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Activation strategies for invariant natural killer T cells

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Abstract

Invariant natural killer T (iNKT) cells are a specialized T cell subset that plays an important role in host defense, orchestrating both innate and adaptive immune effector responses against a variety of microbes. Specific microbial lipids and mammalian self lipids displayed by the antigen-presenting molecule CD1d can activate iNKT cells through their semi-invariant αβ T cell receptors (TCRs). iNKT cells also constitutively express receptors for inflammatory cytokines typically secreted by antigen-presenting cells (APCs) after recognition of pathogen-associated molecular patterns (PAMPs), and they can be activated through these cytokine receptors either in combination with TCR signals, or in some cases even in the absence of TCR signaling. During infection, experimental evidence suggests that both TCR-driven and cytokine-driven mechanisms contribute to iNKT cell activation. While the relative contributions of these two signaling mechanisms can vary widely depending on the infectious context, both lipid antigens and PAMPs mediate reciprocal activation of iNKT cells and APCs, leading to downstream activation of multiple other immune cell types to promote pathogen clearance. In this review, we discuss the mechanisms involved in iNKT cell activation during infection, focusing on the central contributions of both lipid antigens and PAMP-induced inflammatory cytokines, and highlight *in vivo* examples of activation during bacterial, viral, and fungal infections.

Keywords

iNKT; NKT; natural killer T cell; CD1d; lipid antigen

Introduction

The mammalian immune system evolved to protect us against a range of pathogens. Innate immunity provides rapid responses to conserved molecular patterns and danger signals associated with pathogenic encounters. Adaptive immunity provides foreign antigen-specific responses that contribute to eliminate and provide memory against pathogens, but takes days to manifest. Invariant natural killer T (iNKT) cells are a member of a growing family of non-major histocompatibility complex (MHC)-restricted T cells that combine features of both

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innate and adaptive immunity (Godfrey et al. 2015). iNKT cells recognize lipid antigens presented by CD1d, and recognize CD1d-lipid antigen complexes using a limited set of T cell receptor (TCR) rearrangements generated in the thymus by somatic recombination (Bendelac et al. 1995, 2007; Godfrey et al. 2010). Genetically engineered mice deficient in CD1d fail to develop iNKT cells, and such mice have impaired immune responses to many bacteria, viruses, and fungi, demonstrating a broad role for iNKT cells in host defense (Brigl and Brenner 2010; Chandra and Kronenberg 2015; Cohen et al. 2009). iNKT cells bear a resemblance to adaptive T cells with respect to ontogeny and antigen receptor generation, and can be readily activated by signals through their TCRs. However, by the end of their development, NKT cells leave the thymus expressing receptors for inflammatory cytokines such as IL-12, IL-18, IL-25, and IL-23, similar to natural killer (NK) cells and innate lymphoid cells (ILCs), making them highly sensitive to cytokine signals (Cohen et al. 2013; Engel et al. 2016; Watarai et al. 2012). Activation can occur via the steady-state expression of these inflammatory cytokine receptors even in the absence of TCR signals, allowing microbes that are not directly recognized by the iNKT cell TCR to stimulate iNKT cell activation. Activation via the combination of TCR and non-TCR signals results in the rapid and often profound expression of effector functions in a time frame similar to that of innate leukocytes rather than the slower process of clonal proliferation and differentiation of adaptive lymphocytes. To understand the function of iNKT cells in host defense, we will outline how they integrate both TCR and non-TCR signals to activate their diverse effector functions.

Borrelia burgdorferi, *Sphingomonas* spp., and *Streptococcus pneumoniae* all contain specific lipid antigens that can be presented on CD1d and recognized directly by the iNKT cell TCR (Kinjo et al. 2005, 2006, 2011; Mattner et al. 2005; Sriram et al. 2005). Some microbes therefore harbor the potential to drive CD1d-dependent iNKT cell responses by providing a potent TCR signal. In contrast, virtually all microbes contain pathogen-associated molecular patterns (PAMPs) that stimulate innate pathways in antigen-presenting cells (APCs) and can drive iNKT cell activation via cytokine receptor signaling in the absence of recognition of foreign glycolipid antigens by the TCR. For example, *Salmonella* spp. can activate APCs through Toll-like receptor 4 (TLR4) via recognition of lipopolysaccharide (LPS), resulting in the production of IL-12 and IL-18 that in turn stimulates iNKT cells (Brigl et al. 2003). Since many iNKT cells have high baseline expression of cytokine receptors such as IL-12Rβ1 and β2, they are especially sensitive to cytokine-driven activation. Yet, even when iNKT cells are not exposed to foreign lipid antigens, they may still be activated through TCR stimulation via CD1d-lipid antigens derived from the APC itself, referred to as “self lipid” antigens. During most infections *in vivo*, some combination of CD1d-restricted TCR signals and cytokine signals drives iNKT cell activation, though the relative contribution from each is often unclear. iNKT cells are important for controlling infection by a wide range of microbes, and the relative input from cytokine and TCR signals may be different for each microbe.

Rather than being predominantly localized to lymph nodes, iNKT cells are often already localized at tissues in the basal state, and in many cases are tissue-resident with very limited recirculation (Fan and Rudensky 2016; Lynch et al. 2015; Thomas et al. 2011). In contrast to the MHC-peptide-antigen driven presentation pathway that activates naive T cells in the

draining lymph nodes, iNKT cells are poised at steady state to respond rapidly to APC-derived signals at peripheral sites of infection. iNKT cells are triggered early in the course of infection and engage in bidirectional molecular crosstalk with a variety of leukocytes. It is this early dynamic interaction that can profoundly shape the ensuing immune response to bacteria, viruses, and fungi. In this review, we will focus on the molecular interactions between APCs and iNKT cells, and between activated iNKT cells and other effector cells in infected tissues. We will highlight the relevant lipid-driven TCR signals and cytokine-receptor driven signals that lead to iNKT cell activation, and illustrate how iNKT cells orchestrate the immune response mediated by a variety of other effector cells.

