT cells and their synthetic glycolipid agonists in sepsis and endotoxic shock.

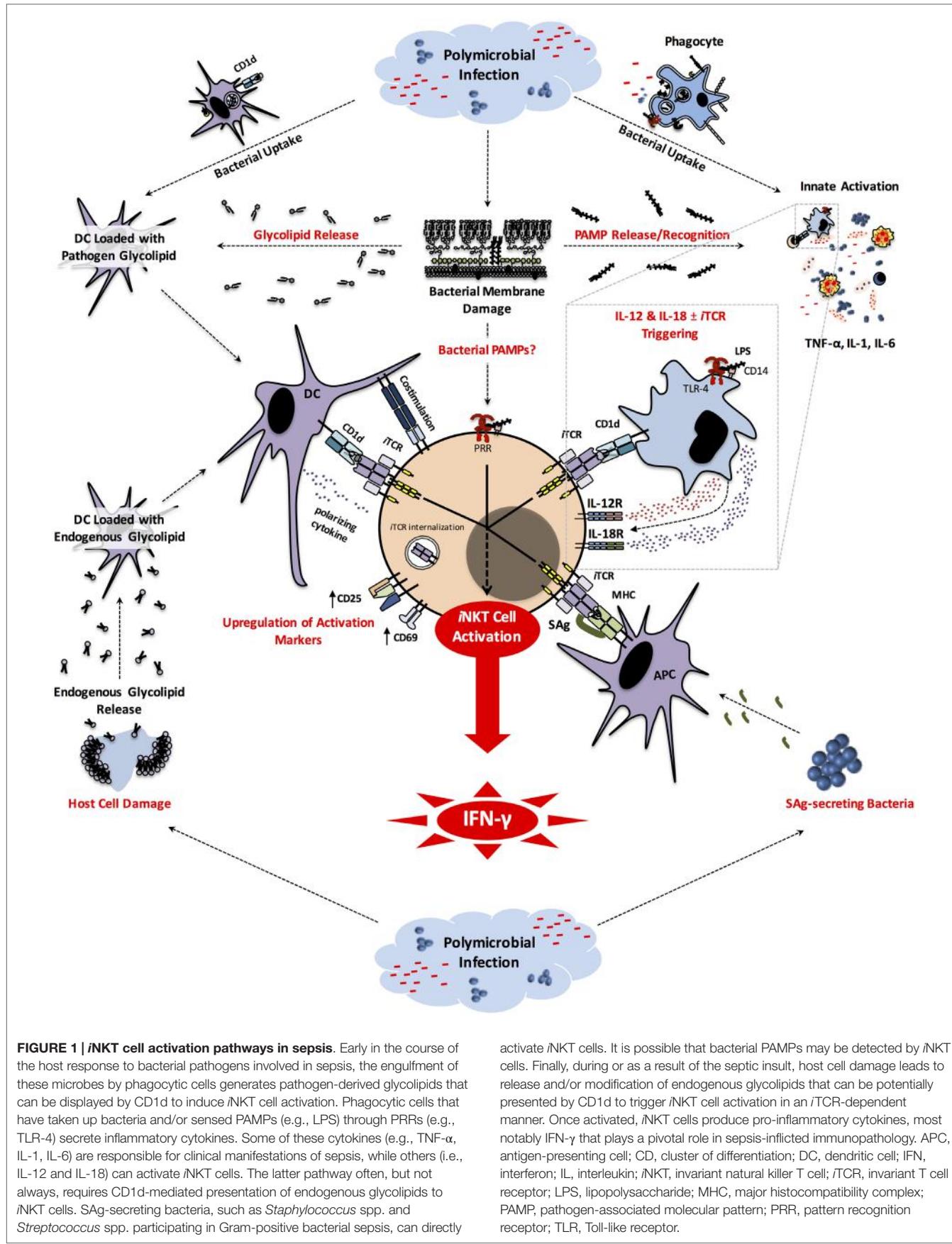
Rhee et al. from Alfred Ayala's laboratory first reported that treating 129S1/SvImJ mice with an anti-CD1d mAb (clone 1B1) before the CLP surgery could reduce plasma and splenic IL-6 and IL-10 levels and prevented sepsis-induced mortality in some of the treated mice (207). They also noted a significant increase in the frequency of cell populations co-expressing T and NK cell markers, which could be reversed by anti-CD1d treatment. It needs to be noted that although the 1B1 mAb has been used extensively to block CD1d interactions with NKT cell TCRs, it

may also potentially activate CD1d⁺ APCs (208). Therefore, the mechanism of action of this mAb could not be definitively determined. More importantly, both *i*NKT and *v*NKT cells interact with CD1d (50). We now know that there are other CD1d-restricted T cell types such as a subpopulation of $\gamma\delta$ T cells (209) that can be affected by anti-CD1d treatment. Nevertheless, the study of Rhee et al. indicated a role for CD1d-restricted T cells in sepsis and set the stage for subsequent important investigations.

Hu et al. extended the above study to other mouse strains (210). They demonstrated that pre-treatment of BALB/c mice with 1B1 before CLP confers upon them a survival advantage. This treatment also prevented the rise in circulating levels of TNF- α , IL-6, monocyte chemotactic protein (MCP)-1 and IL-10. Within the liver, mice receiving 1B1 had lower frequencies of NKT cells capable of producing TNF- α , IL-6, IL-4 or IL-10, indicating no bias toward either a pro- or anti-inflammatory phenotype. Interestingly, however, the percentage of IL-6-producing hepatic macrophages declined whereas that of IL-10-producing cells increased upon anti-CD1d treatment. How CD1d contributes to the immunopathology of sepsis is not clear. It is possible that lipid antigens derived from bacterial pathogens are loaded into CD1d and presented to NKT cells. Alternatively or in addition, recognition of pathogen-associated molecular patterns (PAMPs) by PRRs such as TLRs may lead to the production of IL-12 and IL-18 by APCs during sepsis. Once coupled with CD1d-mediated presentation of endogenous lipids, these cytokines can induce NKT cell activation (42, 43). Sepsis-induced tissue injury may also increase, release and/or modify endogenous lipids that can be displayed by CD1d to trigger NKT cell responses (Figure 1). Consistent with this hypothesis, a previous study reported that serial injections of apolipoprotein E (ApoE), a component of plasma lipoproteins, alters NKT cell compartments and increases CLP-induced mortality in rats (211).

Hu et al. also used the CLP model to examine the contribution of the invariant subset of NKT cells to sepsis (210). They found a marked decline in the frequency of hepatic *i*NKT cells, defined by their reactivity with α -GalCer-loaded CD1d tetramer, in both C57BL/6 and BALB/c mice. This was accompanied by upregulation of CD69 and CD25 on the surface of *i*NKT cells indicating their enhanced activation on a per cell basis. There exist several possibilities to explain the lower percentage of detectable *i*NKT cells in the liver of septic mice. These include iTCR internalization, which is a well-known phenomenon in the context of *i*NKT cell activation by synthetic glycolipids (212), cell death *in situ*, or migration to other locations. To address these possibilities experimentally, one could assay for intracellular iTCRs or quantify mRNA corresponding to the V α 14–J α 18 TCR rearrangement in hepatic non-parenchymal cells, or track *i*NKT cell movements in the body.

To ascertain whether *i*NKT cells play a pathogenic or protective role in sepsis, Hu and coworkers used J α 18^{-/-} mice that lack *i*NKT cells (210). These animals exhibited reduced mortality due to CLP as well as ablated TNF- α , IL-6, MCP-1 and IL-10 systemic responses in comparison with wild-type C57BL/6 mice. It was recently found that the TCR α repertoire of J α 18^{-/-} mice that have been widely available to the research community is shrunk by ~60% (213).



Therefore, the cellular deficiency in $\text{J}\alpha 18^{-/-}$ mice is not exclusive to *i*NKT cells, which necessitates *i*NKT cell reconstitution experiments to validate results obtained using these animals.

More recently, Heffernan et al. demonstrated that while CLP causes a drop in the frequency of *i*NKT cells in the liver, both the absolute number of *i*NKT cells and their frequency among T lymphocytes are elevated in the circulation and within the peritoneal cavity, which is considered the site of polymicrobial infection in this model (214). Furthermore, a much bigger fraction of peritoneal *i*NKT cells expressed CD69 in septic mice in comparison with the sham laparotomy control group. Although *i*NKT cell mobilization by CLP was not directly monitored, these results support the scenario in which *i*NKT cells migrate out of the liver and toward the source of infection, which should account for their decline in the liver. Intriguingly, this migration was mediated by PD-1, which is well known as an anergy/exhaustion marker. Following sepsis, PD-1 $^{-/-}$ mice exhibited a numerical increase in activated hepatic *i*NKT cell populations but intact peripheral blood or peritoneal *i*NKT cell compartments when they were compared with the sham controls. Whether the role of PD-1 in *i*NKT cell migration is intrinsic to these cells was not studied. This question could be addressed by reconstitution of $\text{J}\alpha 18^{-/-}$ mice with PD-1-sufficient or -deficient *i*NKT cells prior to the CLP procedure. These investigators also found that once accumulated in the peritoneal cavity, *i*NKT cells influence the ability of local macrophages to phagocytose bacteria and clear infection. Bacterial load in the cavity was lower in septic $\text{J}\alpha 18^{-/-}$ mice than in wild-type controls. In addition, peritoneal macrophages derived from septic $\text{J}\alpha 18^{-/-}$ mice were more potent than those from septic wild-type animals in engulfing *E. coli*. Collectively, the work of Heffernan and coworkers reveals an interesting interplay between migrant *i*NKT cells and macrophages residing within foci of infection during sepsis. It also suggests that blockade of PD-1 may not only reverse T cell exhaustion to relieve sepsis-induced immunosuppression but also likely benefits the host by modulating the migration capacity of *i*NKT cells to further facilitate microbial clearance.

