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CD1d-restricted iNKT cells, the “Swiss-Army knife” of the immune system

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Summary

Natural Killer T cells are a distinct lymphocyte lineage that regulates a broad range of immune responses. NKT cells recognize glycolipids presented by the non-classical MHC molecule CD1d. Structural insight into the TCR/glycolipid/CD1d tri-complex has revealed an unusual and unexpected mode of recognition. Recent studies have also identified some of the signaling events during NKT cell development that give NKT cells their innate phenotype. Pathogen-derived glycolipid antigens continue to be found, and new mechanisms of NKT cell activation have been described. Finally, NKT cells have been shown to be remarkably versatile in function during various immune responses. Whether these extensive functional capacities can be attributed to a single population sensitive to environmental cues or if functionally distinct NKT cell subpopulations exist remains unresolved.

Introduction

Roughly two decades ago, a unique T lymphocyte population was identified in both mice and humans. These cells were referred to as natural killer T cells (NKT cells) due to their co-expression of phenotypic markers usually found on T and natural killer (NK) cells. Since their discovery, several aspects of the biology of these cells have been uncovered. Not only has the phenotype of these cells been more fully characterized, details of their unique ontogeny and development have started to emerge. In addition, several antigens have been identified and we are beginning to understand how these antigens are recognized by NKT cells. To date, NKT cells have been reported to be critical in the regulation of many different types of immune responses, ranging from self-tolerance and development of autoimmunity to responses to pathogens and tumors. However, it remains unclear how NKT cells conceivably play such apparently diverse roles from one type of immune response to another. With this in mind, we will highlight recent advances in the study of NKT cell function, outlining what is now known about how NKT cells can be activated and what the outcome of their activation on the immune response can be.

What is an NKT cell?

The original definition of NKT cells as T lymphocytes co-expressing an $\alpha\beta$ T cell antigen receptor (TCR) and NK cell receptors, now represents an oversimplification and is a poor definition. While it is true that the vast majority of NKT cells express NK receptors, other, unrelated T cell populations, such as activated conventional CD8 T cells, can also express

them. Furthermore, the expression of NK receptors by NKT cells varies with their developmental stage, their activation state and, at least in mice, their genetic background. Several types of NKT cells have now been defined on the basis of their TCR α chain usage, MHC restriction and expression of various surface molecules. The most studied and best-characterized NKT cell population in mice and humans is referred to as type I NKT cells, or iNKT cells, and is the focus of this review. These NKT cells express a TCR formed by the canonical rearrangement of the V α 14 gene segment (V α 24 in human) to the J α 18 gene segment. These α chains are co-expressed with a limited set of V β chains, predominantly V β 8.2, V β 7 and V β 2 in mice and V β 11 in humans. However, these V β chains are highly diverse in both their CDR3 composition and association with J β segments. iNKT cells recognize glycolipid antigens presented by the non-polymorphic MHC class I-like molecule CD1d. The high degree of conservation of the canonical TCR and CD1d molecules allows for inter-species cross-reactivity, with mouse iNKT cells recognizing human CD1d and vice versa, indicating the likely importance of iNKT cells in the immune system.

The development of CD1d tetramers loaded with the prototypical glycolipid antigen, α -Galactosylceramide (α -GalCer), represented a significant advance in the study of iNKT cells [1]. It made it possible, for the first time, to unambiguously identify iNKT cells on the basis of CD1d/ α -GalCer reactivity alone, irrespective of other phenotypic markers. iNKT cells are found with the highest frequency in the liver and the bone marrow of mice, with significant numbers also in the thymus, spleen and peripheral blood. In humans, the frequency of iNKT cells is usually much lower and a high degree of variability between individuals has been reported [2]. The majority of iNKT cells exhibit an activated or memory phenotype - they are CD69⁺, CD62L^{low}, CD44^{high} and CD122^{high}, and typically express a combination of NK receptors, such as NK1.1, NKG2D and Ly49 markers. In mice, iNKT cells are exclusively CD4⁺ or CD4 CD8 double negative (DN), while they can be CD8⁺ in humans [3].