Lipid uptake and presentation by CD1d

In order to be recognized by the iNKT cell TCR, lipids must first be loaded onto CD1d and presented on the plasma membrane of APCs. After assembly in the endoplasmic reticulum (ER) and delivery to the plasma membrane, CD1 molecules localize to distinct endosomal compartments that they survey to bind antigens and continuously recycle between the endosomal compartment and cell surface (Barral and Brenner 2007). During ribosomal translation, CD1d is translocated into the ER via its signal sequence. There, it undergoes folding and assembly with β2 microglobulin and endogenous lipid ligands with the assistance of calnexin and calreticulin, components also involved in MHC-peptide assembly (Kang and Cresswell 2002). Once folded and stabilized, CD1d-lipid antigen complexes are delivered along the secretory pathway to the cell surface (Bauer et al. 1997; Brutkiewicz et al. 1995). From the cell surface, CD1d is continuously internalized and delivered to lysosomes, as directed by lysine-based sorting motifs in its cytosolic tail. In lysosomes, CD1d complexes may encounter exogenous lipid antigens and exchange their bound endogenous lipids for exogenous lipids before trafficking back to the plasma membrane for recognition by T cells (Barral and Brenner 2007). A study using a mouse model of CD1d with a truncated cytoplasmic tail showed that internalization of CD1d to the endosome was essential for presentation of exogenous lipid antigens (Chiu et al. 2001). Moreover, efficient loading of lipids in CD1d requires lipid transfer proteins, including saposins, which have been shown to play a significant role in the breakdown of glycolipids in lysosomes. Saposin B, in particular, was found to be important for loading lipid antigens in CD1d (León et al. 2012; Yuan et al. 2007). Microsomal triglyceride transfer protein has also been implicated in lipid loading onto CD1d in the ER (Brozovic et al. 2004; Dougan et al. 2007) and may additionally be important for CD1d recycling from the lysosome (Sagiv et al. 2007). Together, these studies show that CD1d has a trafficking pattern that is different from MHC class I and class II, in which it surveys endosomal compartments, exchanges lipids, and continually recycles to present endosomal lipids on the cell surface for recognition by iNKT cells.

Dendritic cells (DCs) and other APCs typically take up exogenous proteins by pinocytosis and phagocytosis and can thereby survey their local environment for antigens. However, in 2005 we found that lipid antigens were inefficiently taken up by DCs from the media unless serum was present. It was noted that when a model iNKT cell lipid antigen was incubated in human serum, it quickly associated with the very low-density lipoprotein fraction, allowing for rapid and efficient receptor-mediated uptake into DCs. The pathway was dependent on

apolipoproteins, in this case apolipoprotein E (ApoE), and the low-density lipoprotein receptor on DCs. We proposed that in addition to the relatively inefficient uptake of lipids by pinocytosis, APCs sample their local environment for lipid antigens and use lipoprotein-binding receptors to facilitate lipid uptake. Moreover, APCs were shown to secrete ApoE as a means to enhance uptake for CD1-based antigen presentation (van den Elzen et al. 2005). Freigang and colleagues used a panel of iNKT cell agonist lipids and different lipoprotein receptor-deficient APCs to test if lipid uptake into cells was dependent on lipid structure. Mice deficient in specific lipoprotein receptors such as CD36 or scavenger receptor A were unable to respond to injection of select iNKT cell agonists, demonstrating that some lipid antigens exclusively utilize one receptor for antigen uptake, whereas other antigens could be taken up via multiple pathways (Freigang et al. 2012). Thus, lipid antigen presentation by CD1 utilizes some of the same machinery utilized for lipid uptake for energy and lipid clearance, adopting this machinery for efficient lipid uptake and delivery into endocytic compartments for loading onto CD1d. These receptor-mediated uptake pathways appear to complement those mediated by phagocytosis of organisms for antigen delivery to lysosomes. A summary review of lipid uptake, processing, and presentation on CD1d can be found elsewhere (Barral and Brenner 2007).

Multiple cell types express CD1d, including hematopoietic and non-hematopoietic cells. Data suggests that the cell types involved in the presentation of lipids, the structures of the specific lipid antigens, and the organ in which antigen presentation occurs may all influence iNKT cell activation. Studies performed with the injection of solubilized lipid antigen have demonstrated an important role for DCs (Arora et al. 2014; Bai et al. 2012; Zajonc et al. 2005). Using a fluorescently labeled lipid and an antibody specific for lipid antigen-CD1d complexes, Arora and colleagues identified $CD8\alpha^{+}DEC205^{+}$ DCs as the major population taking up exogenous glycolipid antigens in mice. Notably, the expression level of CD1d on an APC had little to do with that cell's ability to present antigenic lipids compared to the efficiency of lipid antigen uptake (Arora et al. 2014). During many infections, lipid antigens are likely to be delivered to CD1d on APCs following the uptake of particulate microbes and degradation of these particulates in lysosomal compartments. Barral and colleagues injected mice with silica particles that had been coated with antigenic lipids to mimic bacterial antigens encountered through the endocytic pathway. *In vivo* imaging studies showed that lymph node iNKT cells arrested their movement and colocalized with subcapsular sinus $CD169^{+}$ macrophages, and that these APCs were responsible for the activation iNKT cells in this setting (Barral et al. 2010). Similarly in the liver during *B. burgdorferi* infection, specialized liver macrophages, Kupffer cells, were found to take up whole spirochetes and present their microbial glycolipids to iNKT cells (Lee et al. 2010). Thus, whether lipid antigen is present as a particulate or bound to soluble proteins can determine the relevant APC and the uptake pathway involved in lipid antigen presentation to iNKT cells.

Non-leukocytes also express CD1d, and may present lipid antigens to iNKT cells in certain settings. Adipocytes express high levels of CD1d and have been reported to present antigenic lipids to adipose iNKT cells (Huh et al. 2013; Rakhshandehroo et al. 2014), a unique regulatory iNKT cell subset (Lynch et al. 2015). CD1d is also expressed on the intestinal epithelium (Blumberg et al. 1991; Olszak et al. 2014). By selective ablation of CD1d from intestinal epithelium using a transgenic mouse approach, Olszak and colleagues

showed that ligation of CD1d in the gut epithelium by tissue-resident iNKT cells induced IL-10 expression in intestinal epithelial cells, providing protection in a model of colitis (Olszak et al. 2014). Hepatocytes also express CD1d and have been implicated in iNKT cell activation in multiple contexts (Hua et al. 2010; Zeissig et al. 2012).

Predominantly TCR-driven pathways of iNKT cell activation by lipid antigens

"Invariant" NKT cells are so-named because of their limited TCR repertoire, a striking contrast to MHC-restricted adaptive T cells. In mice, most iNKT cell TCRs use germline Vα14-Jα18 rearrangements without N-region diversity and are rearranged mainly to Vβ8.2, Vβ7, or Vβ2 gene segments (Benlagha et al. 2000; Lantz and Bendelac 1994; Matsuda et al. 2000). In a similar way, the invariant TCR is generated in humans from Vα24-Jα18 rearrangements paired almost exclusively with the Vβ11 chain (Dellabona et al. 1994; Lee et al. 2002; Porcelli et al. 1996). The antigen-presenting element CD1d is itself conserved as it displays little to no polymorphism (Dascher and Brenner 2003), in contrast to the highly polymorphic MHC class I and class II molecules. The combination of a limited TCR repertoire and a monomorphic restricting element suggest a limited set of antigenic targets. The surprising discovery that T cells could recognize lipids as antigens, first reported for mycolic acids from *Mycobacterium tuberculosis* presented on CD1b (Beckman et al. 1994), prompted the search for lipid antigens that could be presented by other CD1 molecules, including CD1d. As described below, lipid structures from both microbial and endogenous sources have been identified as CD1d-presented antigens for iNKT cells.

