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Antigen-Dependent versus -Independent Activation of Invariant NKT Cells during Infection

Keli L. Holzapfel,* Aaron J. Tynznik,† Mitchell Kronenberg, † and Kristin A. Hogquist*

CD1d-reactive invariant NKT cells (iNKT cells) are a distinct lineage of αβ T lymphocytes with an invariant TCR composed in mice of the α-chain V region 14 (Vα14) and the α-chain joining region 18 paired with a restricted subset of TCR β-chains (1). As a result of this TCR expression, iNKT cells are able to recognize several types of glycolipid Ags in the context of CD1d, a nonclassical major histocompatibility class I-like Ag-presenting molecule. Upon glycolipid presentation, and most famously with the strong agonist α-galactosylceramide (αGalCer), iNKT cells are able to rapidly produce cytokines such as IFN-γ and IL-4 (2). This rapid cytokine production contributes to the recruitment and activation of other cell types during an immune response, which has been shown to influence a variety of diseases, including cancer (3), autoimmunity (4, 5), and pathogenic infections (6). Therefore, insight into their activation is crucial for the general understanding of how iNKT cells contribute to immune responses.

One area that remains to be fully elucidated is the extent to which Ag recognition by the iNKT TCR contributes to the activation of iNKT cells during various infections. Previous reports have shown that iNKT cells can be activated through the TCR by certain infectious agents that produce glycolipid Ags (7–11). For example, iNKT cells are activated by glycosylated diacylglycerol Ags from *Salmonella typhimurium* was proposed to induce the presentation of a self-lipid, iNKT cells produced IFN-γ but did not upregulate GFP, consistent with their reported CD1d-independent activation. However, two bacteria known to produce lipid Ags for iNKT cells induced GFP expression and cytokine production. In contrast, although *Salmonella typhimurium* was proposed to induce the presentation of a self-lipid, iNKT cells produced IFN-γ but did not upregulate GFP postinfection in vivo. Even in CD1d-deficient hosts, iNKT cells were still able to produce IFN-γ after *S. typhimurium* infection. Furthermore, although it has been proposed that endogenous lipid presentation is a result of TLR stimulation of APCs, injection of different TLR agonists led to iNKT cell IFN-γ but not increased GFP expression. These data indicate that robust iNKT cell responses to bacteria, as well as viruses, can be obtained in the absence of antigenic stimulation. *The Journal of Immunology*, 2014, 192: 5490–5498.

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Abbreviations used in this article: BMDC, bone marrow–derived dendritic cell; α-Gal A, α-galactosidase A; α-Gal A−/−, α-Gal A–deficient; αGalCer, α-galactosylceramide; βGlCer, β-D-glucopyranosylceramide; GSL, glycosphingolipid; hCD2, human CD2; iGb3, isoglobotrihexosylceramide; iNKT, invariant NKT cell; MCMV, mouse CMV; Vα14, α-chain V region 14; WT, wild type.

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independently of Ag receptor stimulation, sometimes referred to as noncognate activation (19–23). Therefore, it is possible that certain microorganisms are able to activate iNKT cells independently of their TCR (24, 25). This Ag-independent model has been proposed to explain iNKT cell activation during infection with mouse CMV (MCMV) (25). Upon infection with MCMV, iNKT cells demonstrated an activated phenotype and produced IFN-γ. This activation resulted from APC secretion of IL-12 and IL-18, and there was little or no effect on IFN-γ production when CD1d was blocked or deleted (25, 26).