What does it take to make an iNKT cell?

iNKT cells develop in the thymus [4]. They are absent from nude mice, do not develop in thymectomized mice, and first appear in the thymus slightly later than conventional T cells. The unusual phenotype and functional attributes of iNKT cells suggested that they likely derived from a particular developmental program. Originally, two models were proposed to account for the development of this unique lymphocyte population. The first model hypothesized that iNKT cells derive from a pre-committed iNKT cell precursor. The second model proposed that iNKT cells derived from a common T cell precursor but that their developmental program diverged from conventional T cell development upon positive selection mediated by the recognition of CD1d molecules by randomly generated TCRs.

It is now established that iNKT cells segregate from conventional T cells during development at the double-positive (DP) thymocyte stage, coincident with TCR $\alpha\beta$ expression [4]. Generation of the canonical TCR α used by iNKT cells is widely believed to be a random event, for although the amino acids which define the invariant V α 14-J α 18 rearrangement never vary, sequencing analysis has revealed that the nucleotides used to code for these amino acids is diverse [5]. Due to structural constraints on recombination events in the TCR α locus, the numerous V α and J α gene segments become accessible for recombination as a function of their relative location in the locus. And as a result of this, the V α 14 gene segment only starts rearranging with J α 18 within a 24–48 h window before birth [6]. This explains the relatively late appearance of NKT cells in the thymus [4] and is consistent with random generation of the canonical V α 14-J α 18 rearrangement within a common T cell progenitor pool. Furthermore, the frequency of the earliest identified NKT cell precursor was estimated to be 1 cell per 10⁶ thymocytes [7]. Together, these data support the notion that V α 14-J α 18 rearrangement occurs randomly at very low frequency.

As with conventional T cells, iNKT cell development requires recognition of self. The restriction element CD1d is expressed by both DP thymocytes and epithelial cells in the thymus. However, early studies revealed that iNKT cells are selected at the DP stage by CD1d-expressing DP cells themselves as opposed to epithelial cells that drive the selection of conventional T cells. Such a mode of selection was hypothesized to impart the unique developmental program of iNKT cells to the selected thymocytes. Recently, it was demonstrated that homotypic interactions across the DP-DP synapse generated “second signals” that are mediated by the cooperative engagement of the homophilic receptors of at least two members of the signaling lymphocytic-activation molecule (SLAM) family (Slamf1 [SLAM] and Slamf6 [Ly108]) [8λλ–10λλ]. Such engagements lead to the downstream recruitment of the adaptor SLAM-associated protein (SAP) and the Src kinase Fyn, which were previously recognized as essential for the expansion and differentiation of the iNKT cell lineage [4].

Once iNKT cells have been positively selected, they expand in the thymus and undergo an orchestrated maturation process that ultimately leads to the acquisition of their activated NK-like phenotype. This process relies on the proper expression of cytokine receptors, signal transduction molecules (e.g. Fyn, SAP), transcription factors (e.g. NFκB, T-bet, Ets1, Runx1, RORγ, Itk, Rlk, AP-1) (see Ref [4] for reviews), and co-stimulatory molecules such as CD28 and ICOS [11]. Most iNKT cells leave the thymus in an immature stage (as defined by the absence of expression of NK receptors such as NK1.1) and fulfill their terminal maturation in the periphery [12–14]. However, a sizeable fraction of these NK1.1[−] iNKT cells in the peripheral organs do not acquire expression of NK markers and in fact represent mature cells that are functionally distinct from their NK1.1⁺ thymic counterpart [15λ] (see below).

iNKT cells egress from the thymus to the periphery requires lymphotoxin (LT) αβ signaling through the LTβ receptor expressed by thymic stromal cells [16]. Such signaling in turn regulates thymic medullary chemokine secretion [17]. Establishment of iNKT cells tissue residency in the periphery requires expression of the Sphingosine1-Phosphate 1 receptor (S1P1R) by iNKT cells [18] and more specifically expression of CxCR6 for liver localization [19].

However, many iNKT cells remain in the thymus, mature to the NK1.1⁺ phenotype there, and become long-lived residents [20]. The mechanisms responsible for the export/retention of iNKT cells from the thymus at various developmental stages are unknown and clearly require further attention.

What can iNKT cells do?