The first iNKT cell antigen discovered was α-galactosylceramide (α-GalCer), a lipid purified from a marine sponge and then synthetically modified to enhance activity (Kawano et al. 1997; Natori et al. 1997). With the discovery of α-GalCer, Kawano and colleagues also delineated a key structural characteristic that proved to be relevant for many glycolipids that activate iNKT cells, namely an α-stereochemical linkage of the anomeric sugar to the lipid backbone. Since mammals were not known to have the enzymatic machinery for producing α-linked glycosphingolipids, this finding raised the possibility that iNKT cells might recognize α-linked lipids as a microbial motif. In 2005, α-glucuronosylceramide and α-galacturonosylceramide, two *Sphingomonas* cell wall glycosphingolipids (GSLs) were identified as iNKT cell agonists by several groups (Kinjo et al. 2005; Mattner et al. 2005; Sriram et al. 2005; Wu et al. 2005). Since then, other microbial GSLs have been shown to activate iNKT cells, including lipids from the α-proteobacterium *Novosphingobium aromaticivorans* (Mattner et al. 2008) and the human gut microbe *Bacteroides fragilis* (An et al. 2014; Wieland Brown et al. 2013). Diacyglycerol-based lipids bearing α-linked carbohydrate headgroups also have been identified as antigenic for iNKT cells. α-linked galactosyldiacyglycerols from *B. burgdorferi* were the first antigens discovered in this class (Kinjo et al. 2006). Later studies from the same group found that α-linked glucosyldiacylglycerols from *S. pneumoniae* were also recognized by the iNKT TCR (Kinjo et al. 2011). *Helicobacter pylori* has been shown to make α-linked cholesterol glycoside, yet another class of lipid antigen that can be recognized by iNKT cells (Chang et al. 2011).

These landmark studies demonstrate that select microbes produce lipid antigens from several lipid classes, but that all share as a feature α-linked sugar motifs.

The identification of microbial lipid antigens proved to be only a part of the iNKT cell antigen story. Several key observations made it clear that there was an important role for self lipid antigens in iNKT cell biology. It had been known for some time that iNKT cells recognize CD1d-expressing transfectants and tumor cell lines *in vitro* in the absence of microbial products. Additionally, iNKT cells and CD1d play a role in many immune responses where foreign lipid antigens are not present, such as viral infections. iNKT cell recognition of endogenous lipids had also been suggested to be critical for positive selection in the thymus (Bendelac 1995; Coles and Raulet 2000). Gumperz et al., a study from our group, was the first report showing that extracted mammalian cellular lipids loaded onto purified, plate-bound CD1d could induce the activation of CD1d-restricted T cells, including iNKT cells, in the absence of exogenous lipids (Gumperz et al. 2000). Isoglobotrihexocylceramide (iGb3), a GSL present in mice, was proposed to be involved in iNKT cell development and activation (Zhou et al. 2004). iGb3 was shown to activate mouse iNKT cells when presented by CD1d, and the basis for this activation has been structurally defined (Pellicci et al. 2011; Yu et al. 2011; Zajonc et al. 2008). LPS-activated mouse DCs were shown to enhance their production of iGb3, an observation that put forward an important possible mechanism for the activation of iNKT cells by pathogens that lack antigenic lipids themselves (Mattner et al. 2005). However, later studies brought the physiological relevance of iGb3 as an iNKT cell antigen into question. Mice deficient in the iGb3 synthase were found to have normal iNKT cell numbers, indicating that iGb3 is not a critical ligand for positive selection of iNKT cells in the thymus (Porubsky et al. 2007). Since humans lack a functional iGb3 synthase (Christiansen et al. 2008), and iGb3 associated with human CD1d fails to activate most human iNKT cells (Sanderson et al. 2013), the relevance of this lipid in human iNKT cell biology is limited.

A growing body of work has highlighted the importance of the major monohexosylceramides, glucosylceramide and galactosylceramide, as important self lipid antigens for iNKT cells, and recent data suggests that α-linked forms of these lipids are present in small amounts in mammals. Stanic and colleagues reported that glucosylceramide-deficient cells are unable to support iNKT cell autoreactivity (Stanic et al. 2003). Using inhibitors of glycosphingolipid synthesis, we and others have shown that sphingolipids contribute to the natural autoreactivity observed when iNKT cells are co-cultured with CD1d-expressing APCs (Brennan et al. 2011; De Santo et al. 2008; Stanic et al. 2003). By fractionating mammalian lipids, we found that the self lipids that activate iNKT cells co-migrated with the monohexosylceramide fraction, and we initially proposed that β-glucosylceramides were endogenous antigens for iNKT cells (Brennan et al. 2011). Recently, technical advances have revealed previously unknown ‘contaminants’ in this monohexosylceramide fraction that have led to a re-interpretation of these results. Using a biochemical fractionation approach, we found that the lipid activity in the mammalian monohexosylceramide fraction was attributable to a minor component of the total lipids in this fraction. A combination of mass spectrometry, chromatographic behavior, and nuclear magnetic resonance spectroscopy suggested that the activity in the glucosylceramide fraction was likely to be an α-monohexosylceramide (Brennan et al. 2014). Kain and colleagues took

a complementary approach to the same question, using both monoclonal raised against α-GalCer-CD1d complexes and polyclonal antibodies raised against α-GalCer to test if α-GalCer is present in mammalian cells. Monoclonal antibodies specific for lipid antigen-CD1d complexes were shown to block *in vitro* autoreactivity in cultured cells. Immunohistochemical staining of thymocytes with polyclonal antibodies suggested that α-linked glycolipids were present in the thymus, and could thus contribute to the thymic selection of iNKT cells (Kain et al. 2014). These findings supporting the presence of α-linked lipids in mammals were unexpected, as the two mammalian monohexosylceramide synthases, glucosylceramide synthase and ceramide galactosyltransferase, have only been known to produce β-linked anomers (Lairson et al. 2008). How α-linked lipids are produced in mammals is not known. Although these studies are strongly suggestive that α-monohexosylceramides are present in mammals, direct biochemical proof of the presence of α-linked lipids remains elusive, as does genetic evidence for their importance to iNKT cell development and activation.