Further understanding of the indirect activation mode, whereby iNKT cells are activated by endogenous lipids, is important because it may be occurring in diseases such as type I diabetes and asthma (27–29). Greater mechanistic understanding might also provide a profound way to control iNKT cells during infections. However, one challenge to greater understanding is that it remains difficult to distinguish TCR-mediated from non-TCR-mediated activation. The activation parameters typically used in the study of iNKT cells are CD69 upregulation, cytokine production, and reduced recovery of iNKT cells using tetramers, presumed to be secondary to TCR internalization. All of these features, however, can also be a consequence of non-TCR–mediated activation. In this study, we examined the role of Ag receptor stimulation in iNKT cell activation during infections in vivo using Nur77<sup>gfp</sup> BAC transgenic mice, which upregulate GFP in response to Ag receptor but not inflammatory signals (30). Such mice were infected and examined for cytokine production and GFP expression as indicators of iNKT cell activation and iNKT TCR stimulation, respectively.

Materials and Methods

**Mice**

Nur77<sup>gfp</sup> B6 mice were previously described (30). B6 (C57BL/6JNcr) and B6.SJL (B6.LY.5/Cr) mice were obtained from the National Cancer Institute. CD1<sup>d</sup>−/− B6 (B6.129S6-Cd1d1/Cd1d2tm1Spb/J) mice and Vα14 TCR transgenic B6 (C57BL/6-Tg[CD4-69C-aNOS(D32D)]Ai1Bn/J) mice were obtained from The Jackson Laboratory. Fabry mice deficient for the enzyme α-galactosidase A (α-Gal A; B6; 129-Gal<sup>−/−</sup>/J) were obtained from F. Winau (Harvard Medical School). KN2 mice on the B6 background were obtained from M. Mohrs (Trudeau Institute). KN2 Nur77<sup>gfp</sup> B6 mice were generated by the crossing of KN2 and Nur77<sup>gfp</sup> mice. Nur77<sup>gfp</sup> Vα14 TCR transgenic B6 mice were generated by the crossing of Vα14 TCR transgenic B6 and Nur77<sup>gfp</sup> B6 mice. All animal experimentation was approved by and performed according to the guidelines from the Institutional Animal Care and Use Committees at the University of Minnesota or at the La Jolla Institute for Allergy and Immunology.

**Flow cytometry, Abs, and intracellular cytokine staining**

Single-cell suspensions were prepared from spleens, and hematopoietic mononuclear cells were separated by Percoll gradient centrifugation. All cells were resuspended in staining buffer (PBS with 5% FBS). Abs to surface markers were added, and cells were stained on ice for 20 min. Abs used were from eBioscience, BD, or Biolegend. For iNKT cell staining, CD1d monomers loaded with biotinylated PBBS7 or unloaded monomers were obtained from the tetramer facility of the U.S. National Institutes of Health. Cells were analyzed on an LSRII (Becton Dickinson), and data were processed with FlowJo software (Tree Star). A dump strategy (CD11b, F4/80, and CD8) was used to eliminate nonspecific events.

For intracellular detection of cytokines, cells were stained for surface markers as described, then fixed and permeabilized using the Cytofix/ Cytoperm buffer (BD Biosciences) according to the manufacturer’s protocol. Anti–IFN-γ (eBioscience) was added and the cells were stained on ice for 30 min. Cells were washed twice and then resuspended in staining buffer before analysis on an LSRII.

**iNKT cell purification**

For iNKT cell adoptive transfer experiments, a single-cell suspension was prepared from Nur77<sup>gfp</sup> Vα14 TCR transgenic spleens and from hepatic mononuclear cells separated by Percoll gradient centrifugation. This single-cell suspension was depleted of B cells and CD8 T cells using anti-CD19 and anti-CD8α conjugated to magnetic beads and MACS columns (Miltenyi Biotec), according to manufacturer’s protocol. The percentage of iNKT cells in the enriched fraction was ~30%, and the equivalent of ~3 × 10<sup>6</sup> iNKT cells was adoptively transferred per mouse.