Perhaps the single most confounding issue in understanding and predicting iNKT cell function *in vivo* is the fact that iNKT cells have been shown to do so many different kinds of things during an immune response. Not only do they have the capacity to rapidly and robustly produce cytokines and chemokines, they also have the ability, as their name would suggest, to kill other cells (Figure 1A). In addition, they have been shown to influence the behavior of many other immune cells (Figure 1B). In this section, we detail the multitude of functional properties that have been attributed to iNKT cells.

Cytokine and chemokine production

iNKT cells were originally identified as an unusual T cell population with NK markers that had the unique capacity to rapidly and robustly produce IL-4 upon the injection of anti-CD3 antibodies in mice. Later studies revealed that while this robust IL-4 production was a signature of iNKT cells, it certainly was not the only cytokine iNKT cells can produce. To date, iNKT cells have been shown to produce IFN-γ and IL-4, as well as IL-2, IL-5, IL-6, IL-10, IL-13,

IL-17, IL-21, TNF- α , TGF- β and GM-CSF (Figure 1A) [21–26]. iNKT cells are also known to produce a vast array of chemokines [27].

The rapid and dual production of IL-4 and IFN γ by iNKT cells *in vivo* following administration of the α -GalCer antigen has become a trademark feature of iNKT cells. In fact, within 2h of *in vivo* exposure to antigen, intracellular analysis of *ex vivo* iNKT cells from naïve mice revealed that the majority of iNKT cells in the liver produced both IL-4 and IFN γ [1]. How iNKT cells from unsensitized mice produce cytokines so rapidly when activated is unclear. However, the observation that resting iNKT cells have high levels of IL-4 and IFN γ mRNAs provides one potential mechanism [28,29]. Acquisition of this phenotype is a likely consequence of their unique mode of selection in the thymus, because CD4 T cells positively selected by MHC class-II expressed on thymocytes share similar effector functions [30].

Additional studies with antigen-primed “conventional” T cells, which also constitutively express high levels of IL-4 and IFN γ mRNAs, implicated components of the integrated stress response in regulating effector cytokine production [31]. Interestingly, mRNAs encoding for members of the RNA-recognition-motif family of RNA-binding proteins, TIA-1 and TIAR, have been found enriched in iNKT cells [32,33]. mRNAs encoding for the chemokine RANTES (CCL5) have also been found to be constitutively expressed in iNKT cells [32,33], and a similar mechanism of post-transcriptional regulation of RANTES expression in memory “conventional” T cells has been described [34,35]. Altogether, these results suggest the possibility that iNKT might regulate, at least to some extent, their cytokine secretion through such a translational mechanism. Whether it might apply to all cytokines/chemokines that are produced by iNKT cells and perhaps to their cytotoxic apparatus [36], remains to be determined.

iNKT cells also regulate their cytokine production at the transcriptional level. Several transcription factors known to regulate cytokine gene transcription in conventional T cells (T-bet [37], GATA-3 [38], NF κ B [39], c-Rel [40], NFAT [41], AP-1 [42], STATs [41], Itk [43]) have also been implicated in iNKT cells, although their mechanisms of action remain poorly defined. For example, iNKT cells appear to co-express both T-bet and GATA-3 transcription factors leading to the transcription of both IFN γ and IL-4 mRNAs. This is in contrast to conventional T cells where T-bet has been shown to repress the expression of GATA-3 and vice versa [44]. Further studies will be required to understand the transcriptional regulation of cytokine production by iNKT cells. Epigenetic changes in chromatin structures at the IL-4 and IFN γ loci are known to occur during iNKT cell development in the thymus [29]. It was reported that Notch signaling might control the activity of an enhancer located downstream of the IL-4 locus that is responsible for the initial IL-4 expression in iNKT cells [45]. Similarly, identification of regulatory regions that might control IFN γ gene expression by iNKT cells are starting to emerge [46]. Finally, it was reported that GM-CSF signaling, during a window of thymic development, licenses mature iNKT cells to secrete IL-4 and IFN γ after activation in the periphery [47]. In absence of this signaling, iNKT cells can both transcribe and translate IL-4 and IFN γ but fail to secrete the cytokines. Altogether these results illustrate the complexity of cytokine production by iNKT cells.