Lysophosphatidylethenolamine (lysoPE), a lipid that can be generated at sites of inflammation, has been demonstrated to activate human iNKT cells (Fox et al. 2009), and a ternary crystal structure of CD1d-lysoPE-TCR has been solved (López-Sagaseta et al. 2012). Peroxisome-derived ether-bonded mono-alkyl glycerophosphates have also been identified as antigenic for iNKT cells based on lipid fractionation from the thymus, and mice lacking glyceronephosphate *O*-acyltransferase, an enzyme involved in the synthesis of these lipids, have reduced iNKT cell numbers (Facciotti et al. 2012). Taken together, these studies show that several endogenous mammalian lipids can activate iNKT cells when presented by CD1d. However, the physiological role of each of these lipids in iNKT cell development, homeostasis, and activation are not yet understood.

Predominantly cytokine-driven pathways of iNKT cell activation

While some microbes harbor lipid antigens that are capable of inducing iNKT cell activation through cognate recognition by the TCR, most microbes can activate iNKT cells in a manner that is independent of microbial lipid antigens. PAMPs, produced by a majority of microbes, stimulate APCs to secrete pro-inflammatory cytokines that then drive iNKT cell activation. In some cases, a weak CD1d-TCR signal is still required for activation, while in other examples, a signal through the CD1d-TCR axis may not be required at all. We refer to this pathway as the ‘predominantly cytokine-driven’ pathway of iNKT cell activation. We proposed this mechanism following the observation that human iNKT cells were readily activated to secrete IFN-γ when cultured with monocyte-derived DCs in the presence of a range of heat-killed organisms including *Salmonella typhimurium* and *Staphylococcus aureus*, or purified TLR ligands. In this context, iNKT cell activation did not appear to require the presentation of microbial lipid antigens but rather was profoundly dependent on TLR-induced production of IL-12 by APCs (Brigl et al. 2003; Brigl et al. 2011). This pathway is likely more widely applicable in nature than strong lipid antigen, TCR-driven iNKT cell activation since blockade of inflammatory cytokines or TLR signaling in APCs blunts or terminates iNKT cell responses to virtually all microbes.

The predominantly cytokine-driven pathway requires that APCs sense PAMPs through pattern recognition receptors (PRRs) such as the TLRs. All TLRs are thought to use a signaling pathway that culminates in the activation of the transcription factor nuclear factor-κB (NF-κB), leading to the production of pro-inflammatory cytokines such as IL-12 (Akira and Takeda 2004; Barton and Medzhitov 2003). The relevance of IL-12 and IL-18 as powerful mediators of iNKT cell activation is imparted by the constitutive expression of the receptors for both of these cytokines on iNKT cells (Brigl et al. 2011; Kitamura et al. 1999; Leite-De-Moraes et al. 1999). Stimulation of iNKT cells with recombinant IL-12 and IL-18, either alone or in combination, induces their secretion of IFN-γ even in the absence of APCs (Leite-De-Moraes et al. 1999). Importantly, this pathway allows for the activation of iNKT cells by virtually any microbe as long as its PAMPs are sensed by APCs, irrespective of whether or not these microorganisms synthesize foreign lipid antigens recognized by the iNKT TCR. We have highlighted the role of the predominantly cytokine-driven pathway of activation through *in vitro* and *in vivo* studies using APCs deficient in TLR signaling or IL-12 secretion. APCs capable of presenting lipid antigens via CD1d, but incapable of utilizing the cytokine-driven pathway of iNKT cell activation were unable to productively activate iNKT cells following infection with many microbes, even including those known to synthesize lipid antigens that directly activate iNKT cells through their TCRs (Brigl et al. 2011). In cases of bacteria where no foreign lipid-antigen was present to contribute to iNKT cell activation, robust activation of iNKT cells required the combination of an IL-12 signal and a TCR signal, likely resulting from recognition of self lipid antigens presented by CD1d.

CD1d-presented signals have been shown to contribute to iNKT cell activation even in cases where the microbe tested was not thought to produce stimulatory lipids (Brigl et al. 2011). This finding may be explained by the observation that TLR signaling can alter the expression of enzymes involved in lipid metabolism leading to the production or presentation of self lipid antigens (Brennan et al. 2011; Khovidhunkit et al. 2004; Paget et al. 2007; Salio et al. 2007). As an example of this process, Salio and colleagues demonstrated that maturation of human DCs with the TLR agonists LPS (TLR4 agonist) and R848 (TLR 7/8 agonist) induced increased expression of genes encoding enzymes responsible for the synthesis of GSLs. This TLR-mediated activation of iNKT cells was reduced when DCs were treated with the specific inhibitor of GSL biosynthesis, *N*-butyldeoxygalactonojirimycin (*N*B-DGJ) prior to maturation with LPS or R848. In addition, total lipid fractions extracted from R848-matured DCs, in the absence of any microbial lipids, induced stronger iNKT cell activation than lipids extracted from immature DCs (Salio et al. 2007). Similar TLR agonist-induced increases in self lipid stimulating activity have been reported elsewhere (Brennan et al. 2011; De Santo et al. 2008; Paget et al. 2007).

In other studies, iNKT cells have been shown to become activated and secrete IFN-γ in an entirely cytokine-driven fashion, where the interaction between the iNKT TCR and CD1d is completely dispensable (Holzapfel et al. 2014; Leite-De-Moraes et al. 1999; Nagarajan and Kronenberg 2007). In these examples, activation of iNKT cells was thought to be driven by the synergistic action of IL-12 and IL-18 secreted by APCs after recognition of PAMPs through their PRRs. Nagarajan and colleagues demonstrated that IFN-γ production by iNKT cells following intravenous injection of *Escherichia coli* LPS was significantly reduced in IL-12p40-deficient mice and completely absent in IL-18-deficient mice, suggesting that

signaling through the cytokines IL-12 and IL-18 is required to activate iNKT cells during infection. These findings were corroborated *in vitro* in the same study, where iNKT cell activation in response to *E. coli* LPS was also strongly dependent on IL-12 and IL-18 but was not significantly reduced when CD1d-deficient APCs were used (Nagarajan and Kronenberg 2007).