Taken together, the above studies indicate a pathogenic role for *i*NKT cells in CLP-induced sepsis. Several groups have reached the same conclusion using animal models of LPS-inflicted pathology or lethality. Koide et al. established a link between the resistance of D-galactosamine (D-GalN)-sensitized NC/Nga mice to LPS and the presence of fewer NKT cells in these animals (215). This old protocol utilizes the hepatotoxic agent D-GalN to sensitize laboratory animals to very low doses of LPS, and has been used extensively as a model of endotoxic shock and Gram-negative microbial sepsis. We are of the opinion that the D-GalN sensitization model simulates acute hepatic failure more closely. Nevertheless, participation of inflammatory mediators is evident in its immunopathology amid severe liver damage. While co-administration of D-GalN and LPS led to 100% mortality in C57BL/6 mice within 12 h, it failed to kill NC/Nga mice even at 24 h (215). It also raised the activity level of alanine aminotransferase (ALT) in the serum and that of caspase-3 in the liver extract of C57BL/6 mice but not NC/Nga mice (216). Moreover, drastic lesions with hemorrhage and many apoptotic cells were observed in C57BL/6 but not in NC/

Nga liver sections. NC/Nga mice injected with D-GalN and LPS had negligible levels of IFN- γ protein in their serum or IFN- γ mRNA in their liver. This was accompanied by a 10-fold reduction in the size of CD3 ϵ^+ DX5 $^+$ NKT cell compartment in the liver although NKT cells were capable of producing ample IFN- γ on a per cell basis. Finally, administration of recombinant IFN- γ to D-GalN-sensitized NC/Nga mice rendered them susceptible to LPS-induced mortality. In this body of work, frequency analyses were performed on NKT cells co-expressing T and NK cell markers. However, *i*NKT cells are the likely culprits and the early triggers of pathology in D-GalN/LPS-prone mice. This is because *i*NKT cells comprise the vast majority of hepatic NKT cells in mice (217). Second, when Koide and coworkers injected NC/Nga mice with the *i*NKT cell superagonist α -GalCer a few hours before the D-GalN/LPS challenge, endogenous IFN- γ production was restored leading to increased expression of inducible nitric oxide synthase (iNOS), appearance of apoptotic cells in the liver, and 100% mortality (215). It was therefore concluded that the resistance of NC/Nga mice to the LPS-mediated lethality with D-GalN sensitization is due to impaired IFN- γ production caused by a shortage of *i*NKT cells and reduced nitric oxide production in these animals. An additional confirmatory approach would have been to adoptively transfer a large number of syngeneic *i*NKT cells into NC/Nga mice to increase their frequency before testing the susceptibility of the recipients to D-GalN/LPS.

In a different model of endotoxic shock, α -GalCer injection sensitized wild-type mice, but not $\text{J}\alpha 18^{-/-}$ mice, to LPS-mediated lethality (218). Interestingly, shock in these animals was accompanied by severe lesions and hemorrhage, marked accumulation of polymorphonuclear leukocytes and mononuclear cells, and significant cell death almost exclusively in the lungs. Although serum ALT levels were elevated, hepatic lesions were focal and mild, and other organs showed no signs of overt injury or other changes except for congestion. Pulmonary manifestations and lethal shock in this model could not be induced by simultaneous administration of α -GalCer and LPS, and required an interval period of 3–24 h between α -GalCer sensitization and the LPS challenge. This is consistent with the kinetics of IFN- γ secretion in response to α -GalCer, which is potentiated by *i*NKT cells and largely contributed by transactivated NK cells (219). Ito et al. found that α -GalCer injection gives rise to high blood levels of IFN- γ within the above timeframe and augments the subsequent production of TNF- α , a major mediator of endotoxic shock, in response to LPS (218). They further demonstrated that neutralizing IFN- γ or genetic deficiency of TNF- α abolishes the systemic lethal shock in this model. Therefore, it was proposed that IFN- γ and TNF- α play pivotal roles in preparation and execution of LPS-mediated lethality, respectively, in α -GalCer-primed mice. Following up on these findings, Tumurkhuu et al. found that priming with α -GalCer increases the frequency of NKT cells among pulmonary non-parenchymal leukocytes and induces local IFN- γ production (220). This resulted in expression of several adhesion molecules, most notably vascular cell adhesion molecule-1 (VCAM-1), on vascular endothelial cells of the lungs, which in turn promoted the accumulation of very late activating antigen-4 (VLA-4) $^+$ cells among inflammatory cell recruits in the lungs. This was significant because an anti-VCAM-1

mAb partially averted LPS-mediated lethal shock in α -GalCer-sensitized mice.

In the above studies, the relative contributions of *i*NKT and NK cells to IFN- γ production was not determined. There currently exists no commercially available antibody for selective depletion of *i*NKT cells although online literature search through the World Wide Web indicates that a mAb called NKT14 may serve this purpose in the future. Until this or similar antibodies become available, one could employ anti-asialo GM1 antiserum and an anti-NK1.1 mAb (clone PK136) in parallel cohorts of mice to address this question. The former depletes NK cells without affecting the NKT cell population, and the latter depletes both NK and NKT cells (45, 221).

Another important question is why LPS-induced pathology in α -GalCer-sensitized mice is restricted to the lungs while the liver is largely spared. This is particularly interesting in light of the observation that α -GalCer induces IFN- γ production by both hepatic and pulmonary *i*NKT cells and that IFN- γ is readily detectable at mRNA and protein levels in both organs. It has been argued that IFN- γ signaling is fully operational in the lungs but not in the liver of α -GalCer-primed mice (222). Augmented expression of phosphorylated STAT1 was more sustained in the lungs than in the liver. In addition, IFN regulatory factor 1 (IRF1) was upregulated in the lungs but not in the liver of α -GalCer-treated mice. Second, pulmonary NKT cells reportedly produce IFN- γ as their main cytokine, whereas hepatic NKT cells produce IFN- γ , IL-4 and IL-10. Neutralization of IL-4 enhances STAT1 activation, exacerbates the hepatic injury, and increases the number of apoptotic cells in the liver. Therefore, IL-4 has been proposed to inhibit IFN- γ signaling in the liver while its absence promotes IFN- γ -mediated pathology in the lungs (222). Finally, one might wonder why a potentially similar mechanism is not at play to protect NC/Nga livers in the D-GalN/LPS model (215). It is possible that the cytokine profile of α -GalCer-primed NC/Nga mice differs from that of C57BL/6 and BALB/c mice. The hepatotoxic nature of the D-GalN insult may also mask the influence of other factors involved. These possibilities are not mutually exclusive.

Several studies have focused on the role of *i*NKT cells in systemic Shwartzman reaction. IFN- γ is considered a key cytokine in the pathogenesis of Shwartzman reaction because it induces massive production of TNF- α , IL-1 and other inflammatory mediators. Dieli et al. found $J\alpha 18^{-/-}$ mice on either C57BL/6 or BALB/c background to be resistant to the LPS-induced mortality of Shwartzman reaction (223). $J\alpha 18^{-/-}$ mice had lower serum levels of IFN- γ and TNF- α in comparison with wild-type animals, and administration of recombinant IFN- γ was sufficient to prime these animals. In two more recent studies, Sierci et al. tested the effect of α -GalCer treatment at different time points before or after LPS priming (224, 225). When α -GalCer was given approximately 6, 9, or 12 days prior to the first injection of LPS, mice survived the subsequent LPS challenge and their protection was associated with reduced serum levels of IFN- γ and TNF- α and hepatic level of MCP-1 (224). In stark contrast, when administered 1 or 3 days before priming, α -GalCer failed to protect the mice from lethal endotoxic shock. It appears likely that earlier α -GalCer injection induces *i*NKT cell anergy (226),

thus hampering their IFN- γ production capacity. It would be interesting to examine the expression level of PD-1 on *i*NKT cells obtained from α -GalCer-pretreated mice or to test whether blockade of the PD-1:PD-L1 interaction restores Shwartzman reaction. In a separate study, Sierci et al. found that α -GalCer administered within 2 h before or after the LPS challenge rescues the mice (225). This timeframe is consistent with the period in which IL-4 production by α -GalCer-stimulated *i*NKT cells reaches its peak while only minute amounts of IFN- γ are detectable in the serum. Accordingly, Sierci and coworkers noted increased IL-4 and IL-10 responses and decreased levels of IFN- γ and TNF- α in protected mice. In addition, blood levels of ALT and aspartate aminotransferase (AST) were lower in these animals indicating milder injury to the liver. The beneficial effect(s) of Th2-type cytokines were confirmed when mice receiving either an anti-IL-4 or an anti-IL-10 mAb succumbed to endotoxic shock. Therefore, inducing Th2-skewed *i*NKT cell responses may have potential therapeutic applications in sepsis. We recently put this hypothesis to the test by using Th2-promoting *i*NKT cell agonists in the FIP model of sepsis (read below).

In a prospective study, we demonstrated that patients with sepsis have a significantly elevated ratio of peripheral blood *i*NKT:T cells in comparison with non-septic trauma patients (37). The patient cohorts were similar in age and in severity of illness that was calculated based on their Acute Physiology and Chronic Health Evaluation II (APACHE II) scores in the initial 24-h period post-diagnosis (227). Next, we compared wild-type and $J\alpha 18^{-/-}$ mice receiving a fecal slurry i.p. for severity of FIP using a murine sepsis score (MSS) that we recently developed (114) and also for mortality from sepsis. The severity of sepsis was significantly lower in $J\alpha 18^{-/-}$ mice than in wild-type controls. In addition, intra-abdominal fecal challenge resulted in 100% mortality in wild-type animals but no death in septic $J\alpha 18^{-/-}$ mice within 24 h. Importantly, reconstitution of $J\alpha 18^{-/-}$ mice with *i*NKT cells before the septic insult increased the severity of their symptoms. Together, these results confirm the pathogenic nature of *i*NKT cells in the FIP model. In the next series of experiments, we explored the therapeutic potentials of OCH, a Th2-polarizing analog of α -GalCer (34), in FIP. We found that a single i.p. injection of OCH within 20 min after the fecal challenge reduced the MSS scores and prolonged the survival of septic mice compared with vehicle- or α -GalCer-treated animals. These changes were associated with elevated blood levels of the Th2-type cytokine IL-13 and reduced levels of the pro-inflammatory cytokine IL-17. Furthermore, OCH treatment decreased the number of apoptotic T cells, B cells and macrophages in the spleen. Anti-inflammatory mechanisms are known to contribute to sepsis-induced immunosuppression, which may make a septic individual susceptible to opportunistic infections (93). Therefore, we asked whether OCH treatment worsens the microbial load in septic mice. Much to our satisfaction, this was not the case, and blood and tissue homogenates prepared from the heart, lungs, kidneys, liver and spleen of vehicle-, OCH- and α -GalCer-treated septic mice had comparable numbers of microbial colony-forming units. Finally, administration of C20:2, another glycolipid that is even more potent than OCH in inducing a Th2 bias (228, 229) and that additionally suppresses downstream NK cell function (230),

also mitigated the severity of FIP-induced sepsis. However, this effect was only transient, which may be explained by the relatively short half-life of C20:2 compared with OCH (228, 230). Based on these results, we propose that Th2-skewing agonists of *i*NKT cells may be employed to treat the hyperinflammatory phase of sepsis without compromising the patient's immunity to microbial pathogens.

Finally, during polymicrobial sepsis, common bacterial pathogens, such as *Staphylococcus* spp. and *Streptococcus* spp., are likely to release the SAGs they harbor (Figure 1). We recently discovered that staphylococcal and streptococcal exotoxins belonging to phylogenetic group II SAGs can directly activate mouse and human *i*NKT cells leading to IFN- γ production (45). However, anticipating the net effect is not simple because: (i) the type of microbial pathogens involved in sepsis may vary substantially among different individuals; (ii) how multiple SAGs released by multiple bacterial pathogens may cross-regulate the response to each other is far from clear; (iii) host responses to SAGs may be modulated by cell wall components of the very bacteria that release SAGs as we previously described (231).