Cytolytic activity of iNKT cells

iNKT cells express high levels of granzyme B, perforin, and FasL, consistent with a cytolytic function for these cells. *In vitro* assays have demonstrated that iNKT cells have the ability to kill antigen-pulsed APCs in a CD1d-dependent manner. In addition, several mouse models have revealed that iNKT cells play an important role in tumor surveillance and tumor rejection. However, it remains unclear if iNKT cell cytolytic activity *in vivo* is a critical aspect of successful tumor surveillance and rejection. In some tumor models, IFN γ production by iNKT cells is clearly instrumental in the activation of NK cells, which in turn mount a robust anti-

tumor response [48]. Similarly, recent work has shown that iNKT cells recognize and respond to bacterial antigens and participate in bacterial clearance [49–51]. IFN γ production by iNKT cells is again critical to this process. In studies with *Listeria monocytogenes*, adoptive transfer of iNKT cells into Rag $^{-/-}$ γ c $^{-/-}$ recipients, which lack both iNKT cells and NK cells, profoundly reduced bacterial burden in these mice. This could suggest a cytolytic function for iNKT cells in bacterial clearance in this model. However, the most significant contribution of iNKT cells in these models is likely to be the ability of iNKT cells to drive NK cell activation early in infection.

Regulation of other immune cells

Early studies demonstrated that iNKT cell-derived cytokines can activate several other cell types, including NK cells, conventional CD4 $^{+}$ and CD8 $^{+}$ T cells, macrophages and B cells and recruit myeloid dendritic cells (see ref [52] for review). iNKT cells can also modulate the recruitment of neutrophils through their secretion of IFN γ [53]. Recent studies have extended the number and cell types that are influenced by iNKT cell response (Figure 1B). A cross-talk between CD4 $^{+}$ CD25 $^{+}$ regulatory T cells (Treg) and iNKT cells has been described, where activated iNKT cells quantitatively and qualitatively modulate Treg function through an IL-2 dependent mechanism, while Treg can suppress iNKT cell functions by cell-contact-dependent mechanisms [54]. A similar cross-regulation between iNKT cells and other CD1d-restricted NKT cells that do not express the invariant TCR- α chain that characterize iNKT cells (type II NKT cells [55]), has also been observed [56,57]. Type II NKT cells appeared to suppress the activation of iNKT cells through IL-12 secreted by dendritic cells [57], a signaling event usually associated with stimulation rather than suppression of iNKT cells (see below).

iNKT cells have also been reported to synergize with $\gamma\delta$ T cells in a model of allergic airway hyperresponsiveness [58], although the mechanism of cooperation between these two cell types remains unknown.

Finally, it has been recognized for some time that systemic iNKT cell activation by α -Galcer injection induces activation of B cells non-specifically. New data now show that purified iNKT cells from lupus-prone NZB/W F1 mice can spontaneously increase antibody secretion by B-1 and marginal zone B cells but not follicular zone B cells [59]. Direct interactions between iNKT cells and the B cell subsets were necessary and the effect could be blocked by anti-CD1d and anti-CD40L mAbs [59]. However, this activity was not found with C57BL/6-purified iNKT cells. Nevertheless, iNKT cells seem to play an important role in antibody responses. C57BL/6 mice immunized with proteins and α -Galcer developed antibody titers 1–2 logs higher than those induced by proteins alone and increased the frequency of memory B cells generated [60]. The mechanism was mediated through the combined action of CD40-CD40L interactions and cytokine secretion. CD1d expression by B cells is also absolutely required for the iNKT cell-enhanced response, suggesting cognate interaction between iNKT cells and B cells [61]. Interestingly, while the effector antibody response is unaffected in iNKT-deficient mice, a significantly faster decay of circulating antibodies was observed, pointing to a potential role of iNKT cells in the homeostasis of plasma cells [60].

What antigens do iNKT cells recognize?

The first described iNKT cell ligand was α -Galactosylceramide (α -GalCer), which was identified from a panel of marine extracts for its anti-tumor activity [62]. Since then, many more iNKT cell antigens have been discovered, including both endogenous and exogenous antigens. Unlike conventional T cell antigens that are predominantly peptides presented by MHC molecules, iNKT cell antigens have a distinct lipid component to them. Most iNKT antigens defined to date share a common structure: a lipid tail that is buried into CD1d and a sugar head group that protrudes out of CD1d and makes contact with the NKT TCR. The main

exception to this is the iNKT antigen phosphatidylethanolamine, which lacks a sugar head group.