To study the role of TCR signaling in iNKT cells during infection, Holzapfel and colleagues used Nur77 promoter-driven green fluorescent protein (GFP) transgenic reporter mice that express GFP in response to TCR stimulation, but not cytokine signaling. iNKT cells expressed GFP *in vivo* following infection with microbes known to synthesize foreign lipid antigens that directly activate iNKT cells, including *S. pneumoniae* and *Sphingomonas paucimobilis*. However, infection with microbes lacking a foreign lipid antigen, such as *S. typhimurium*, did not induce detectable expression of GFP in iNKT cells. Furthermore, iNKT cells in this case were activated similarly when transferred into CD1d-deficient mice as they were in wild type mice. These results suggest that robust iNKT cell responses to microbes can be obtained in the absence of strong TCR signals (Holzapfel et al. 2014). It is possible that prior engagement of the iNKT cell TCR by CD1d-presented self lipid antigens contributes to the apparently purely cytokine-mediated activation of iNKT cells. This possibility is supported by the finding that even weak stimulation of the iNKT cell TCR induces histone H4 acetylation at specific regions near the *IFNG* gene locus, priming iNKT cells to rapidly produce IFN-γ in response to IL-12 and IL-18 without the additional need for concomitant TCR stimulation (Wang et al. 2012). This epigenetic state was shown to remain stable over hours or even days following the initial weak TCR stimulation, and the ability of iNKT cells to rapidly respond to IL-12 and IL-18 without concurrent TCR signals waned thereafter. Together, these studies on the cytokine-driven pathway demonstrate that cytokine-mediated activation of iNKT cells is a major contributor to their activation in response to infection with many microbes.

iNKT cell effector responses

Although predominantly TCR-driven and predominantly cytokine-driven pathways for iNKT cell activation are discussed separately above, combinations of these pathways are likely to be involved in iNKT cell activation during infection. These activation signals are integrated by iNKT cells, which then carry out effector responses including cytokine production, chemokine production, and cytotoxicity (Figure 1). iNKT cells have been shown to contribute to remarkably diverse immune responses through the production of Th1, Th2, Th17, and regulatory-type cytokines including IL-2, IL-3, IL-4, IL-5, IL-9, IL-10, IL-13, IL-17A, IL-21, IFN-γ, TNF, and GM-CSF (Coquet et al. 2008). How this range of effector cytokines is selectively regulated is not completely understood. Specific model lipid antigens have been shown to differentially elicit either Th1 or Th2 type cytokine responses (Im et al. 2009; Li et al. 2009; Miyamoto et al. 2001), but the physiological relevance of differential cytokine responses based on lipid antigen type in nature is unclear. However, emerging data suggests that chemically-modified antigens that can elicit polarized responses may prove to be useful in vaccine development or as therapeutic agents (Carreño et al. 2014; Cerundolo et al. 2009). A compelling explanation for differential cytokine production by iNKT cells stems from the recognition that iNKT cells are not a homogeneous population of cells, but

rather exist as polarized subsets. In mice, NKT1, NKT2, and NKT17 subsets, analogous to helper T cell subsets and ILC subsets, have been well-defined (Engel et al. 2016; Lee et al. 2013; Watarai et al. 2012). These subsets have tissue-specific and mouse strain-specific distributions that may explain the differential effector functions of iNKT cells in different settings (Lee et al. 2015; Watarai et al. 2012). In addition to having specific anatomic locations, cells from each subset express characteristic receptors for inflammatory cytokines, with NKT1 cells expressing receptors for IL-12 and IL-18, NKT2 cells expressing IL-25 receptor, and NKT17 cells expressing IL-23 receptor (Brigl et al. 2003; Doisne et al. 2011; Kitamura et al. 1999; Leite-De-Moraes et al. 1999; Nagarajan and Kronenberg 2007; Rachitskaya et al. 2008; Stock et al. 2009; Terashima et al. 2008; Watarai et al. 2012; Yamagishi et al. 2016). Cytokine-driven activation through these receptors can selectively activate specific iNKT cell subsets, thus eliciting polarized iNKT cell effector cytokine and/or chemokine responses. During pulmonary infection with *S. pneumoniae* or *Pseudomonas aeruginosa*, macrophage inflammatory protein-2 and IFN-γ, presumably from NKT1 cells, have been implicated in the recruitment and activation of neutrophils and macrophages (Kawakami et al. 2003; Nakamatsu et al. 2007; Nieuwenhuis et al. 2002). IL-17A from NKT17 cells has been proposed to recruit neutrophils to sites of inflammation such as the lungs and the skin (Doisne et al. 2011; Michel et al. 2008). A common theme that emerges in these studies and others reviewed elsewhere is that iNKT cells shape immune responses to microbes by leading to the recruitment and/or activation of multiple other immune cell types including T cells, B cells, NK cells, macrophages, DCs, eosinophils, and neutrophils (Brennan et al. 2013; Brigl and Brenner 2010; Chandra and Kronenberg 2015; Cohen et al. 2009; Van Kaer et al. 2011).

NKT1 cells play a critical role during infection, driving strong Th1 responses that are characterized by robust production of IFN-γ, and in certain contexts, cytotoxicity. During thymic development, NKT1 cells are enriched for cytotoxicity factors perforin, granzymes, and Fas ligand (FasL) (Engel et al. 2016), a phenotype thought to be driven by the transcription factor T-bet. Similar to iNKT cells from T-bet-deficient mice, gene expression profiles in iNKT cells from IL-15-deficient mice showed reduced levels of granzyme B and canonical NK cell receptors, suggesting that signaling through IL-15 receptors on iNKT cells may support their cytotoxic functions (Gordy et al. 2011; Townsend et al. 2004). Although iNKT cell cytotoxicity during host defense against intracellular pathogens and viruses remains largely unstudied, the role of this effector mechanism has been investigated in antitumor responses. *In vivo* administration of either IL-12 (Cui et al. 1997) or α-GalCer (Kawano et al. 1998) demonstrated that iNKT cells can have potent antitumor activity independent of NK cells and MHC-restricted, peptide-specific adaptive T cells. Adoptive transfer of iNKT cells, in the absence of exogenous stimulation, into mice with methylcholanthrene-induced fibrosarcoma showed that iNKT cells can also play a critical role in tumor immunosurveillance to prevent the appearance of tumors by working in concert with NK cells and $CD8^+$ T cells (Smyth et al. 2002). Other *in vivo* models carried out in the absence of exogenous iNKT cell activation have demonstrated direct CD1d-dependent iNKT cell killing of tumor cells (Bassiri et al. 2014) or indirect control of tumor growth resulting from the killing of tumor-associated macrophages by iNKT cells (Song et al. 2009). *In vitro* studies have illustrated perforin (Bassiri et al. 2014; Dao et al. 1998) and

FasL-dependent (Leite-De-Moraes et al. 1999; Wingender et al. 2010) cytotoxic activity of activated iNKT cells toward target cells. In addition, emerging evidence suggests that iNKT cells can utilize the activating NK cell receptor NKG2D to target cells, subsequently killing these cells through perforin-mediated cytolysis even in the absence of CD1d (Kuylenstierna et al. 2011). Taken together, these studies show that both TCR-driven and non-TCR-driven activation of iNKT cells can stimulate the cytotoxic potential of iNKT cells. Whether iNKT cells utilize FasL, perforin, or other killing mechanisms is likely to depend on both their activation state and the ability of target cells to express the relevant set of molecules, including CD1d and Fas, above a certain threshold. It is likely that iNKT cell cytotoxicity plays an important role in controlling some infections, and this will be an important area of future study for the field.