To summarize, the studies highlighted in this section all point to a pathogenic role for *i*NKT cells in sepsis regardless of the experimental model employed, and IFN- γ is a major mediator of *i*NKT cell-inflicted damage in this context.

vNKT Cells and Sepsis

The extent to which vNKT cells contribute to or regulate sepsis is unknown. However, *in vivo* treatment with sulfatide, a CD1d-restricted ligand of vNKT cells, has been demonstrated to attenuate the magnitude of the septic response, thus providing indirect evidence for a protective role of activated vNKT cells in sepsis.

The beneficial effect of sulfatide was initially noted in two relatively old studies on LPS-induced sepsis with a focus on how this glycolipid influences leukocyte adhesive properties and transendothelial migration as opposed to NKT cell functions (232, 233). Higashi et al. reported that while 75% of C3H/HeN mice died within 2 days of injection with a large dose of LPS, only 20% of mice that were pretreated with bovine brain-extracted sulfatide succumbed even after 7 days (232). Administration of sulfatide to either C3H/HeN or C57BL/6 mice also partially inhibited their TNF- α response to a sublethal dose of LPS. Finally, using a mouse model of endotoxin-induced hypotension, these investigators demonstrated that treatment with sulfatide prior to the LPS challenge prevents an otherwise progressive decline in systolic blood pressure. Squadrito and coworkers explored the effect of sulfatide on acute lung injury in a rat model of endotoxic shock (233). When administered shortly after the LPS injection, sulfatide was able to partially offset hypotension, revert leukopenia, and diminish myeloperoxidase activity in the lungs that was used as an indication of neutrophil accumulation in this tissue. Also importantly, sulfatide treatment caused a near complete prevention of LPS-induced lethality.

The only published report to date addressing a link between CD1d-restricted, sulfatide-reactive T cells and sepsis has utilized an experimental mouse model of *S. aureus* infection (234). In this work, Kwiecinski et al. found that TCR β^+ NK1.1 $^+$ α -GalCer/CD1d tetramer $^-$ cells, which should contain a sizeable population

of vNKT cells, have an activated phenotype as judged by their upregulated expression of CD69 in infected mice. They also showed that treating wild-type C57BL/6 mice with porcine sulfatide before bacterial inoculation lowers their blood levels of TNF- α and IL-6 without altering the staphylococcal burden in blood, liver or kidneys. Therefore, sulfatide treatment does not impede the ability of the immune system to combat this pathogen. In fact, wild-type mice receiving sulfatide 1 h before and 3 days after bacterial inoculation were partially protected. Importantly, the survival advantage conferred by sulfatide treatment could be recapitulated in $J\alpha 18^{-/-}$ (vNKT-sufficient, *i*NKT-deficient) mice but was missing in $CD1d^{-/-}$ (vNKT- and *i*NKT-deficient) mice. The significance of this finding is two-fold. First, it strongly suggests that activated vNKT cells mediate the protective effect of sulfatide in septic wild-type mice. Second, unlike in other models where sulfatide treatment induces *i*NKT cell anergy to ameliorate inflammation and injury (235, 236), its beneficial effect in *S. aureus* sepsis does not require the presence of *i*NKT cells. Of note, late injection of sulfatide in this model (i.e., on day 3 post-bacterial inoculation) failed to improve survival. This may be viewed as an impediment to the possibility of sulfatide therapy in staphylococcal sepsis once the symptoms have developed. However, more comprehensive studies are warranted to possibly find a window of opportunity during which sulfatide-based interventions may be effective in staphylococcal and other forms of sepsis.

MAIT Cells and Sepsis

MAIT cells are relatively frequent among human innate-like T cells and capable of responding to a wide variety of bacterial and fungal pathogens. The ability of MAIT cells to rapidly produce inflammatory cytokines, together with their strategic positioning at the host-pathogen interface, makes them an ideal candidate to fulfill the role of emergency responders to infection and/or regulators of the septic response.

Grimaldi et al. recently explored how sepsis may change the frequency and absolute number of MAIT cells in the circulation (237). In a prospective study, they recruited a relatively large number of patients with severe sepsis or septic shock and compared their peripheral blood MAIT, *i*NKT, and $\gamma\delta$ T cell compartments with those of critically ill patients with non-septic (mostly cardiogenic) shock, and age-matched healthy volunteers. Septic patients exhibited an early and dramatic decrease in their MAIT cell count compared with non-infected critically ill patients or healthy controls. This was unlike *i*NKT or $\gamma\delta$ T cell counts that remained unaltered in different groups. Also interestingly, there was no association between MAIT cell and total lymphocyte counts, suggesting that MAIT cells follow an independent kinetic pattern in sepsis. By the same token, the frequency of MAIT cells among CD3 $^+$ TCR $\gamma\delta^-$ conventional T cells was significantly lower in septic patients than in healthy subjects.

The above investigation also led to other potentially important observations. First, the early drop in MAIT cell frequencies was more pronounced in septic patients with non-streptococcal infections than in those with streptococcal infections. In addition, in a small cohort of patients with severe viral infections in the absence of concomitant bacterial infections, MAIT cell values were similar to those of healthy controls. *Streptococcus* spp. and

viruses are known not to activate MAIT cells (238). Therefore, the above findings are consistent with the hypothesis that the observed numerical change in the MAIT cell compartment of septic patients is dictated by the type of pathogen(s) encountered. Second, Grimaldi et al. found a higher cumulative incidence of ICU-acquired infections in patients with a persistent decline in peripheral blood MAIT cells. In fact, patients who did not develop secondary infections showed a gradual return to normal MAIT cell values. Therefore, sepsis-induced changes in the MAIT cell compartment seem to be reversible by nature.

It is not clear why MAIT cell numbers drop early in sepsis. Apoptotic cell death, TCR internalization, and migration to peripheral tissues, for instance toward the infectious focus/foci, may provide an explanation for this phenomenon. The latter possibility is supported by the observation that in a few patients registered in the above study, a higher proportion of MAIT cells was detectable in unspecified biological fluids than in blood (238). This is reminiscent of previous reports that MAIT cell numbers drop in the peripheral blood but increase in the lungs of patients with tuberculosis (238, 239). Monitoring MAIT cell frequencies in individual septic patients and future mechanistic studies to uncover the cause of MAIT cell decline in the blood circulation will be informative.

Outstanding Questions and Concluding Remarks

Despite decades of active research and numerous clinical trials, sepsis continues to take its Toll on human lives and cause significant morbidity, thus imposing a heavy burden on human populations and healthcare systems worldwide. Standardized management protocols and better ICU facilities have improved sepsis outcomes. However, there is currently no “cure” for this devastating syndrome. Targeting conventional T cells, APCs or individual inflammatory cytokines has not met with success. We propose that NKT and MAIT cells provide attractive targets for immunotherapy of sepsis. This is because: (i) they are abundant in certain anatomic locations where microbial pathogens are first encountered. For instance, iNKT cells are enriched in the human omentum (16), for which the term “policeman of the abdomen” was coined by a British surgeon, Rutherford Morison, in 1906 (240). Human MAIT cells may serve as “gate-keepers” in mucosal layers and within the liver where they are highly abundant (66); (ii) NKT and MAIT cells can be activated early in the course of sepsis; (iii) they produce large quantities of immunomodulatory cytokines that control the function of downstream innate and adaptive effector cells, thus setting the tone for subsequent host responses; (iv) NKT and MAIT cells are restricted by monomorphic Ag-presenting molecules (i.e., CD1d and MR1, respectively) as opposed to distinct HLA allomorphs. Therefore, they can be stimulated and potentially manipulated by universal ligands in many, if not most, septic patients. This saves time and allows for therapeutic interventions to be implemented speedily; (v) using Th1- or Th2-polarizing ligands, typically in the case of iNKT cells, provides flexibility in tailoring therapies to sepsis stages in which hyper- or hypoinflammatory responses predominate.

Evolutionary conservation of CD1d and MR1 recognition across mammalian species makes animal models of sepsis particularly useful for studying NKT and MAIT cells. There is an urgent need for more investigations in models that mirror aging and various co-morbidities. Humanized mouse models should also shed mechanistic light on how clinical sepsis is initiated, perpetuated, or regulated. Such models could also potentially address some of the discrepancies noted between the results of rodent and human studies on NKT/MAIT cell frequencies, effector functions and homing properties.

Currently available $\text{J}\alpha 18^{-/-}$ mice that have been used extensively by many investigators including us were recently found to lack T cells other than iNKT cells (213). Therefore, if based merely on $\text{J}\alpha 18^{-/-}$ mice, the findings of preclinical studies on sepsis need to be revisited. Antibody-mediated depletion of NKT and NK cells in parallel (45, 221) and functional inactivation of iNKT cells by carefully timed α -GalCer treatment (226) are among other experimental options to study these cells *in vivo*. Using $\text{J}\alpha 18^{-/-}$ and $\text{CD}1d^{-/-}$ mice reconstituted with iNKT cells should help solidify our knowledge of the roles that these cells play in sepsis. Reconstitution with $\text{CD}4^+$ or double-negative subsets or with iNKT cells isolated from different tissues will enable functional studies on these cells in the context of sepsis. This is particularly important in light of reported functional differences between various iNKT cell subpopulations in other conditions, such as cancer (17). Even if/when mouse models of iNKT cell deficiency allow for relatively convincing conclusions to be drawn, one has to remain cognizant of the possibility that iNKT cells may behave differently in their steady and activated states (241). Much remains to be learned about direct activation of iNKT cells by microbial glycolipids and SAGs likely secreted during polymicrobial sepsis. Future studies should also explore the therapeutic potentials of iNKT cell glycolipid agonists when used in combination with antibiotics.

There is a paucity of information on the role of vNKT cells in sepsis. This is due, at least largely, to a lack of powerful or stable experimental tools to study these cells. $\text{CD}1d^{-/-}$ mice are devoid of both vNKT and iNKT cells (50). Once exclusively iNKT cell-deficient mice become available, they can be used in parallel with $\text{CD}1d^{-/-}$ animals to address the relative contribution of iNKT and vNKT cells to the septic response. Sulfatide-loaded CD1d tetramer reagents invented by Vipin Kumar's laboratory provide a very useful tool for detection of vNKT cells (46) but are not very stable. Treatment with native sulfatide has been used as a means of vNKT cell activation *in vivo*. However, it is likely that sulfatide exerts other effects and engages other cell types in the body. Sulfatide-reactive, CD1d-restricted $\gamma\delta$ T cells have been described in human (242, 243), but their presence in mice is not completely clear. Future studies will need to test the effect of other vNKT cell ligands on sepsis. One such ligand is lysophosphatidylcholine whose levels are in fact altered during inflammatory processes (244). Finally, vNKT and iNKT cells are known to cross-regulate each other in tumor models (245). Whether a similar cross-talk exists during sepsis remains an open question.