Exogenous iNKT cell antigens

The majority of naturally occurring exogenous iNKT cell antigens derive from microbes. Whereas the ability of some previously described ligands of microbial origin - *Plasmodium* (and *Trypanosoma*) glycosylphosphatidylinositol (GPI) and *Mycobacterium* phosphatidylinositol mannoside (PIM) - to activate iNKT cells has been questioned, recent work has rigorously demonstrated that GSL-1 from *Sphingomonas* and BbGL-II from *Borrelia* undoubtedly stimulate NKT cells in a CD1d-dependent manner [49,63–65]. It remains to be determined whether direct recognition of these glycolipids by iNKT cells is responsible for the iNKT cell-dependent anti-bacterial activity noted during infection with these bacteria [49,63].

Endogenous iNKT cell antigens

Although α -GalCer is undeniably a potent antigen for iNKT cells, it was clear that any endogenous ligands for iNKT cells would differ significantly in structure, given that mammals cannot synthesize α -linked glycolipids. The identification of endogenous antigens is of paramount importance to further our understanding iNKT cell selection and development. Moreover, iNKT cells are autoreactive. Determining the self-antigens that might drive this autoreactivity will be crucial to understanding the implications of iNKT cell autoreactivity *in vivo*.

When the lysosomal glycosphingolipid (GSL) isoglobotrihexosylceramide (iGb3) was described as an endogenous, and perhaps the selecting, ligand for iNKT cells [66], it faced a good deal of skepticism, mainly because humans are believed to lack functional α 1,3 Galactosyltransferase [67,68], an enzyme thought to be critical to the synthesis of iGb3. Two papers published last year raised some doubts regarding the role of iGb3 as a self-antigen. First, iGb3 could not be detected in mouse and human thymocytes and dendritic cells using a high-performance liquid chromatography (HPLC) assay with a sensitivity of 200 molecules per cell [69]. Second, iNKT cells developed normally in mice lacking the iGb3 synthase, the enzyme responsible for iGb3 synthesis [70]. In addition, a previous report revealed that several strains of genetically-modified mice, all lacking in enzymes of the GSL metabolism pathway, showed various iNKT cell defects [71], raising the possibility that GSL storage *per se*, and not a specific enzyme deficiency, disrupted iNKT cell development. In contrast, two papers this year have shown the successful detection of iGb3 in human cells using a sensitive ion-trap mass spectrometry method to detect GSLs of the globo and isoglobo series [72,73].

The controversy surrounding iGb3 and its importance as an endogenous iNKT cell ligand is currently one of most disputed topics in the iNKT cell field. Although its role as the singular, or at least the predominant, selecting ligand has been challenged, it remains the most potent endogenous agonistic antigen described so far. Although other β -linked GSLs such as β -Galcer have been shown to stimulate iNKT cells [74,75], these results could not exclude the potential contamination by α -linked GSLs that can occur during the synthesis of these compounds, and further experiments are needed to clarify whether β -Galcer (and other β -linked GSLs) are bona fide low affinity iNKT cell ligands.

How do NKT cells recognize their antigens?

The unique antigen specificity of iNKT cells is dictated by the expression of the semi-invariant TCR. How this TCR, which was known to have a similar overall structure to known peptide/MHC reactive TCRs, might instead recognize glycolipid antigens in the context of CD1d was

the subject of constant speculation. Recent crystallographic success and mutational analyses have exposed how this TCR recognizes CD1d/glycolipid complexes. First, the crystal structure of a human iNKT TCR in complex with CD1d/ α -GalCer revealed a unique docking strategy that differed from known TCR/MHC/peptide interactions [76 $\lambda\lambda$]. Compared with conventional TCR-MHC interactions, where TCR engages the distal portion of the MHC in a diagonal orientation, the iNKT TCR docked at the very end of, and parallel to, the CD1d- α -GalCer complex. In the structure, the binding surface between the iNKT TCR and CD1d- α -GalCer complex was composed primarily of three out of the six complementarity-determining region (CDR) loops: CDR1 α , CDR3 α and CDR2 β , with the invariant TCR α chain dominating the interaction with both the glycolipid and CD1d, while the role of the TCR α chain was restricted to the CDR2 β loop interacting with the α 1 helix of CD1d. CDR3 β , the only hypervariable region of the iNKT TCR, which usually mediates antigen specificity together with CDR3 α for conventional TCR, did not make any contact with the antigen. Thus, recognition of α -GalCer-CD1d by the iNKT TCR is entirely mediated by germline-encoded surface on the iNKT TCR.