Examples of iNKT cell activation mechanisms during infection *in vivo*

iNKT cell activation in *Borrelia burgdorferi* infection

iNKT cells have been shown to play a role in the immune response to many prokaryotic microbes. Infection with the spirochete *B. burgdorferi* can cause Lyme disease, the most common vector-borne illness in the United States (Hu et al. 2016). Similar to gram-positive bacteria, *B. burgdorferi* is composed of an inner cytoplasmic membrane associated with a peptidoglycan cell wall. However, *Borrelia* is further encapsulated by an outer cell membrane that lacks LPS, encircling a distinct flagellar structure in the periplasmic space (Kelesidis et al. 2014). *Borrelia* also harbors membrane-associated lipoproteins, including the outer surface proteins (Osp) A and OspB, that bind to TLR1/2 and stimulate inflammation through NF-κB (Tilly et al. 2008). *B. burgdorferi's* membranes are dominated by two lipids, BbGLI (23%), cholesteryl 6-O-acyl-β-galactoside, and BbGLII (12%), 1,2-diacyl-3-O-α-galactosyl-sn-glycerol. As discussed above, the α-linked lipid BbGLII activates both human and mouse iNKT cells to produce IFN-γ in a CD1d-dependent fashion (Kinjo et al. 2006). Thus, *Borrelia* harbors both a stimulatory lipid antigen capable of TCR-driven iNKT cell activation and TLR agonists that may drive cytokine-driven indirect activation of iNKT cells.

Following dermal infection, tissue-resident macrophages and iNKT cells are among the first cells to recognize and respond to invading *Borrelia* spirochetes. TLR2-deficient mice have greater ankle swelling and weaker antibody responses against the Osp proteins, neutrophilic infiltrates of tendon sheaths and joint synovium, and exhibit higher bacterial titers in peripheral tissues compared to wild type controls (Wooten et al. 2002). Mice that lack iNKT cells display higher spirochete numbers in peripheral organs, similar to TLR2-deficient mice, following challenge with *Borrelia* infected ticks, demonstrating the importance of iNKT cells in preventing spirochete dissemination. Not surprisingly then, mice lacking iNKT cells also develop pathological thickening of the knee and tibiotarsal joints (Kumar et al. 2000; Tupin et al. 2008). iNKT cells have been implicated as extravascular patrollers in the joint itself. Following *B. burgdorferi* infection, activated iNKT cells surrounding the joint vasculature have been shown to directly kill the invading *Borrelia* through granzyme B, but independently of CD1d, preventing the spirochete from entering the joint. The precise

molecular cues that trigger this direct granzyme-mediated microbicidal pathway in iNKT cells have yet to be determined (Lee et al. 2014).

During systemic infection, *B. burgdorferi* spirochetes drain to the hepatic vasculature where they are phagocytosed by Kupffer cells, liver-resident macrophages. Strategically localized in the hepatic sinusoids, Kupffer cells have been visualized using intravital microscopy to capture spirochetes within hours after infection (Lee et al. 2010). iNKT cells ignored spirochetes that adhered to sinusoidal endothelial cells, demonstrating that the uptake and processing of spirochetes was important for their recognition by iNKT cells. Following the uptake of spirochetes by Kupffer cells, as many as 10-15 iNKT cells were seen to cluster around infected, but not uninfected Kupffer cells. These interactions and the resulting activation of iNKT cells were dependent on CD1d. Although the PAMPs involved in the recruitment of iNKT cells were not specifically identified, CXCL9, a potent chemokine downstream of NF-κB activation and the ligand for CXCR3, was increased in Kupffer cells in *Borrelia* infected mice. Blocking CXCR3 signaling using antibodies or pertussis toxin, to abrogate G protein-coupled receptor signaling, prevented iNKT cell clustering and activation, suggesting that this chemokine has an essential role in attracting iNKT cells to the APC to initiate inflammation (Lee et al. 2010). These studies highlight critical contributions for both TCR-driven and non-TCR-driven signals to elicit a robust iNKT cell response to control *B. burgdorferi* infection.

iNKT cells and viral infection

Viruses are not known to induce the expression of virus-specific lipids, yet iNKT cells are prominently activated during viral infection. This suggests that during viral infections, iNKT cells are activated either in a cytokine-driven manner or through a combination of cytokines and self lipid antigens presented by CD1d. Tyznik and colleagues showed that engagement of endosomally expressed TLR9 by CpG oligodeoxynucleotide, a model TLR9 agonist, results in the activation of iNKT cells through a combination of IL-12 and type I interferons (Tyznik et al. 2008, 2014). In this context, both IL-18 secretion and CD1d expression on DCs were found to be dispensable. However, another group reported that CpG induced *de novo* production of charged GSL(s) by DCs, although the specific activating lipid(s) was not identified (Paget et al. 2007). Whether or not iNKT cells are activated by a combination of IL-12 and IL-18 as discussed above, IL-12 and type I interferons, or cytokines and self lipid antigens is likely to depend on the nature of the relevant PAMP(s) and other contextual factors. Type I interferons, for example, are not induced upon engagement of all TLRs, but rather primarily by those that localize to intracellular endosomal compartments commonly associated with viral infection. Although it is not known if self lipid antigens are presented by APCs during most viral infections, there is evidence that recognition of viral nucleic acids by endosomal TLR 3, 7 and 9 enhances the expression of enzymes involved in GSL biosynthesis, and inhibition of GSL biosynthesis abolishes the responsiveness of iNKT cells toward APCs stimulated with viral-associated TLRs (Brennan et al. 2011; Salio et al. 2007).

In mice where CD1d expression is lacking or blocked, iNKT cells are activated normally in response to infection with the herpesvirus murine cytomegalovirus (MCMV), suggesting iNKT cells are activated in a TCR-independent, cytokine-driven manner in this infection

(Tyznik et al. 2008; Wesley et al. 2008). iNKT cell activation following MCMV infection *in vivo* is driven by a combination of IL-12 and type I interferons induced upon recognition of MCMV-derived viral DNA by TLR9 and TLR3 in plasmacytoid DCs and myeloid DCs (Krug et al. 2004; Tabeta et al. 2004). The same observations were made *in vitro*, where infection of DCs with MCMV resulted in induction of iNKT cell production of IFN-γ in a manner that required IL-12 and type I interferons, yet was independent of CD1d and IL-18 (Tyznik et al. 2014).