Exploration of MAIT cell roles in sepsis is still in its infancy. Mouse and human MAIT cells have distinct tissue distribution

and frequencies. However, inducing sepsis in MAIT cell-deficient MR1^{-/-} mice may still provide useful clues toward understanding the role of these cells in sepsis. In addition, mouse and human MR1 tetramer reagents (68), once more widely available, will undoubtedly advance the field of MAIT cell immunology. They will enable mechanistic and functional studies on MAIT cells and elucidate their effector and/or regulatory functions during sepsis.

CD1d- and MR1-restricted T cells have become a focus of intense investigation in recent years. The advent of novel and reliable tools, techniques and models by which to study these cells will better our understanding of their basic biology and therapeutic

potentials in various disorders including sepsis. We remain optimistic that the remarkable, quick-acting and wide-ranging immunomodulatory functions of these cells can be harnessed to invent efficacious treatments for different stages of sepsis.

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Adoptive T cell therapy targeting CD1 and MR1

Tingxi Guo^{1,2}, Kenji Chamoto² and Naoto Hirano^{1,2*}

¹ Department of Immunology, University of Toronto, Toronto, ON, Canada, ² Princess Margaret Cancer Centre, University Health Network, Toronto, ON, Canada

Adoptive T cell immunotherapy has demonstrated clinically relevant efficacy in treating malignant and infectious diseases. However, much of these therapies have been focused on enhancing, or generating *de novo*, effector functions of conventional T cells recognizing HLA molecules. Given the heterogeneity of HLA alleles, mismatched patients are ineligible for current HLA-restricted adoptive T cell therapies. CD1 and MR1 are class I-like monomorphic molecules and their restricted T cells possess unique T cell receptor specificity against entirely different classes of antigens. CD1 and MR1 molecules present lipid and vitamin B metabolite antigens, respectively, and offer a new front of targets for T cell therapies. This review will cover the recent progress in the basic research of CD1, MR1, and their restricted T cells that possess translational potential.

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Toshinori Nakayama,
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Sid P. Kerkar,
National Institutes of Health, USA

*Correspondence:

Naoto Hirano,
Princess Margaret Cancer Centre,
University Health Network, 610
University Avenue, Toronto, ON M5G
2M9, Canada
naoto.hirano@utoronto.ca

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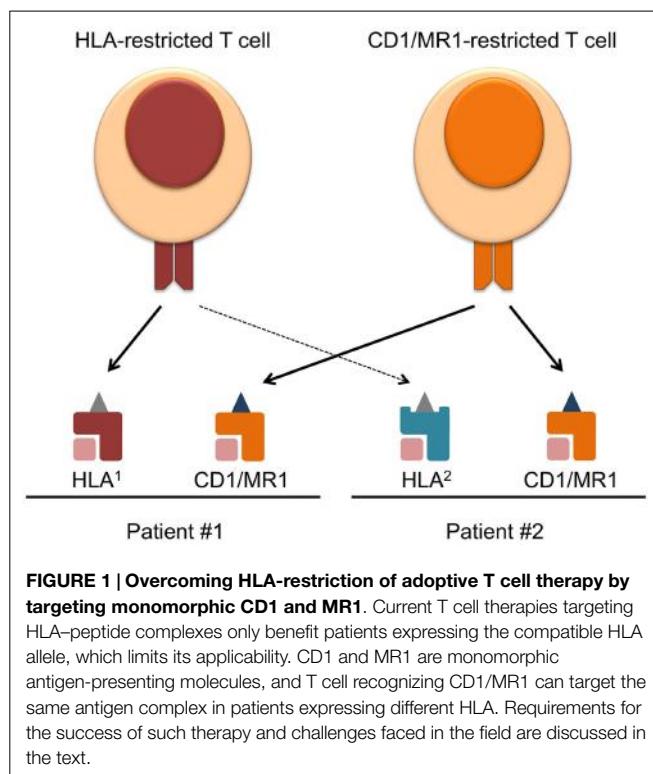
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Introduction

Given their central role in numerous diseases, T cells have become the focus and mediators of many immunotherapies. T cell immunotherapy has had impressive success in treating malignant and infectious diseases. Currently, there are several methods of using T cells as therapy. T cells are cultured and/or engineered *ex vivo* and adoptively transferred into the patient, or T cells are directly targeted *in vivo* by vaccination or biological compounds. Regardless of the approach taken, these immunotherapies generate a *de novo* T cell-mediated immune response and/or enhance preexisting functions, which are often suppressed in patients. Adoptive T cell transfer therapy offers unique advantages and has been considerably tested in numerous trials. The modern approach to this method allows the personalization of T cells through the desired *ex vivo* activation, expansion, and genetic modification, followed by infusion back into the patient (1). As part of this method, we are able to produce a large number of long-lived memory T cells with defined functions, which last up to years in the patients after infusion depending on the expansion protocol (2). The T cells can also be genetically engineered to express recombinant T cell receptors (TCRs) or chimeric antigen receptors (CARs) to specifically target tumor or pathogen-associated antigens. Whereas CARs are only able to target surface molecules, TCRs recognizing peptide antigens presented on HLA are able to target the large repertoire of intracellular antigens.

In the past and current TCR-directed adoptive T cell transfer therapies, most trials have been focused on conventional T cells restricted to one HLA allele. The human HLA gene locus is vastly varied between individuals (3), and although conventional T cell therapies have aimed to target common alleles such as HLA-A2, a significant portion of HLA-mismatched patients cannot benefit from this type of treatment. Therefore, the heterogeneity of HLA alleles represents a major barrier to the applicability of current TCR-directed adoptive T cell therapies. With the recent advancements in the field of CD1, MR1, and their, respectively, restricted T cells, these molecules are becoming attractive targets of immunotherapy. These molecules offer the advantage of being monomorphic



antigen-presenting molecules that are conserved across humans, as well as the ability to present completely different classes of antigens other than peptides (4). Therefore, targeting CD1 and MR1 will broaden the applicability of adoptive T cell therapy (**Figure 1**).

The MHC class I homolog CD1 family of molecules contains four antigen-presenting members in humans, CD1a-d, and only one in mice, CD1d (5). Many of the CD1 studies have been focused on invariant natural killer T (iNKT) cells (type I NKT) found in both humans and mice. These cells are defined by their invariant TCR α and semi-variant TCR β gene usage, and the recognition of the canonical ligand, α -galactosylceramide (α -GalCer) (6). The nature of type II NKT cells, which comprise the remainder of CD1d-restricted T cells that do not recognize α -GalCer, and CD1a-c-restricted T cells have become better understood in recent years. MR1 is also an MHC class I homolog presenting vitamin B metabolites. MR1-antigens complexes are recognized by mucosal-associated invariant T (MAIT) cells, which are another group of evolutionarily conserved $\alpha\beta$ T cells found in high numbers in humans (7). Like iNKT cells, they express an invariant TCR α chain that is paired with an oligoclonal TCR β chain repertoire (8). These molecules and cognate T cells will be discussed further in details below.

To date, the only clinical trials involving CD1 and MR1 have been utilizing iNKT cells as a cellular adjuvant by activating them via α -GalCer. Many mouse studies implicated roles for iNKT cells in tumor regression (9) and antimicrobial immunity (10). Unfortunately, many of these findings have not been translated well to humans. In the published clinical trials, when cancer or chronically infected patients were treated with iNKT cells activated by α -GalCer, alone or pulsed on antigen-presenting

cells (APCs), only limited efficacy was observed (11–19). Based on the experiences that led to effective adoptive T cell therapy targeting HLA, T cell therapies targeting CD1 and MR1 can be improved. In this review, we will address how CD1 and MR1 can be targeted more effectively in diseases by examining the three constituents of successful adoptive T cell transfer therapy, which are the knowledge of (1) disease-associated target antigen complexes, (2) TCRs that recognize these complexes specifically without eliciting harmful autoimmunity, and (3) the optimal function of the responding T cells.

The Targets

To target CD1 or MR1 in diseases, their expression on the pathological tissue of interest is necessary. However, the presence of antigen-presenting molecule alone is not enough. An effective T cell therapy should ideally target diseased tissue specifically, with minimal autoimmune response against healthy tissues. Therefore, understanding the nature of antigens presented during pathological and steady state is required to safely and efficiently target CD1 and MR1 in diseases (**Table 1**).

Pattern of Expression of CD1 and MR1

The CD1 family of lipid-presenting molecules can be separated based on patterns of expression into two groups. Group 1 includes CD1a, CD1b, and CD1c, and is mainly found on professional APCs and developing thymocytes, with CD1a more strictly restricted to Langerhans cells (20, 25, 50). Group 2 only includes CD1d and is expressed widely on many tissues (31, 32). Similar to CD1d, MR1 is expressed at the transcript level in many tissue types (45), but the detection of surface MR1 expression has been challenging. A newly developed mAb against murine MR1 was able to stabilize and enhance its transient surface expression (51). However, the MR1 surface expression pattern on human healthy and pathological tissues is largely unknown.

All CD1 molecules can be found on various leukemia and lymphomas, although the exact expression pattern varies between patients (29, 52, 53). In addition, CD1d can be found on subsets of medulloblastoma, multiple myeloma, and renal cell carcinoma patients (54–56). Other cells in the tumor microenvironment that suppress anti-tumor immunity and/or promote tumor growth also express CD1 molecules. For example, tumor-associated monocytes and macrophages, which are associated with poor prognosis in neuroblastoma patients, were found to express CD1d and could be targeted by iNKT cells in a mouse model (57). Targeting CD1d on both the tumor cells and the supporting stromal cells could be an effective approach.

In infections, CD1d expression can be downregulated by viral immune evasion mechanisms of human immunodeficiency virus and herpes simplex virus (58, 59). Similar to MHC class I, surface CD1d expression on epithelial and immune cells is upregulated in inflammatory conditions and can be induced by interferons (60). Yakimchuk et al. recently demonstrated that CD1b and CD1c molecules are upregulated on Langerhans cells of Lyme disease patients compared to healthy samples. *In vitro*, all group 1 CD1 molecules on monocytes could be upregulated by stimulation with extracted *Borrelia burgdorferi* lipids via TLR-2 as well as IL-1 β (61). The expression of CD1 and MR1 molecules need to be

TABLE 1 | Characteristics of CD1 and MR1 antigen-presenting molecules and their, respectively, restricted T cells in humans.