These results were confirmed and extended through an extensive mutational analyses of both mouse and human iNKT TCRs [77 λ ,78 λ]. The results confirmed an energetic ‘hot-spot’ formed by residues within the CDR1 α , CDR3 α and CDR2 β loops of the TCR that were critical for the recognition of the α -GalCer-CD1d complex and provided the basis for the extremely biased TCR repertoire of iNKT cells. In the mouse system, this ‘hot-spot’ was similarly required for recognition of structurally different glycolipid antigens such as α -GalCer and iGb3. Because recognition of diverse glycolipid antigens used the same germline-encoded residues, these observations suggest that the iNKT TCR functions as a pattern-recognition receptor [77 λ]. In this way, different NKT cell clones have overlapping antigen specificity despite diversity in the TCR β chain.

The iNKT TCR interacts most extensively with the region of CD1d directly above the F’ pocket and distal to the protruding head group of loaded glycolipid antigen [78 λ]. Interestingly, the length of the lipid chain occupying this F’ channel was shown to modulate the affinity of the iNKT TCR [79 λ]. Because conformational changes between empty and antigen-loaded CD1d molecules have been observed in this region of CD1d [80], it is possibility that the iNKT TCR might essentially recognize a change in CD1d conformation following antigen loading.

How do iNKT cells get activated?

Cognate recognition and activation of iNKT cells by foreign antigen

Microbial glycolipids presented as cognate antigens that activate iNKT cells have been identified. iNKT cells have been shown to directly recognize α -linked glycosphingolipids and diacylglycerol antigens that are expressed by bacteria such as *Sphingomonas*, *Ehrlichia* and *Borrelia burgdorferi* in a CD1d-dependent manner [49,63–65] (Figure 1C). The biological response to these glycolipid antigens includes the production of IFN γ and IL-4 by iNKT cells. Because these bacteria lack lipopolysaccharide (LPS), it has been suggested that these glycolipids might be acting as surrogates for ‘conventional’ pathogen-associated molecular pattern (PAMPS) molecules, such as LPS, and might trigger the immune system through iNKT cell recognition. This would be in good agreement with the postulated role of the iNKT cell population as an ‘innate’ sensor of the immune system that links innate and adaptive immunity. Indeed, upon antigenic stimulation, iNKT cells expand for a few days and then quickly contract without generating memory cells [81].

Indirect recognition and activation of iNKT cells

Albeit no cognate glycolipid antigens that are recognized by iNKT cell TCRs have been found in the main Gram-negative and Gram-positive bacterial pathogens that are prominent in human

disease, alternative modes of iNKT cell activation have been reported for such bacteria. For example, LPS-positive bacteria like *Salmonella* or *Escherichia* have been shown to activate iNKT cells indirectly (Figure 1D). These indirect means of recognition fall into two main groups, those that depend, at least partially, upon CD1d/TCR interactions in conjunction with the activation of antigen presenting cells, and those that appear to be CD1d-independent.

First, it was shown that Gram-negative bacteria (such as *Salmonella typhimurium*) or Gram-positive bacteria (such as *Staphylococcus aureus*) cultured with dendritic cells can stimulate iNKT cells in absence of specific cognate foreign glycolipids [49,82]. Such stimulation is blocked by either anti-CD1d or anti-IL-12 mAbs *in vitro* and *in vivo*. These results suggest that a vast array of microorganisms might be able to induce iNKT activation indirectly through APC stimulation. This mechanism is dependent on TLR engagement of the APC as *S. typhimurium*-exposed wild-type derived bone marrow-derived dendritic cells (DCs), but not TLR-signaling molecules-deficient DCs, were able to stimulate iNKT cells *in vitro* [49]. It is also likely dependent upon recognition of a self glycolipid by the iNKT TCR because CD1-deficient DCs are unable to stimulate iNKT cells in when stimulated similarly. Furthermore, APC activation by TLR ligands was shown to modulate the lipid biosynthetic pathway and to induce the specific upregulation of CD1d-bound ligand(s), as demonstrated with the use of multimeric iNKT TCRs as a staining reagent [83]. Whether the upregulated self-ligand(s) correspond to the GSL iGb3, as originally postulated [49], remains a matter of contention.