**In vivo infections**

MCMV-K181 was obtained from C. Benedict (La Jolla Institute for Allergy and Immunology). Mice were infected i.p. with 1 × 10<sup>3</sup> PFU diluted in 300 µl PBS. S. paucimobilis cultured in Tryptic Soy Broth (BD) at 37°C were collected at a midlog phase and washed with PBS. Mice were inoculated i.v. with ~1 × 10<sup>2</sup> CFU diluted in 200 µl PBS. S. pneumoniae UPR918 (clinical isolate, serotype 3) cultured in Todd Hewitt Broth (BD) at 37°C were collected at a midlog phase and then washed with PBS. Mice were inoculated i.v. with ~1 × 10<sup>2</sup> CFU diluted in 200 µl PBS. Salmonella typhimurium (SL1344) was obtained from S. McSorley (University of California, Davis). S. typhimurium cultured in Todd-Hewitt broth (BD) at 37°C were collected at a midlog phase and then washed with PBS. Mice were inoculated i.v. with ~1 × 10<sup>6</sup> CFU diluted in 200 µl PBS. As a control for all infections, 2 µg αGalCer diluted in 200 µl PBS was injected i.v., and spleen and liver were harvested 2–4 h later.

**TLR agonists**

Mice were injected i.v. in a final volume of 200 µl with 50 µg E. coli–derived LPS diluted in PBS, or with 10 µg oligodeoxynucleotide 1826 diluted in endotoxin-free water. Spleen and liver were analyzed at the indicated time points after injection.

**Lipid-pulsed bone marrow dendritic cells**

Bone marrow cells from the femurs of mice were cultured for 7 d (5 × 10<sup>5</sup> cells/well) at 37°C in 6-well cell culture dishes with complete RPMI 1640 medium in the presence of recombinant murine GM-CSF (50 ng/ml; PeproTech) and IL-4 (10 ng/ml; PeproTech). On day 6, cells were pulsed with either 100 ng to 1 µg/ml αGalCer (KRN7000; Avanti Polar Lipids), 1 µg/ml OCH (Alexis Biochemicals), 1 µg/ml GLucCer (C24:1 Glucosyl[β] Ceramide [d18:1/24:1(15Z)]; Avanti Polar Lipids), 1 µg/ml iGb3 provided by D. Zhou (MD Anderson), or 1 µl/ml solvent (2:1 methanol:chloroform) for 12–15 h at 37°C. Bone marrow–derived dendritic cells (BMDCs) were i.v. injected into Nur77<sup>gfp</sup> mice (0.5–1.0 × 10<sup>6</sup> cells/mouse), and endogenous splenic iNKT cells were analyzed 16 h later.

**Mixed bone marrow chimeras**

Total bone marrow cells were prepared from the femurs of Nur77<sup>gfp</sup> B6.SJL (CD45.1<sup>−</sup>/CD45.1<sup>−</sup>), B6 (CD45.2<sup>−</sup>/CD45.1<sup>−</sup>), or Fabry B6 (CD45.2<sup>−</sup>/CD45.2<sup>−</sup>) donor mice, and samples were depleted of mature T cells with anti-Thy1.2 (30-H12; Biolegend) and complement. Recipient mice were collected at a midlog phase and then washed with PBS. Mice were inoculated i.v. with ~1 × 10<sup>6</sup> CFU diluted in 200 µl PBS. As a control for all infections, 2 µg αGalCer diluted in 200 µl PBS was injected i.v., and spleen and liver were harvested 2–4 h later.

**Statistical analysis**

 Prism software (GraphPad) was used for statistical analysis. Unpaired two-tailed t tests were used for data analysis.

**Results**

**iNKT cells from the Nur77<sup>gfp</sup> reporter mouse upregulate GFP in response to antigenic lipids**

Nur77<sup>gfp</sup> BAC transgenic mice encode GFP in the locus of Nur77 (Nra1), an immediate early gene downstream of the TCR. Previous work showed that T lymphocytes from these mice expressed GFP only after Ag receptor stimulation, and not after inflammatory signals, cytokines, or stimuli that promote cell survival or trafficking (30). iNKT cells in this reporter mouse exhibited a very low level of GFP in the periphery at steady-state, suggesting that iNKT cells are not continuously receiving stimulation through their TCR (30). However, when injected with potent TCR agonist αGalCer, iNKT cells greatly increased expression of GFP (30). Therefore, we used the Nur77<sup>gfp</sup> mouse to examine iNKT TCR activation in vivo.