Antigen-presenting molecule	Pattern of surface expression	Nature of antigens presented	Cognate TCRs	Frequency of cognate T cells
CD1a	Restricted [thymocytes, professional APCs, Langerhans cells, Ref. (20)]	Mycobacterial lipopeptide, and self apolar lipids (21, 22)	Diverse TCRs	~Up to 20% of CD4 ⁺ and CD4 ⁻ CD8 ⁻ T cells (23, 24)
CD1b	Restricted [thymocytes, professional APCs, Ref. (25)]	Mycobacterial lipids (26, 27)	GEM [TRAV1-2-TRAJ9, Ref. (28)], and diverse TCRs	~Up to 1.5% of CD4 ⁺ and CD4 ⁻ CD8 ⁻ T cells (23)
CD1c	Restricted [thymocytes, professional APCs, Ref. (25)]	Mycobacterial lipids and self lysophospholipid (29, 30)	Diverse TCRs	~Up to 7% of CD4 ⁺ and CD4 ⁻ CD8 ⁻ T cells (23)
CD1d	Widely expressed [e.g., hematopoietic, gastrointestinal, and reproductive tissues, Ref. (31, 32)]	Bacterial and self glycolipids, plasmalogens, phospholipids (33–44)	iNKT (mostly TRAV10–TRAJ18 paired with TRBV25), and diverse TCRs (38)	~Up to 3% of CD4 ⁺ and CD4 ⁻ CD8 ⁻ T cells (23)
MR1	Unknown [widely expressed at the mRNA level, Ref. (45)]	Small molecule metabolites (46, 47)	TRAV1-2 paired with TRBV20 or TRBV6 TCRs (48)	~1–10% of total T cells (48, 49)

Antigens listed only include those that have been identified and validated. Frequency is among peripheral blood T cells in healthy humans.

better characterized for different diseases, since understanding the surface expression pattern on infected or transformed cells *in vivo* is essential for CD1- or MR1-restricted immunotherapy.

Antigens Presented by CD1 and MR1

Several studies have shed light on the disease associated and natural antigens presented by CD1 molecules. The ability of group 1 CD1 molecules to present foreign mycobacterial antigens, such as dideoxymycobactin, glucose monomycolate, and mycolic acids, has been well established (21, 26, 27, 30). These antigens are uniquely derived from the bacteria and are not found in the absence of infection. Lepore et al. discovered a novel tumor-associated self-lipid antigen presented by CD1c. Methyl-lysophosphatidic acid was found 100-fold higher in acute B lymphoblastic leukemia and acute myeloid leukemia cells compared to normal B cells or monocytes, and stimulated T cells in a CD1c-dependent manner (29). Although this lipid was also found at elevated levels in dendritic cells, it nevertheless demonstrated the existence of tumor-associated lipid antigens. In addition, some studies have characterized changes in the lipidome of transformed cells (62, 63). Therefore, it is likely that more tumor-associated lipid antigens exist and possibly shared across different patients and cancers. The nature of antigenic self-lipids presented by CD1a were also recently elucidated by de Jong et al. Using a CD1a-restricted T cell line, the group showed that apolar lipids lacking hydrophilic functional groups such as squalene and triacylglyceride were antigenic when presented by CD1a (22).

CD1d is able to present several microbial derived α -linked glycolipids, which are potent activators of iNKT cells (33, 34). For virus-derived lipid antigens, none have been identified to date. However, Zeissig et al. demonstrated that hepatitis B virus-infected human hepatocytes stimulated iNKT cell lines significantly more than non-infected, and this was attributed to the enrichment of virus-induced endogenous antigenic lysophospholipids (35, 36). Several new stimulatory lipid ligands recognized by type II NKT cells have also been discovered. Tatituru et al. demonstrated that bacterial and mammalian phosphatidylglycerol and diphosphatidylglycerol were able to stimulate murine type II NKT hybridomas, but not iNKT cells (37). Nair et al. identified β -glucosylceramide and glucosylsphingosine lyso-glucocerebroside as antigens for human and mouse type II NKT

cells. The circulating levels of these two lipids are elevated in patients with Gaucher's disease. The group stained PBMC with CD1d tetramer loaded with either of the two lipids, and found that the tetramer positive cells did not express the invariant NKT TCR, indicating that they were not type I iNKT cells. Their frequencies were, in fact, much higher than α -GalCer tetramer positive cells. Monocyte-derived DCs pulsed with each antigen were able to expand the respective tetramer positive population (38), validating their stimulatory capacity for type II NKT cells.

In terms of steady state self-lipid antigens, iNKT cells recognize phospholipids, plasmalogens, and glycolipids (39–41). Until recently, it was thought that endogenous mammalian and foreign bacterial glycolipids differed in the β or α linkage between the sugar moiety and the lipid. This was due to the fact that mammals lack the enzyme required to form α -linked glycolipids, which allowed iNKT cells strongly recognizing α -linked glycolipids to easily distinguish between self and foreign antigens. However, two independent groups have reported that a previously identified endogenous β -linked glycolipid, β -glucopyranosyl ceramide (42), was contaminated with the rare, but strongly stimulatory α -linked version (43, 44). Kain et al. demonstrated using α -linked glycolipid specific antibody that mammalian cells likely produced this class of lipids (44). These findings strongly influence the choice of antigen for CD1d targeted therapy, since the previously assumed structural exclusivity of foreign and self-glycolipids may not be so strict.

Mucosal-associated invariant T cells are activated by bacteria but the nature of microbial antigens presented by MR1 remained elusive until recently. Kjer-Nielsen et al. demonstrated vitamin B metabolites as putative ligands presented by MR1, and solved the x-ray crystal structure of a folic acid derivative (6-FP) complexed with MR1. This study also showed that, although 6-FP was not immunogenic in stimulating MAIT TCR transfectants *in vitro*, a riboflavin-derivative isolated from the supernatant of *Salmonella* culture was able to upregulate MR1 expression on an MR1-expressing target cell line and activate primary human MAIT cells (46). The same group also identified byproducts of an intermediate of the riboflavin synthesis pathway as MAIT antigens (47). Given the strong influence of gut microbiota on MAIT cells (64), addressing whether these identified antigens are

presented only during infection or also at steady state dictates their therapeutic potential.

The Receptors

The responsible molecule that targets CD1 or MR1 is the TCR. Clinical trials have confirmed that T cells engineered to express a recombinant TCR can effectively target cells expressing cognate antigens in humans. Therefore, understanding the repertoire of CD1 and MR1-restricted TCRs (**Table 1**) and their molecular mechanism of antigen recognition are important for developing therapy directed at the molecule of interest.

CD1- and MR1-Restricted Invariant T cell Receptors

Invariant NKT TCRs are the CD1-restricted TCRs that have been most extensively characterized. In humans, iNKT TCRs are largely composed of the TRAV10–TRAJ18 invariant TCR α chain paired with TRBV25 TCR β chains with a hypervariable CDR3 β region. In mice, they are mainly TRAV11–TRAJ18 TCR α paired with TRBV13, 29, or 1 TCR β chains (4). Non-TRAV10 and non-TRAV11 iNKT cells have also been identified in humans and mice, respectively, but are generally rare (65, 66). As mentioned above, successful T cell therapy relies on maximizing on-target effect, while minimizing off-target autoreactivity. Having disease-specific antigen is only half of the requirement, since antigen-specific receptors are also required to distinguish them from normal tissue antigens. Several studies demonstrated that iNKT TCRs act as pattern-recognition receptors, unable to distinguish the lipid antigen presented by CD1d (67–69). This is attributed to its conserved docking mode on CD1d, where only the germline-encoded regions of the TCR are involved in recognizing the lipid antigen, while the single variable region, CDR3 β , interacts with the antigen-presenting molecule (67, 68, 70). Therefore, the diversity in the TCR is supposed to only impact the overall affinity to the lipid-CD1d complex, but not antigen selectivity. This would prevent the isolation of tumor or pathogen-specific iNKT TCR. However, our group has recently characterized a large panel of natural human iNKT TCRs and demonstrated selective antigen recognition of different lipid-CD1d complexes. Furthermore, it appears that most of the peripheral human iNKT cells express antigen-selective TCRs (in revision, Chamoto et al.). Given the many crystal structures of iNKT TCR-CD1d complexes showing the same docking mode, these newly identified human iNKT TCRs unlikely possess an alternate docking mode. It is possible that the hypervariable CDR3 β adjust the conformation of the antigen-recognizing germline portion of the TCR (71), in a sequence-dependent manner, thus allowing for distinction between antigens. Intriguingly, mouse and human iNKT TCRs possess cross-species reactivity for human and mouse CD1d, respectively (72). It remains to be seen whether mouse iNKT TCRs are able to distinguish lipid antigens presented by human CD1d. HLA-restricted mouse TCRs have been already tested in the clinic without causing any toxic xenoreaction in cancer patients (73).

Two other subsets of T cells expressing an invariant TCR α chain have been characterized. Recently identified germline-encoded, mycolyl lipid-reactive (GEM) T cells are a subset of

CD1b-restricted T cells expressing a TRAV1-2–TRAJ9 TCR α chain. These TCRs possess a fixed CDR3 α length and minor amino acid variations across different tuberculosis patients. Clonotypic GEM TCRs recognized either mycobacterial antigen glucose monomycolate or mycolic acid presented by CD1b. Structural analysis demonstrated that the footprint of GEM TCRs on CD1b-antigens resembled conventional TCRs, which explains their ability to distinguish the two antigens. Furthermore, GEM TCRs recognized foreign antigens with high affinity and did not display baseline autoreactivity (28). Thus, they represent a viable option in targeting these mycobacteria-derived antigens.

Mucosal-associated invariant T cells represent the third group of T cells with a biased TCR repertoire. Recently developed MR1 tetramer loaded with the stimulatory riboflavin-derivative demonstrated that MAIT TCRs also utilize the TRAV1-2 gene, which is mostly rearranged to TRAJ33, and pairing mostly with TRBV20 or TRBV6-4 (48). Functional and structural studies on MAIT TCRs suggested that they possess antigen selectivity, where stimulation by MR1 $^+$ target cells infected by different genus of microbes specifically enriched different clonal populations of MAIT cells *ex vivo* (74). This is consistent with the crystal structures of MAIT TCR-MR1-antigen complexes, where the TCR takes a more perpendicular docking mode similar to GEM and conventional TCRs (75, 76). In these studies, no interactions were identified between the hypervariable CDR3 β of the MAIT TCR and the two vitamin B metabolite antigens previously identified. Interestingly, however, this docking mode permitted the hypervariable region of the MAIT TCR to interact with novel antigens (47) and a derivative of 6-FP presented by MR1 (77). In the two subsequent studies, the crystal structures of several MAIT TCRs-MR1-antigen complexes were solved, showing that the CDR3 β loop directly interacted with the antigen. Collectively, these evidences support an antigen-selective mode of recognition by MAIT TCRs.