In striking contrast with these results, it was reported that *Escherichia coli* LPS induces the stimulation of iNKT cells in an APC-dependent but CD1d-independent manner [84]. In these experiments, IFN γ -production by iNKT cells did not require the CD1d-mediated presentation of an endogenous antigen and exposure to a combination of IL-12 and IL-18 was sufficient to activate them (Figure 1E).

Finally, it was reported that in addition to the LPS-detecting sensor TLR4, activation of the nucleic acid sensors TLR7 and TLR9 in DCs also leads to the stimulation of iNKT cells, as measured by their production of IFN γ [85]. In this system, iNKT cell activation required the co-stimulation by type I interferon and the presentation of a yet-to-be-determined charged glycosphingolipid that may be distinct from iGb3 [85] (Figure 1F).

The 'Swiss' army knife of the immune system: how can iNKT cells do so many things?

It is becoming increasingly clear that iNKT cells can and do respond differently under different circumstances. Yet how do iNKT cells achieve this? Do iNKT cells represent a single population of cells with the plasticity to perform multiple tasks depending upon environmental cues? Or, do iNKT cells comprise a collection of many distinct subpopulations, each with unique functional attributes? Due to space limitations we will not detail the putative roles of iNKT cells in the numerous disease models studied to date (see reviews [21,86]). We will, however, summarize some of the factors that might account for the differences in iNKT cell responses that have been reported.

The heterogenous phenotype of iNKT cells

While iNKT cells are defined by their TCR specificity for α -Galcer/CD1d, the population itself is clearly heterogenous in its expression of other cell surface markers. Ongoing studies are revealing the increasing complexity of studying iNKT cells, as different functional attributes have been linked to phenotypic differences. Differential expression of the CD4 co-receptor and NK markers have been shown to discriminate between functionally distinct subpopulations of iNKT cells.

iNKT cells in mice are either CD4⁺ or DN. The physiological significance of CD4 expression on CD1d-restricted iNKT cells is unclear. However, several observations have identified functional differences in human CD4⁺ and CD4⁻ iNKT cells. *In vitro* studies with human iNKT cells have shown that activated CD4⁺ iNKT cells tend to produce both Th1 and Th2-type cytokines, while CD4⁻ iNKT cells appear to be more Th1-like in their response, [26,87]. However, these findings have not been confirmed in the mouse system. Recent studies with human iNKT cell clones have demonstrated that CD4 engagement in conjunction with TCR/CD3 signaling enhances both cytokine secretion and increased calcium flux as compared with CD4⁻ iNKT cell clones [88,89], suggesting that CD4 might be able to modulate the potency of an iNKT cell response.

Like CD4, the distribution and expression of NK markers on iNKT cells is heterogenous. Currently, the effect of NK receptor expression on iNKT cell function is not known. However, given the influence of NK receptors on other immune cell types, it is likely that NK receptor expression on iNKT cells will modulate their function. Since thymic iNKT cells lacking in the expression of NK1.1 and other NK markers can give rise to iNKT cells that express NK1.1 and other NK markers, NK1.1⁻ iNKT cells are generally perceived as being 'immature' and NK1.1⁺ iNKT cells as 'mature'. However, a recent report demonstrated the existence of a functionally competent subpopulation of NK1.1⁻ iNKT cells in the lung, which preferentially produces IL-17 [22].

The influence of the microenvironment on the iNKT cell response

iNKT cells form a substantial proportion of the T cells in the liver, spleen, thymus, bone marrow, and peripheral blood. Tissue specific microenvironments, from the local cytokine milieu to the composition and activation status of other lymphocytes, likely influence the behavior of resident iNKT cells.