Using OT-I TCR transgenic CD8 T lymphocytes, the Nur77 reporter was shown to respond to very weak TCR stimuli, even those that support positive selection and are incapable of inducing
proliferation of mature T cells. Therefore, we sought to test the sensitivity of GFP upregulation after iNKT cell TCR stimulation. Most antigenic lipids have lower affinities for the iNKT TCR compared with αGalCer (31, 32), and we tested OCH, βGlcCer, and iGb3, in addition to αGalCer. OCH is an analog of αGalCer, and is reported to also strongly activate iNKT cells, albeit with a slightly weaker affinity for the iNKT TCR than αGalCer (31, 33). iGb3, and more recently βGlcCer, were identified as putative endogenous self-lipids capable of activating iNKT cells through the TCR (17, 18). iGb3 is a weak activator of iNKT cells with a much lower affinity for the iNKT TCR than αGalCer, in the low micromolar range compared with as low as 11 nM for complexes of αGalCer bound to CD1d (34, 35). βGlcCer, when loaded into CD1d tetramers, is at least capable of binding to a proportion of iNKT cells, suggesting that it is a stronger ligand than iGb3, although an affinity measurement per se has not been reported (17).

We tested the ability of these lipids to activate the iNKT TCR by pulsing BMDCs and then i.v. injecting them into Nur77 gfp mice. After 18 h, iNKT cells were isolated and examined directly ex vivo by flow cytometry for GFP upregulation. All four lipids induced an upregulation of GFP as compared with iNKT cells from a control mouse injected with unpulsed BMDCs (Fig. 1). αGalCer consistently induced the strongest increase, although induced only slightly less. Of the putative self-lipids, βGlcCer induced a higher upregulation of GFP than iGb3. CD69 was also upregulated, showing the same order of expression (αGalCer > OCH > βGlc > iGb3; Fig. 1), although its use as a marker of iNKT TCR stimulation during infections is limited by the fact that it can also be upregulated by inflammatory stimuli (30, 36). These results suggest that in the Nur77 gfp mouse, GFP expression is a sensitive tool for assessing iNKT TCR stimulation.

**MCMV infection does not stimulate iNKT cells through their TCR**

MCMV does not contain a stimulatory lipid that activates the iNKT TCR; however, MCMV has been shown to activate iNKT cells during infection in vivo, as demonstrated by increased CD25 and CD69 expression, and by IFN-γ production detected by intracellular cytokine staining as early as 24 h postinfection (25). This activation is thought to be predominantly a result of IL-12 secretion and IL-18 and/or type I IFN secretion from APCs that were activated through TLR9 (26, 37). Importantly, there was little to no effect on iNKT cell IFN-γ production in experiments where CD1d was blocked and/or deleted (25). Because CD1d-mediated Ag is not considered a key player in the MCMV-induced activation of iNKT cells, we tested this putatively cytokine-mediated model of iNKT cell activation in vivo using the Nur77gfp mouse. Similar to previous reports, iNKT cells from Nur77gfp mice infected with MCMV showed a statistically significant increase in the percentage of hepatic iNKT cells that produce IFN-γ 36 h postinfection (Fig. 2B). Interestingly, the level of GFP expression remained the same at all time points examined, suggesting that iNKT cells did not receive TCR stimulation during MCMV infection (Fig. 2A), and confirming that other inflammatory stimuli produced during infections do not cause GFP upregulation (30).