Studies using CD1d-deficient mice have suggested that iNKT cells can contribute to the control of pulmonary influenza virus infection (De Santo et al. 2008; Paget et al. 2011). Early during infection with influenza A virus, myeloid-derived suppressor cells (MDSCs), which encompass immature DCs, immature macrophages, and granulocytes, are recruited to the lung where they can suppress $CD8^+$ T cells that are critical for the clearance of influenza virus. iNKT cells were found to play a role in preventing the suppression of $CD8^+$ T cells mediated by MDSCs during infection with influenza A virus (De Santo et al. 2008). This study demonstrated that iNKT cells block the expansion of MDSCs and reduce their suppressive activity via CD40-CD40L interactions through a mechanism that required cognate interactions between the iNKT cell TCR and CD1d on MDSCs following their exposure to TLR agonists. Interestingly, iNKT cells incubated with MDSCs that had been matured with TLR ligands but treated with the specific inhibitor of GSL biosynthesis *N*B-DGJ failed to secrete IFN-γ and had a reduced ability to relieve the suppressive activity of MDSCs. Therefore, it is likely that exposure of MDSC to TLR ligands may result in the upregulation of endogenous lipids that serve as antigens for the iNKT cell TCR, and initiate the crosstalk between iNKT cells and MDSCs. This example also illustrates an important theme where CD1d-iNKT TCR interactions not only contribute to iNKT cell activation, but also promote effector functions in the APCs that activate the iNKT cells.

iNKT cells and fungal infection

Fungi represent unique pathogens since the main species that cause disease are generally not pathogenic to the host and are ubiquitous in the environment. TLR2, TLR4, and TLR9 are the main TLRs involved in sensing fungal components and fungal DNA (Romani, 2011). Like most fungi, *Aspergillus fumigatus* is a heterotrophic eukaryote that can be morphologically classified into yeast and filamentous forms, with clear differences in PAMP expression. The specific fungal PAMPs that activate PRRs depend on growth stage and also on the host responder cells that sense these PAMPs (Romani, 2011). Another unique feature of fungi is the extensive carbohydrate structures that constitute the fungal cell wall. The β-glucan and mannan polymers that coat the outside of fungi are recognized by C-type lectin receptors (CLRs). Genetic deficiency in Dectin-1, a member of the CLR receptor family that recognizes β-glucans, results in increased susceptibility to fungal infections (Maródi and Erdös 2010; Taylor et al. 2007). Ultimately, recognition and clearance of fungi by these PRRs induces a Th1-type response characterized by the production of IL-12, IFN-γ, and TNF *in vivo* (Hole and Wormley 2016; Livonesi et al. 2008; Souto et al. 2000). In addition, IL-17 acts as a critical mediator of fungal clearance. Mice deficient in IL-17 are highly susceptible to fungal infections, in particular at mucocutaneous surfaces, and rare inborn

errors of IL-17 immunity have been associated with increased *Candida albicans* susceptibility in humans (Bär et al. 2014; Cypowyj et al. 2012).

Consistent with their broad role in orchestrating host defense, iNKT cells have been shown to play a beneficial role in antifungal responses. With their high steady state expression of the IL-12 receptor, iNKT cells are poised to respond to IL-12 and robustly secrete IFN-γ upon activation (Brigl et al. 2011). iNKT cells accumulate in the lungs of mice inoculated intratracheally with *Cryptococcus neoformans*, and Jα18-deficient mice that lack a TCR segment necessary for iNKT cell development exhibit higher *C. neoformans* infectious burden in the lungs than do wild type controls. The accumulation of iNKT cells following *C. neoformans* infection relies on monocyte chemoattractant protein-1 and mice deficient in this chemoattractant show hampered NKT cell recruitment and lower IFN-γ production at the peak of fungal infection (Kawakami et al. 2001).

A. fumigatus is a ubiquitous mold responsible for a spectrum of human illnesses, including allergic disease and fatal invasive disease in immune-compromised hosts. We showed that *A. fumigatus* was capable of activating iNKT cells *in vivo* and *in vitro* in a CD1d-dependent manner (Cohen et al. 2011). Biochemical fractionation of *A. fumigatus* showed that the majority of the observed activity was not attributable to fungal lipids, but rather to the fungal polysaccharides. β1,3-glucans, glucose polymers integral to the cell wall of most fungi and particularly abundant in *A. fumigatus* hyphae, were found to drive the activation of iNKT cells. β1,3-glucans are recognized by the C-type lectin, Dectin-1, on the APC and this facilitates phagocytosis and Syk/CARD9 signaling. IL-12p35-deficient DCs were unable to support iNKT cell activation this setting, suggesting a major role for cytokine-driven activation. Expression of the TLR-associated adaptor molecule MyD88 was also required, indicating collaborative signaling between Dectin-1 and TLR(s) in this pathway. Interestingly, IL-12 secretion by APCs stimulated by *A. fumigatus* did not occur when the fungi alone were recognized by the APC. Rather, IL-12 secretion required the presence of both iNKT cells and CD1d, illustrating a striking example of bidirectional activation between iNKT cells and APCs. In this case, initial activation of the iNKT cell by the APC enabled the iNKT cells to cross-activate the APC to secrete high levels of IL-12. In turn, the IL-12 and a CD1d-TCR signal combined to drive a strong iNKT cell activation response. Importantly, this pathway likely represents a broadly applicable mechanism for driving antifungal iNKT cell responses (Cohen et al. 2011).

In addition to causing invasive disease in immunocompromised hosts, non-infectious *A. fumigatus* exposure can trigger allergic responses in humans that lead to asthma and a severe form of allergic airways disease termed allergic bronchopulmonary aspergillosis (Agarwal et al. 2013). Mouse airway hyperreactivity (AHR) can also be triggered by exposure to *A. fumigatus* and is dependent on the activation of iNKT cells (Akbari et al. 2003). This process was found to be independent of both MyD88 and Dectin-1 signaling, suggesting that TCR-driven, rather than cytokine-driven signaling may be important for the AHR response to fungi (Albacker et al. 2013). Albacker and colleagues isolated an *A. fumigatus*-derived glycosphingolipid, asperamide B, that stimulates iNKT cell IL-4 and IL-13 in a CD1d-dependent manner *in vivo*, and intratracheal administration of synthesized asperamide B was sufficient to worsen AHR (Albacker et al. 2013; Chaudhary et al. 2013). This lipid was

reported to have a β-linked sugar head group. As recent data suggests that mammalian β-GSLs are not active, it is possible that the fungal-specific lipid backbone imparts activity, or alternatively that this lipid contained trace amounts of α-linkages similar to mammalian self lipids. The identification of a lipid antigen in *A. fumigatus* suggests that this fungus also may be able to support TCR-driven iNKT cell activation in some infectious settings. Taken together these studies on the iNKT response to fungi provide a clear example of how a single organism can lead to distinct responses stemming from various combinations of TCR-driven and cytokine-driven activation of iNKT cells, depending on the context.