Cytokines can modulate the iNKT cell response. IL-12, which is produced at sites of inflammation, has been shown to preferentially drive IFN γ production in iNKT cells [90]. However, IL-12 has also been shown to enhance IL-4 production by iNKT cells [91]. IL-7, on the other hand, appears to promote iNKT cell secretion of IL-4. Other cytokines, such as IL-21, might affect iNKT cell proliferation and enhance cytokine production [24].

In addition, the type and/or status of antigen presenting cells encountered in the microenvironment may alter the response. Effective co-stimulation via CD28 or 4-1BB on iNKT cells has been shown to be required for optimal iNKT cell production of both IL-4 and IFN γ [92]. On the other hand, CD154-CD40, OX40L-OX40 interactions or engagement of CxCR6 cells seem important only for IFN γ production [93–95]. APCs activated by TLR signaling or by LPS can induce IFN γ but not IL-4 production by iNKT cells.

Tissue specific differences in iNKT cell function are perhaps most compelling as demonstrated by Crowe and colleagues [96]. iNKT cell-mediated tumor rejection was most potent with iNKT cells from the liver, as compared with iNKT cells from the spleen or thymus. In another example of phenotypically similar iNKT cells behaving in distinctly different ways depending on their resident tissue, a stable population of NK1.1⁻ iNKT cells has been identified in the liver and spleen, which appear to behave similarly to other peripheral NK1.1⁺ iNKT cells and unlike NK1.1⁻ iNKT cells from the thymus [15]. Altogether these observations underscore the difficulty in effective prediction of iNKT cell function. Further studies will be required to discriminate between circumstantial and direct associations of phenotype and function.

The type of iNKT cell stimulation

Qualitative differences in TCR recognition of different glycolipid antigens could also possibly alter iNKT cell cytokine responses. For example, several synthetic agonist analogs of α -Galcer have distinct effects on iNKT cell function. The OCH [97] and C20:2 [98] analogs have both been shown to elicit a predominantly Th2-like response while α -C-Galcer drives a Th1 response [99]. The reason why these analogs induce different functional responses following iNKT cell stimulation is unclear. Altered TCR binding is a possibility. The length of lipid chain of exogenously loaded lipids occupying the F' channel of CD1d can fine tune the affinity of the TCR for the complex, resulting in altered TCR binding kinetics and modulating the strength of activation signals [79]. However, differences in solubility, resistance/sensitivity to enzymatic degradation *in vivo*, binding to various lipid-binding proteins [100], compartmentalization in the cells, affinity, and kinetics of CD1d loading could also affect both the quantitative and qualitative nature of the iNKT cell response. This, in turn, would be expected to translate into differences in the cross-talk between iNKT cell and other immune cells, such as NK cells, and therefore the overall tone of the immune response.

Whatever their mechanism of action, the development of iNKT-cell-based adjuvants that promote distinct immune responses holds great promise in the development of potent immunotherapies. In concert with this, understanding how iNKT cells can orchestrate the tone of the ensuing immune response will be critical to accurately predict the outcome of using these diverse glycolipid adjuvants in different immune settings.

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Figure 1. Activated iNKT cells, the “Swiss-army knife” of the immune system.

(A) CD1d-restricted iNKT cells exert potent killing activity and promptly release an impressive array of cytokines and chemokines that (B) influence many other cells of the innate and adaptive immune systems. Several known mechanisms for iNKT cell activation by pathogens have been identified (C) Microorganisms containing CD1d-restricted glycolipids (*Sphingomonas*, *Borrelia*) directly activate iNKT cells through TCR engagement, in the absence of co-stimulation. (D) Alternatively, LPS-positive bacteria (*Salmonella*) activate TLR4-expressing APCs through LPS and can indirectly induce iNKT cell activation through presentation of self-glycolipid(s) in conjunction with IL-12 co-stimulation, or (E) by an IL-12/IL-18-dependent, CD1d-independent mechanism. (F) Finally, intracellular pathogens may stimulate TLR7/9 to induce the presentation of CD1d-restricted self-glycolipid(s) to iNKT cells. In this setting, co-stimulation by IFN- α/β is required.