CD1-Restricted Diverse T Cell Receptors

Non-invariant TCRs recognizing CD1 represent the majority of the total CD1-restricted TCR repertoire in humans (23, 38). These diverse TCRs do not appear to possess the conserved parallel docking mode seen with iNKT TCRs. The crystal structures of murine type II NKT TCRs recognizing CD1d-self-antigens demonstrated an orthogonal docking mode similar to the one classically seen with MHC-restricted TCR (78, 79). The CDR3 β made direct interactions with the antigens, indicating that type II NKT TCRs, if all similarly possess this docking mode, would potentially be able to discriminate antigens depending on the hypervariable CDR3 sequences. Roy et al. performed alanine scanning on the CD1c molecule presenting mycobacterial phosphomycoketide (PM) and measured the dissociation constant for the mutants against a panel of CD1c-PM reactive clones (80). The group observed that different point mutations affected the strength of interaction differently for different TCRs. This would not be expected if all the TCRs recognized the CD1c complex in a conserved manner. Whether this variable docking mode holds true for CD1c presenting other foreign and self-antigens remains to be tested, and examining the antigen selectivity of this unpredictable docking mode requires experiments involving

more antigens. The crystal structure of TCR-CD1a-self ligand was recently solved with a clonotypic CD1a-restricted TCR. In this study, although the TCR docked orthogonally onto CD1a, the recognition of the antigen complex relied on contacts with CD1a only. This allowed the TCR to recognize various “permissive” self-ligands that did not disrupt the TCR-CD1a interaction (81). It will be of interest to see if this is also the case with other CD1a-restricted TCRs. How diverse CD1b-restricted TCRs recognize the cognate CD1-antigen complex and their ligand selectivity remains uncharacterized. CD1c- and CD1d-restricted $\gamma\delta$ TCRs have also been identified (82–84), and offer a separate repertoire from which to isolate disease-specific receptors. Structural analysis demonstrated that CD1d-restricted $\gamma\delta$ TCRs recognized the antigen complex also similarly to conventional MHC-restricted TCRs (85). The germline-encoded CDR1 and CDR2 recognized the monomorphic CD1d, while the hypervariable CDR3 δ was positioned on top of the ligand. This is highly suggestive of an antigen-selective mode of recognition. The wealth of potentially antigen-selective $\alpha\beta$ and $\gamma\delta$ TCRs recognizing CD1- or MR1-antigen complexes hold great therapeutic potential for cancer and infection-specific T cell therapy restricted by CD1 or MR1.

The Cells

Functional Phenotype of CD1- and MR1-Restricted T Cells

In the cases of cancer and infection, the ultimate goal of immunotherapy is to induce cell death in the malignant or infected cells to control the disease. As mentioned above, iNKT cells have been targeted with the aim to jump-start the ensuing immune response that ultimately leads to a cytotoxic cellular response. However, in mice, multiple functional subsets of iNKT cells paralleling MHC class II-restricted T cells have been discovered. Lee et al. identified NKT1, NKT2, and NKT17 subsets that preferentially secrete IFN- γ , IL-4, and IL-17, respectively, based on the lineage transcription factor expressed (86). It is unknown whether the NKT1, NKT2, and NKT17 functional subsets exist in humans, and if so, the variation in the frequency of the different functional lineages between individuals needs to be addressed. A small fraction of suppressive IL-10 secreting iNKT cells was reported in humans, and their frequency in peripheral blood ranged over one order of magnitude between individuals (87). Activation of one or a few particular functional subsets of iNKT cells by α -GalCer in some of the clinical trials might explain the lack of efficacy, especially if the activated subset antagonizes a favorable response. As for the other CD1-restricted T cells and MR1-restricted MAIT cells, their functions *in vivo* are largely unknown but likely resemble Th1 and/or Th17 phenotypes. *Ex vivo* or *in vitro* stimulation studies demonstrated that non-iNKT CD1-restricted T cells are capable of producing IFN- γ and MAIT cells producing both IFN- γ and IL-17, both with some capacity for cytotoxicity and IL-2 production (22, 23, 28, 61, 74, 88–90). CD1a-restricted T cells are also able to produce IL-22, consistent with their role in dermal immunity (24). Nevertheless, the fidelity of much of these functions *in vivo* remains to be examined before CD1-restricted T cells and MAIT cells can be used to combat the appropriate disease. Although a Th1 functional profile is

generally preferred for optimal anti-tumor and viral immunity, the multifaceted functionality of CD1- and MR1-restricted T cells can potentially expand their applicability to other diseases.

Large Scale Production of Effector Cells

Once the function of these cells has been established, other aspects of making adoptive therapy successful need to be considered. Obtaining a large number of effector cells is important for efficacy. For example, typically 10–100 billion HLA-restricted T cells are infused to a single cancer patient, although not all are antigen-specific, it still represents a sizable number. Depending on the type of function and the aim of the therapy, fewer cells could be required. Nevertheless, given the limited number of iNKT cells in the periphery, it will be necessary to expand this population for therapy. α -GalCer-based stimulation has been traditionally used and serves as an effective method to expand iNKT cells, either pulsed on APCs or in a cell-free system. However, this method expands all iNKT cells independent of antigen selectivity, avidity, and functional profile. There is also evidence indicating that α -GalCer-expanded iNKT cells possess an anergic phenotype (91). Although other CD1-restricted T cells and MAIT cells are more numerous in the peripheral blood (23, 24, 48, 49), they will likely also require *ex vivo* expansion for therapeutic use (Table 1). Cell-based artificial antigen-presenting cells (aAPCs) have been highly effective in expanding conventional MHC-restricted T cells in preclinical and clinical settings (2, 92–94). Similar aAPCs expressing different CD1 molecules have been developed and could stimulate, respectively, restricted T cells (24). It is possible to improve the capacity of these aAPCs to expand and stimulate CD1 or MR1-restricted T cells by co-expressing the necessary costimulatory molecules along with CD1 or MR1, and culturing in the appropriate cytokine milieu (92).

Memory and Longevity

The longevity of infused T cells *in vivo* is also an important factor in clinical success. It has been demonstrated that central memory T cells are more effective than terminally differentiated T cells in adoptive T cell models of cancer and chronic viral infection, owing to their prolonged survival and effector output (95). iNKT cells are well-known for their pre-primed effector memory phenotype, immediate after maturation and in the absence of antigen exposure, which allows their rapid response. Majority of iNKT cells do not express L-selectin (6), a key marker of naïve and central memory phenotype. Although the *in vivo* turnover rate of peripheral and adoptively transferred iNKT cells is unknown, their effector memory phenotype does not suggest prolonged persistence to the extent of central memory conventional T cells. By contrast, a significant portion of type II NKT cells were recently described to possess a naïve-like phenotype, expressing L-selectin and CD45RA (38). This strongly suggests that they could potentially differentiate to long-lived central memory T cells. In fact, CD1-restricted T cells in general, excluding iNKT cells, seem to possess a naïve T cell phenotype (23). Likewise, MAIT cells in PBMC express central memory markers and start to demonstrate a memory phenotype as early as the age of 3 months in humans (89). Whether the expression pattern of memory phenotype markers on these unconventional T cells confer the same cellular longevity as

conventional MHC-restricted T cells need to be evaluated. Furthermore, the memory phenotype of CD1- and MR1-restricted T cells can be altered during *ex vivo* expansion, as seen with conventional T cells, where they lose their survival capacity after repeated stimulation and subsequent expansion (96). The aAPC system developed can be a useful tool to expand these cells, while maintaining the desired memory phenotype (2, 97).

Mix and Match – Building the Best Therapy

In the age of genetic engineering reaching ever higher levels of feasibility and safety, cellular immunotherapies are no longer limited by the inherent constraints of the naturally existing immune system. The aforementioned problems of iNKT cells in therapy (e.g., apparent lack of antigen and functional specificity, and shortage of numbers) can be overcome by extrinsically modifying cell-intrinsic properties. Heczey et al. recently demonstrated that introducing a CAR targeting GD2, a tumor-associated surface ganglioside, to sorted and expanded human iNKT cells can redirect their specificity independent of CD1d. Importantly, the CAR contained the 4-1BB signaling domain and biased the iNKT cells to a Th1 phenotype upon antigen engagement (98). This approach is an example of how to overcome the inherent limitations of iNKT cells. Conversely, CD1 and MR1 can be targeted by redirecting conventional MHC-restricted T cells with TCRs recognizing CD1/MR1-antigen complexes of interest. This would be one of the most practical and translatable methods of targeting CD1 and MR1 currently, since transducing T cells with recombinant TCRs is a fairly well-established methodology to redirect T cell reactivity and has been used in many trials (99). The major barrier to this would be to identify the appropriate TCRs capable of selectively recognizing diseased tissues, as discussed above, which will represent the rate-limiting step to target CD1 and MR1 through conventional T cells. Combining cell-based therapy targeting CD1 and MR1 with small-molecules (100), checkpoint blockade

reagents, or other biologics could also prove to be beneficial. Anti-CTLA4 mAb treatment combined with adoptive HLA-restricted T cell therapy indeed demonstrated greater efficacy than adoptive T cell therapy alone (2). Lastly, CD1, MR1, or MHC-presented antigens, if they are expressed simultaneously or in combinations on the target cells, can be targeted together to minimize the immune escape routes of the malignant or infectious agents, since these three classes of antigen-presenting molecules likely present ligands derived from non-overlapping molecular pathways.

Concluding Remarks

Adoptive cell therapy of MHC-restricted T cells has undoubtedly produced impressive clinical responses in chronically infected and cancer patients. Use of T cells targeting lipids and small molecule metabolites presented by CD1 and MR1 as a T cell graft will broaden applicability of T cell therapy to more diseases and patients without the limitation of HLA restriction. The research areas pivotal for successful adoptive CD1- and MR1-restricted T cell therapy, which are already underway, are to better characterize the pattern of expression of CD1 and MR1 molecules, identify disease-associated antigens processed and presented by CD1 and MR1 molecules, and isolate cognate TCRs or T cells with the desired function that recognize these antigen complexes but not others. Although the biology of CD1- and MR1-restricted T cells and their receptors require further study before being tested in clinical trials, they represent an exciting venue of therapeutic potential in the near future.

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iNKT and MAIT cell alterations in diabetes

Isabelle Magalhaes^{1,2,3}, **Badr Kiaf**^{1,2,3} and **Agnès Lehuen**^{1,2,3,4*}

¹ INSERM U1016, Institut Cochin, Paris, France, ² UMR8104, CNRS, Paris, France, ³ Laboratoire d'Excellence INFLAMEX, Université Paris Descartes, Sorbonne Paris Cité, Paris, France, ⁴ Département de Diabétologie, Hôpital Cochin, Assistance Publique-Hôpitaux de Paris, Paris, France

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S. M. Mansour Haeryfar,
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Colin C. Anderson,
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Richard S. Blumberg,
Brigham and Women's Hospital, USA
Marika Falcone,
San Raffaele Scientific Institute, Italy

*Correspondence:

Agnès Lehuen,
CNRS8104, INSERM, Institut Cochin,
Hôpital Cochin-Port-Royal, Bâtiment
Cassini, 123, Boulevard de
Port-Royal, Paris 75014, France
agnes.lehuen@inserm.fr

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Type 1 diabetes (T1D) and type 2 diabetes (T2D) are multifactorial diseases with different etiologies in which chronic inflammation takes place. Defects in invariant natural killer T (iNKT) cell populations have been reported in both T1D and T2D patients, mouse models and our recent study revealed mucosal-associated invariant T (MAIT) cell defects in T2D and obese patients. Regarding iNKT cells many studies in non-obese diabetic mice demonstrated their protective role against T1D whereas their potential role in human T1D is still under debate. Studies in mouse models and patients suggest that iNKT cells present in adipose tissue (AT) could exert a regulatory role against obesity and associated metabolic disorders, such as T2D. Scarce data are yet available on MAIT cells; however, we recently described MAIT cell abnormalities in the blood and ATs from obese and T2D patients. These data show that a link between MAIT cells and metabolic disorders pave the way for further investigations on MAIT cells in T1D and T2D in humans and mouse models. Furthermore, we hypothesize that the gut microbiota alterations associated with T1D and T2D could modulate iNKT and MAIT cell frequency and functions. The potential role of iNKT and MAIT cells in the regulation of metabolic pathways and their cross-talk with microbiota represent exciting new lines of research.