**S. pneumoniae and S. paucimobilis stimulate iNKT cells through their TCR**

We next infected Nur77gfp mice with microbes that are known to produce lipid Ags that activate iNKT cells (7, 10). *S. pneumoniae* is an extracellular Gram-positive pathogen, which produces glycosylated diacylglycerol Ags (9, 10). These Ags are highly abundant in the bacteria, they can stimulate the iNKT TCR, and iNKT cells were shown to be important for clearance and host survival (10). *S. paucimobilis* is a Gram-negative bacterium that does not produce LPS but does produce a GSL with an α-linked glucuronic acid, which also has been shown to directly stimulate the iNKT TCR when presented by CD1d (7, 8). *S. paucimobilis* also produces another GSL with a tetrasaccharide head group, which is either weakly antigenic (38) or not antigenic (39).

We infected Nur77gfp mice with *S. pneumoniae* systemically by i.v. injection, harvested iNKT cells 6 or 20 h later, and analyzed cytokine by intracellular staining directly ex vivo without re-stimulation (Fig. 3A). As expected, iNKT cells produced IFN-γ (Fig. 3B) and upregulated CD69 (data not shown). Consistent with a TCR-dependent activation mode, the expression of GFP increased in iNKT cells as well (Fig. 3A). This increase was statistically significant compared with the uninfected control mice, but was well below that seen after injection of αGalCer, indicating that although lipids are presented during microbial infections that are able to activate iNKT cells through their TCR, they are weaker agonists than αGalCer. This result is consistent with previous reports that lipid Ags from *S. pneumoniae* presented by CD1d have an affinity for the iNKT TCR in the low micromolar range, compared with the low nanomolar range for αGalCer complexes with CD1d (10, 40). Notably, the GFP level was higher in the subset of iNKT cells producing IFN-γ, suggesting that a TCR signal activated cytokine production.

Similarly, iNKT cells upregulated GFP expression 18 h post-infection with *S. paucimobilis* (Fig. 4A). This increase was statistically significant compared with the uninfected controls, but again was well below that seen after injection of αGalCer (Fig. 4A), which is consistent with previous reports (7). For these experiments, we used Nur77gfp/KN2 mice that have a human CD2 (hCD2) gene engineered into one allele of the IL-4 locus, because
it was shown that Sphingomonas GSLs also induce IL-4 production by iNKT cells (7). S. paucimobilis infection resulted in the production of IL-4 by iNKT cells, as judged by increased hCD2 staining, which interestingly was primarily produced by cells that had upregulated GFP (Fig. 4B), again supporting the idea that a TCR signal activates IL-4 in iNKT cells. Overall, these data indicate that S. paucimobilis and S. pneumoniae infections, in which the bacteria have glycolipid Ags for the iNKT TCR, activate iNKT cells through TCR stimulation.

S. typhimurium infection does not stimulate iNKT cells through their TCR

Having analyzed a cytokine-mediated model of activation and two direct activation models, we next analyzed a third proposed model of iNKT cell activation. In this case, we infected mice with a microbe that has not been shown to produce a microbial Ag for iNKT TCR stimulation, but one that putatively causes APCs to present an endogenous self-lipid that stimulates the iNKT TCR, namely, S. typhimurium (11, 13). S. typhimurium is a Gram-negative, LPS+ pathogen in which a microbial lipid Ag has not been detected, but which causes an activation of iNKT cells that has been shown to be partially CD1d dependent, but which is also dependent on MyD88 expression by the APCs (11) and on their ability to produce IL-12 when activated (13). Unexpectedly, when Nur77gfp mice were infected with S. typhimurium, the iNKT cells did not upregulate GFP expression (Fig. 4B), again supporting the idea that a TCR signal activates IL-4 in iNKT cells. Overall, these data indicate that S. paucimobilis and S. pneumoniae infections, in which the bacteria have glycolipid Ags for the iNKT TCR, activate iNKT cells through TCR stimulation.