Concluding Remarks

In the 20 years since their discovery, iNKT cells have been shown to contribute to host defense against multiple microbes, including bacteria, viruses, and fungi. Their remarkable ability to be activated in a broad range of infections, despite having a semi-invariant TCR and using a monomorphic antigen presenting molecule results from their ability to be activated by self lipid antigens as well as microbial lipid antigens and, at the same time, sense PAMP-stimulated inflammatory cytokines via constitutively expressed receptors. This complex of inputs allows iNKT cells to respond to a vast array of microbes, even those lacking lipid antigens. The combination of signaling inputs that activate iNKT cells and their multifaceted effector toolbox are summarized in Fig. 1. Once activated, iNKT cells function as immune orchestrators by participating in bidirectional activation with antigen presenting cells and by transactivation of other effector leukocytes. Here, we have highlighted examples of crosstalk with Kupffer cells (Lee et al. 2010), DCs (Arora et al. 2014; Bai et al. 2012; Brigl et al. 2003; Cohen et al. 2011; Tyznik et al. 2008), MDSCs, $CD8^+$ T cells (De Santo et al. 2008), macrophage populations (Barral et al. 2010; Kawakami et al. 2003; Lee et al. 2010; Nieuwenhuis et al. 2002), and non-hematopoietic cells (Hua et al. 2010; Huh et al. 2013; Olszak et al. 2014; Rakhshandehroo et al. 2014; Satoh et al. 2016; Zeissig et al. 2012). In other examples that were not discussed here, iNKT cells have been shown to activate or modulate the function of granulocytes (De Santo et al. 2010), B cells (Chang et al. 2012; Galli et al. 2007; King et al. 2012; Leadbetter et al. 2008), adaptive T cells (Fujii et al. 2004; Hermans et al. 2003), and NK cells (Carnaud et al. 1999; Schmieg et al. 2003). Thus, iNKT cell effector functions are often mediated through their transactivation of other powerful effector cells and pathways. In this review, we have focused on the mechanisms by which iNKT cells sense and integrate microbe-derived stimuli through a combination of TCR-driven signals and cytokine-driven responses, elicited by microbial lipid antigen or microbial PAMP-triggered inflammatory cytokines and self lipid antigen. It will be important to identify the self and foreign lipid antigens, PAMPs, and tissue-specific APCs that lead to iNKT cell activation in distinct settings to fully understand the roles that iNKT cells play in host defense and immunopathology in different infectious contexts. Precise understanding of the mechanisms of activation will provide insights into how the iNKT cell response is focused on infected cells and tissues while at the same time limiting nonspecific systemic inflammation.

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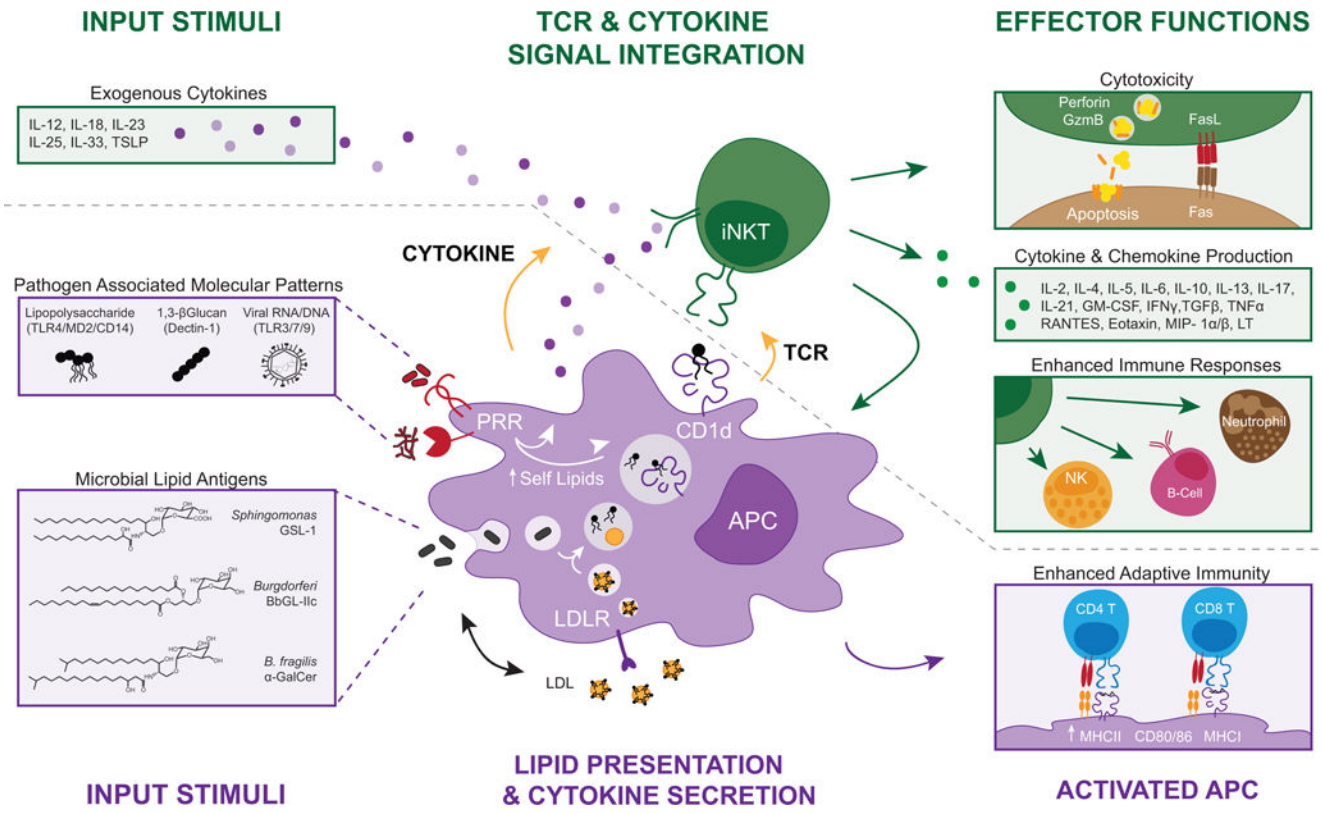

Figure 1.
Mechanisms of iNKT cell activation.