Keywords: T1D, T2D, iNKT cells, MAIT cells, obesity, microbiota

Introduction

According to the WHO diabetes will be in 2030 the seventh leading cause of death (1). Type 1 diabetes (T1D) is a chronic autoimmune disease characterized by the destruction of the insulin-producing pancreatic β -cells, resulting in insulin deficiency and hyperglycemia. Type 2 diabetes (T2D) that accounts for 90–95% of all cases of diabetes, is characterized by insulin resistance, hyperglycemia, and decreased β -cell function and mass. The immune system is known to play a deleterious role in T1D as evidenced already in 1965 by Gepts who described insulitis in patients with T1D (2). More recent studies have shown that insulitis also occurs in patients with T2D, and support the notion that inflammation may participate in the pathogenesis of T2D (3, 4).

Invariant natural killer T (iNKT) and mucosal-associated invariant T (MAIT) cells are evolutionary conserved T cell subsets. iNKT and MAIT cells express semi-invariant T cell receptor (TCR) α chains: V α 24J α 18 and V α 7.2J α 33 in humans (V α 14J α 18 and V α 19J α 33 in mice), respectively (5, 6). Both display a memory phenotype, can readily produce cytokines, and thus, represent a bridge between innate and adaptive immunity. Based on mouse models, iNKT cells exert a regulatory role in T1D, while their role in T2D is still matter of debate. Regarding MAIT cells and diabetes, virtually nothing is known and the first insights regarding MAIT cells in T2D patients (7) have only recently been published.

Genetic and environmental factors are crucial in the development of T1D and T2D with growing evidence supporting an important role of the gut microbiota. This review will focus on iNKT and MAIT cells in the context of diabetes and discuss the potential impact of altered gut microbiota on these immune cells.

iNKT Cell Defects in Non-Obese Diabetic Mice

The implication of iNKT cells in diabetes was first demonstrated in non-obese diabetic (NOD) mice that spontaneously develop T1D. Numerical and functional iNKT cell defects in NOD mice have been identified: reduced iNKT cell frequency and IL-4 production (8, 9). When compared with 37 other inbred mouse strains, in NOD mice iNKT cell numbers are at the low end of the spectrum in different tissues (i.e., peripheral blood, spleen, and thymus) (10). In NOD mice, defects in the expression of SLAM by double positive thymocytes that are responsible for the positive selection of NKT cells (11) and by myeloid dendritic cells (DCs) (12) are sought to play a role in the reduced iNKT cell number and impaired iNKT cell IL-4 production, respectively. Several other loci modulating iNKT cells in NOD mice have been identified (13, 14). More recently, Tsaih and colleagues demonstrated that a locus in chromosome 13 inversely regulates CD1d expression on double positive thymocytes and iNKT cell frequency, with the NOD allele shown to promote high CD1d expression on thymocytes and subsequent low iNKT cell frequency (15).

Of note, NOD mice have elevated frequency and number of an iNKT cell subpopulation producing IL-17, namely, iNKT17 cells, in the thymus and periphery (16). In the pancreatic lymph nodes, iNKT17 cells represent 13% of total iNKT cells in NOD mice as compared to 2% in C57BL/6 mice (16, 17).

Regulatory Role of iNKT Cells in T1D in Mice

The accelerated development of T1D in CD1d-deficient NOD mice (18, 19), and the prevention of T1D development in NOD mice with increased iNKT cell number (20, 21) have suggested that iNKT cells play overall a protective role in T1D. T1D protection mediated by iNKT cells after cell transfer, upon cyclophosphamide treatment, or activation by α -galactosylceramide (α -GalCer), was shown to rely on IL-4 and/or IL-10 production (20, 22) and inhibition of pathogenic autoimmune responses (23, 24). Repetitive stimulation with α -GalCer-induced tolerogenic myeloid DCs (25) and plasmacytoid DCs that in turn converted naive BDC2.5 diabetogenic T cells into regulatory T (Treg) cells in pancreatic lymph nodes (26). Our group also demonstrated that iNKT cells could induce BDC2.5 T cell anergy in a cytokine-independent (i.e., IL-4, IL-10, IL-13, and TGF- β) (27) fashion, but required cell-cell contact and was independent of CD1d expression in the periphery, suggesting that molecular interactions other than CD1d/TCR are involved (28).

Environmental factors, such as viral infections, can be either deleterious or protective in T1D. Upon lymphohochoriomeningitis virus infection, in pancreatic lymph nodes iNKT cells-induced tolerogenic plasmacytoid DCs, which converted naive T cells into

Treg cells that migrated to the pancreatic islets and inhibited anti-islet T cells, thereby providing protection against T1D (29). Of note, even though a single injection of α -GalCer at the time of infection increased the frequency of Treg cells in pancreatic islets, and further promoted the protection against T1D, such protection was even seen in the absence of α -GalCer injection in wild type mice, but not in CD1d and J α 18 deficient NOD mice. Thus, iNKT cells are key in the induction of Treg cells and the protection against T1D in this infectious setting. We have also analyzed the role of iNKT cells upon another viral infection that is relevant to the human disease. Coxsackievirus B4 has been proposed as an etiologic agent that could promote the development of T1D in patients as well as in the diabetes susceptible NOD mouse. Coxsackievirus B4 infection accelerated T1D in NOD mice, whereas α -GalCer injection at the time of infection activated pancreatic iNKT cells that produced rapidly large amount of IFN- γ and upregulated indoleamine 2,3-dioxygenase production by macrophages recruited in the pancreas. These suppressive macrophages inhibited pancreatic anti-islet T cells and subsequently prevented T1D development (30). These data together showed that in both viral infections, through two different mechanisms, iNKT cells exert an efficient regulatory role.

However, not all iNKT cell subsets are protective. We showed that iNKT17 cells infiltrate the pancreas of NOD mice and promote diabetes development. α -GalCer treatment suppresses IL-17 (and to a lesser extend IFN- γ) produced by iNKT cells, which could also contribute to the protective role of α -GalCer in T1D (16). Of note, the presence of IL-1 and IL-6 in inflamed pancreatic islets of NOD mice may contribute to the activation of iNKT17 cells (31).

Putative Role of iNKT Cells in Human T1D

The first data obtained in patients with T1D showed a decreased frequency of iNKT cells as well as a defect in IL-4 production (32) but since, contradictory results from clinical studies have been published; some following reports have supported this finding (33, 34), while one report has shown increased numbers of iNKT cells (35), and others did not find differences in iNKT cell numbers (36–39).

While the frequency of iNKT17 cells might be extremely low in the peripheral blood of healthy controls and patients with T1D, iNKT17 cells could be expanded *in vitro* in the presence of IL-1 β . These cells were only obtained from the blood of T1D patients but not from healthy controls (40), suggesting that iNKT17 cells could also be involved in T1D pathogenesis in patients.

Altogether, despite converging evidence that iNKT cells play a regulatory role in T1D using mouse models, their role in human T1D remains controversial urging more clinical studies with well defined T1D patient cohorts.

iNKT Cells in T2D and Obesity

Type 2 diabetes is a progressive disease resulting from the insulin resistance that develops with advancing age and lifestyle factors, such as inactivity, diet, and obesity (most patients with T2D are obese or overweight), but those factors are not the only trigger. It is now recognized that T2D results from the interaction between

different genetic events and with environmental factors (41). The detection of TNF- α in obese rat adipose tissue (AT) provided the first evidence that tissue inflammation was correlated with insulin resistance and T2D (42). In the lean state M2 macrophages with an anti-inflammatory phenotype accumulate in AT, whereas obesity leads to the preferential accumulation in AT of proinflammatory M1 macrophages known to participate in insulin resistance development. Other immune cells infiltrate AT, and iNKT cells are particularly enriched in white AT.

In obese mice, iNKT cell frequency in white AT is decreased while weight loss reverses decreased AT iNKT cell frequency (43). Several studies have analyzed the impact of iNKT cells in metabolic control with contradictory results. The use of CD1d $^{-/-}$ or J α 18 $^{-/-}$ mice lacking all NKT cells (iNKT and variant NKT cells) or only iNKT cells, respectively, and other factors, such as different diets, or experimental procedures have been implicated to explain the protective, the absence, or the negative impact of iNKT cells on weight gain or metabolic control (44, 45). In a recent review, Lynch argues that despite the divergent results obtained using iNKT-deficient mouse models, most experiments using transferred or activated α -GalCer iNKT cells converge to support a protective role of iNKT cells in obesity and she proposes that AT iNKT cells via IL-4 and IL-10 production regulate anti-inflammatory cytokines and adipocyte function (46). The regulatory role of AT iNKT cells is supported by recent findings showing that in murine AT, iNKT cells did not express the PLZF transcription factor, characteristic of iNKT cells, but instead the transcription factor E4BP4, and via IL-10 and IL-2 expression control the homeostasis of macrophages and Treg cells, respectively (47).

In obese patients as compared to lean individuals, iNKT cell frequency is decreased in omental AT and peripheral blood (7, 48). Conversely, iNKT cell frequency in peripheral blood is restored after bariatric surgery of obese patients (43).

MAIT Cells in T1D

Due to the lack of specific antibodies directed against the murine V α 19 TCR chain, limited data on murine MAIT cells are available. However, the recent development of mouse MR1-antigen loaded tetramers detecting specifically MAIT cells (49) will most likely soon shed a new light on the role of MAIT cells in different mouse disease models, such as diabetes.