S. typhimurium infection does not stimulate iNKT cells through their TCR

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FIGURE 2. MCMV infection induced iNKT cell cytokine production, but not GFP expression. Nur77gfp B6 mice were infected with MCMV i.p., and tet+ liver iNKT cells were analyzed by flow cytometry 36 h later for GFP expression (A) and IFN-γ production (B). Results show uninfected (gray), MCMV-infected (red), and αGalCer–treated animals 3 h after infection (black). Data are representative of two independent experiments, with at least three animals per time point. The histogram in (B) shows GFP expression of the IFN-γ− iNKT cell population (black) versus the IFN-γ+ iNKT cell population (red) from an animal 36 h postinfection. In graphs, each dot represents one mouse (uninfected: n = 6; 12 h postinfection: n = 3; 24 h postinfection: n = 3; 36 h postinfection: n = 6; 3 h post-αGalCer injection: n = 3). **p = 0.00026, percentage uninfected IFN-γ− iNKT cells versus percentage 36 h postinfection IFN-γ− iNKT cells (unpaired two-tailed t test).

FIGURE 3. S. pneumoniae infection induced cytokine production and GFP expression. Nur77gfp B6 mice were infected with S. pneumoniae i.v., and tet+ iNKT cells were analyzed by flow cytometry for GFP expression (A) and IFN-γ production (B). Results show uninfected mice (gray), mice 6 h post–S. pneumoniae infection (red), and αGalCer–treated animals 2.5 h after injection (black). The histogram in (B) shows GFP expression in the IFN-γ− iNKT cell population (black) versus the IFN-γ+ iNKT cell population (red) from 20 h postinfection. Data show representative dot plots of two independent experiments with six animals per time point. For percentage of GFP+ iNKT cells graph (A), ***p = 0.0006, uninfected versus 6 h postinfection, and ***p = 0.0007, uninfected versus 20 h postinfection. For percentage of IFN-γ+ iNKT cells graph (B), *p = 0.0478, uninfected versus 6 h postinfection, and *p = 0.0483, uninfected versus 20 h postinfection (all using unpaired two-tailed t test).
earlier (4 h) and later (72 h) times, but did not observe a GFP increase (Fig. 5A). However, at all of the tested time points, there was a statistically significant increase in the percentage of iNKT cells producing IFN-γ (Fig. 5B). These data suggest that iNKT cells were not receiving stimulation through their TCR during *S. typhimurium* infection, despite being activated to produce IFN-γ.

Given that this result contradicts previous reports (13, 15), we tested whether iNKT cells could produce IFN-γ in the absence of CD1d, leading to the stimulation of iNKT cells through endogenous self-lipid Ag, during *S. typhimurium* infection.

*TLR stimulation does not cause TCR-dependent activation of iNKT cells*

The presentation of endogenous self-lipids by CD1d leading to the stimulation of iNKT cells has been proposed to occur as a result of TLR stimulation of APCs (14–16). Specifically, stimulation of TLR4 or TLR9 was suggested to lead to increased synthesis and/or presentation of endogenous self-lipids (11, 14–16). To test this, we injected Nur77 GFP mice with either LPS to stimulate TLR4 or CpG oligodeoxynucleotides to stimulate TLR9, and examined iNKT cell activation 4, 24, or 36 h later. There was no increase in GFP in iNKT cells at any time point after TLR activation, despite robust production of IFN-γ and elevated expression of CD69 at 4 h for LPS and 36 h for CpG (Fig. 7 and data not shown).
iNKT cells did not have increased GFP when recovered from α-Gal A−/− hosts (Supplemental Fig. 2). Lastly, we performed a direct comparison with the reported data, but analyzed GFP expression as a measure of iNKT TCR stimulation in addition to the decrease in the αGalCer-loaded CD1d-tetramer− TCRβ+ population used in the previous report as a measure of activation (14). Similar to the prior study, we transferred α-Gal A−/− BMDCs, βGlCer-pulsed α-Gal A−/− BMDCs, or WT BMDCs into Nur77+ recipient mice. iNKT cells from animals injected with α-Gal A−/− BMDCs failed to show increased GFP expression in comparison with animals injected with WT BMDCs, whereas increased GFP expression was seen in iNKT cells from animals injected with βGlCer-pulsed α-Gal A−/− BMDCs (Supplemental Fig. 3).