To date, only scarce data on the role of MAIT/MAIT-like cells in T1D are available. The observation that the expression of V α 19J α 33 TCR as a transgene in NOD mice delays the onset of T1D (50) suggests that MAIT cells may play a protective role. In humans, MAIT cells are identified using anti-V α 7.2 TCR chain and anti-CD161 antibodies. A recent report analyzed that the CD161 $^{\text{bright}}$ CD8 $^{+}$ T cell subset in juvenile T1D patients (51), with the CD161 $^{\text{bright}}$ CD8 $^{+}$ T cells displaying a phenotype, IL-18R α $^{+}$, CD127 $^{+}$, CD45RA $^{-}$, and CCR7 $^{-}$, suggestive of MAIT cells. No difference in the CD161 $^{\text{bright}}$ CD8 $^{+}$ T cell frequency was observed in juvenile T1D patients as compared to age-matched controls. As described previously for MAIT cells (52), the frequency of CD161 $^{\text{bright}}$ CD8 $^{+}$ T cells increased with age in juvenile controls and new-onset T1D patients but not in juvenile

long-standing (≥ 1 year) T1D patients. These results suggest that in long-standing T1D patients the circulating CD161 $^{\text{bright}}$ CD8 $^{+}$ T cells may be depleted. The CD27 $^{-}$ CD161 $^{\text{bright}}$ CD8 $^{+}$ T cells (a subset enriched in IL-17 producing cells) were increased in patients with T1D as compared to controls. Further studies of T1D patients using anti-V α 7.2 TCR chain and anti-CD161 antibodies and/or with human MR1-antigen loaded tetramers specifically directed toward MAIT cells are needed in order to accurately decipher their role in T1D.

Additionally, whether MAIT cells as seen for IL-17-producing $\gamma\delta$ T cells exit the thymus as CD27 $^{-}$ cells (53), or acquire the CD27 $^{-}$ phenotype in the periphery upon activation and differentiation as observed for Th17 cells (54) and the understanding of the underlying mechanisms would be of utter interest.

MAIT Cell Defects in T2D and Obese Patients

Our group has shown that MAIT cells exhibit several defects in T2D and obese patients (7). MAIT cell frequency was dramatically reduced in patients with T2D, and particularly in obese patients. In 12/69 severe obese patients study, we could not detect circulating MAIT cells. Higher frequencies of MAIT cells producing IL-17 were detected in T2D and obese patients, as compared to lean control individuals, and this was even more pronounced in T2D patients. Furthermore, when stimulated *in vitro* with MAIT cell ligand, a higher frequency of T2D patient MAIT cells produced IL-17. We showed that MAIT cells are present in the omental and subcutaneous AT, with comparable frequencies between lean control individuals and obese patients. Interestingly, in five obese patients for whom we could not detect circulating MAIT cells, MAIT cells were present in omental AT. In AT, and particularly subcutaneous AT, a vast majority of MAIT cells from obese patients, but not from lean control individuals, produced IL-17. AT from obese patients promoted MAIT cell activation (upregulation of CD25 and CD69 expression), and as compared to circulating MAIT cells, AT MAIT cells displayed higher Ki67 expression, altogether suggesting a recruitment, and local activation of MAIT cells in the AT. Bariatric surgery of obese patients restored circulating MAIT cell frequency and decreased their production of IL-2 and granzyme B. However, up to 12 months post-surgery high frequencies of MAIT cells still displayed an augmented Th17 profile. A recent report by Carolan et al. confirms our observation on MAIT cell alteration in adult obesity, showing decreased MAIT cell frequency and increased IL-17 production (55). However, the frequency of circulating MAIT cells in obese children was increased as compared to lean children, and this increased frequency in patients was associated with hyperinsulinemia and insulin resistance. Their analysis of adult obese patients' AT confirms the increased production of IL-17 by MAIT cells and shows a decreased frequency of IL-10 producing MAIT cells.

The mechanisms underlying MAIT cells defects and increased IL-17 production in T2D and obesity remain to elucidate. For instance, whether the expression of IL-1 β (56) and IL-6 (57) in the AT of T2D patients plays a role in MAIT cell activation and production would be of interest. Altogether, these data show alterations of the MAIT cell compartment in T2D and obese patients

and paves the way for further studies assessing the role of MAIT cells in diabetes and metabolic disorders.

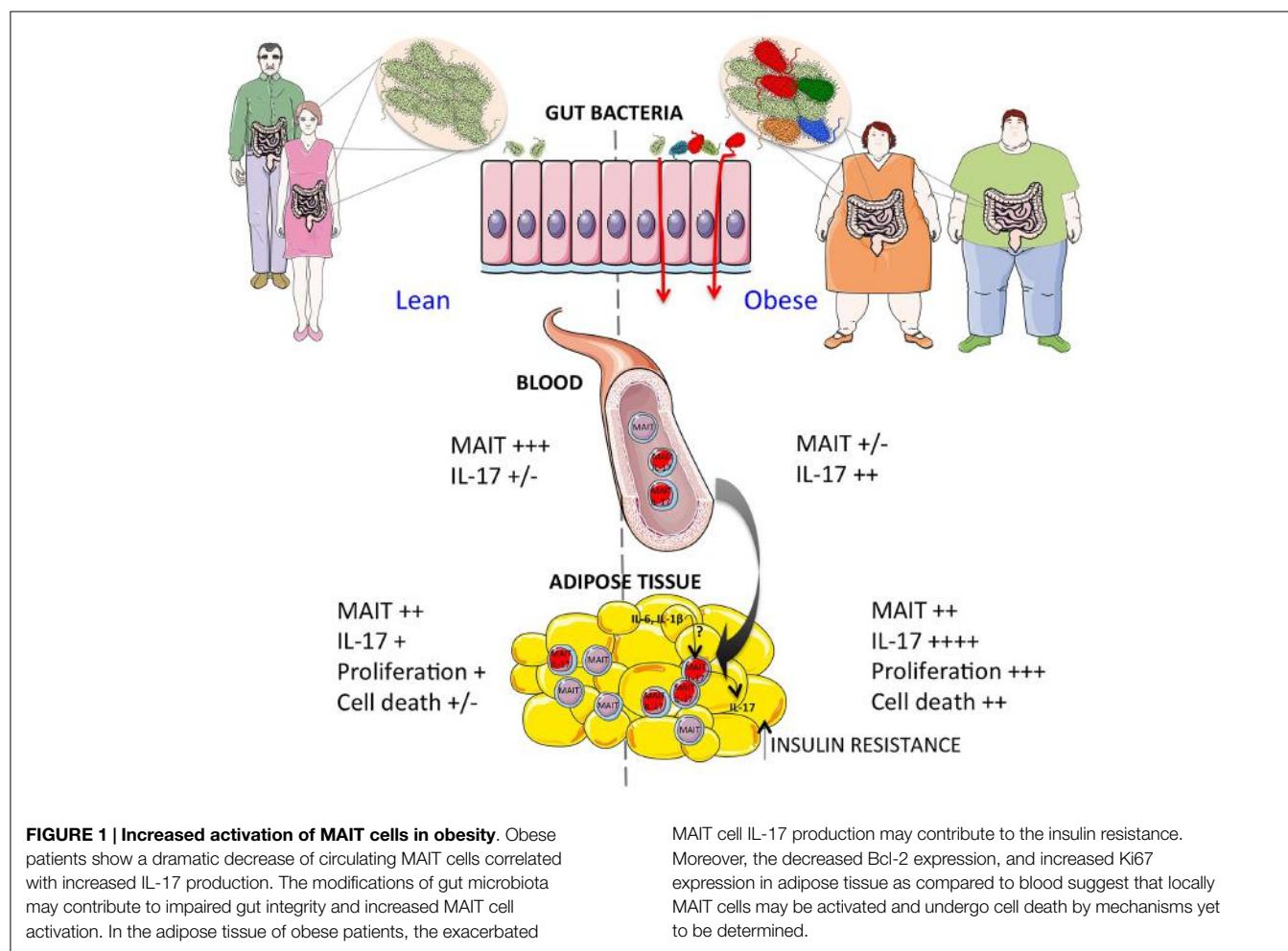
iNKT and MAIT Cells and Impact of Microbiota in T1D and T2D

Type 1 diabetes and T2D are linked to genetic predisposition but non-genetically determined factors, such as the gut microbiota, also impact their development. We will discuss below how altered gut microbiota may impact iNKT cells and MAIT cells in T1D and T2D.

Invariant natural killer T and MAIT cells are present in human and murine AT and intestine. In mice, microbial exposure early in life impacts iNKT cell numbers (58), and in germ-free mice MAIT cells are absent in peripheral tissues (59). Altogether, these results show that commensal bacteria impact intestinal iNKT cell homeostasis, and are essential for MAIT cell expansion. The microbial lipids activating intestinal iNKT cells remain to be elucidated, but recent reports have shown that, in a CD1d-dependent fashion, some sphingolipids from the gut commensal *Bacteroides fragilis* activate (60) while other sphingolipids inhibit iNKT cells (61). MAIT cell ligands are MR1-restricted derived bacterial products of vitamin B metabolism (62). The 6-formyl pterin, a folic acid (vitamin B9) metabolite is a non-activating ligand, while ligands

derived from the riboflavin (vitamin B2) synthesis pathway, such as ribityllumazines and pyrimidines, activate MAIT cells. Pyrimidines that represent the most potent MAIT cell activating ligands are formed from the condensation of an intermediate of riboflavin synthesis (5-amino-6-D-ribitylaminouracil) with the glucose-derived methylglyoxal or glyoxal (63).

Alterations in the gut microbiota of NOD mice have been shown to be strongly associated with the development of T1D. This was supported by gut microbiota transfer experiments from T1D-protected animals into young diabetes-prone mice that upon transfer showed delayed T1D or protection from T1D (64, 65). The impact of the human intestinal microbiota on different diseases including T1D development is a field of intensive investigation and cohort studies designed to address its role on T1D are currently underway (66). Several studies analyzing the fecal bacteria composition and metagenomic support the association between changes in intestinal microbiota and risk of T1D (67, 68). Similarly, alterations of the gut microbiota composition have been reported in obese mice, obese patients, and patients with T2D (69). In turn, those alterations in microbiota may impact gut permeability in T1D, obesity, and T2D; increased bacterial translocation is thought to contribute to the establishment of AT microbiota (70). Two metagenome studies of T2D patients and healthy individuals have revealed differences in microbial



functions related to vitamin metabolism (including riboflavin) (71, 72). Interestingly, in T1D and T2D patients the production and plasma levels of methylglyoxal are elevated (73).

The gut microbiota plays a crucial role on iNKT and MAIT cell development, and iNKT and MAIT cells activating and inhibiting microbial-derived antigens have been identified. Therefore, it is tempting to speculate that in T1D and T2D alterations of the gut microbiota, and possibly also AT associated microbiota, impact iNKT and MAIT cells homeostasis in the gut and AT. We hypothesize that in diabetes and obesity MAIT cells are recruited to the gut and/or AT therefore depleting the circulating compartments, and in the AT of obese patients the exacerbated IL-17 production by MAIT cells participate to the local inflammation and insulin resistance (**Figure 1**).

Invariant natural killer T cells have been shown to influence the gut microbial colonization (74). It would be very interesting to assess if MAIT cells can also modulate the gut microbiota. A recent report showed that circulating MAIT cell deficiency observed in patients with systemic lupus erythematosus (SLE) and patients with rheumatoid arthritis (RA) was associated with circulating iNKT cell deficiency in patients with SLE but not in patients with RA (75). Furthermore, the authors showed that iNKT cell activation by α -GalCer induces MAIT cell activation. In obese patients, we did not find a correlation between the decreased iNKT and MAIT cell frequency (unpublished data).

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