Activation of iNKT cells can lead to a decreased recovery when using tetramer, because of TCR internalization (41) and/or activation-induced cell death (42, 43). This reduced recovery has been used as a measure of iNKT cell activation in vivo. However, mice from α-Gal A−/− BMDC-injected groups exhibited variability in the percentage of αGalCer-loaded CD1d-tetramer+ TCRβ+ populations recovered (Supplemental Fig. 4). Overall, our data are not consistent with the hypothesis that TLR stimulation of APCs leads to the presentation of endogenous self-lipid for the stimulation of iNKT TCRs.

### Discussion

iNKT cells are able to rapidly produce cytokines not only in response to bacterial, viral, and fungal infections, but also in response to cancers, various inflammatory conditions, and autoimmune diseases (44). The invariant nature of the iNKT cell TCR α-chain must restrict the diversity of lipids that are capable of stimulating iNKT cells, but despite this, diverse structures have been reported for both the microbial and self-lipids recognized by these cells including GSLs, diacylglycerols, phospholipids, and cholesterol-containing compounds (45). Also, several TCR-independent modes of activation of iNKT cells have been reported, including the cytokine-mediated activation described earlier, but also stimulation mediated by engagement of activating NK receptors such as NKG2D (46) and activation of iNKT cells in the liver by en-
gagement of β-adrenergic receptors (47). This ability to respond in such diverse contexts raises the question as to the importance of TCR-dependent pathways in those situations in which iNKT cells play a role in the immune response, and also, when the TCR is involved, the relative weight of recognition of self versus foreign glycolipids.

In this study, we used the Nur77gfpmouse as a sensitive tool to examine the role of iNKT cell TCR stimulation during various infections. We showed that this reporter mouse can detect iNKT cell responses to self glycolipid Ags presented by CD1d that have an affinity for the iNKT cell TCR of ~5 μM or weaker, nearly 10^2-fold weaker than the affinity for αGalCer presented by CD1d. Using this system, we showed that iNKT cells are stimulated through their TCR when infected with pathogens known to have antigenic lipids, such as in S. pneumoniae infection and S. paucimobilis infection.

Using infection with MCMV, we showed that iNKT cells became activated in the absence of TCR stimulation, consistent with other results showing that cytokines from APCs were sufficient to activate a protective response by iNKT cells after this infection (25, 37). Further evidence showing that iNKT cells can undergo TCR-independent stimulation was recently reported. Using a transgenic mouse in which the iNKT TCR could be conditionally ablated, the authors showed that TCR^-iNKT cells could still mount a response to LPS with similar amounts of IFN-γ produced compared with iNKT cells that retained TCR expression (48).

More surprising was our finding that iNKT cell TCR stimulation could not be detected after either S. typhimurium infection or TLR stimulation of APCs, because these observations run counter to a currently popular model (45). It could be argued that the reporter mouse is not sufficiently sensitive, and that although we have tested some of the weakest reported TCR agonists, S. typhimurium induces the presentation of a self-Ag that has a very low abundance, and/or TCR affinity, that it cannot give an activation signal in the Nur77gfpmouse. We consider this unlikely, however, because when transferred to CD1d^-/-deficient hosts, we found that the iNKT cell IFN-γ response to S. typhimurium was not impaired. Regarding the reported TCR dependence of the iNKT cell response to S. typhimurium, much of the data relied on in vitro studies. For example, in vitro experiments showing that CD1d^-/- BMDCs were ineffective at inducing IFN-γ production by tetramer sorted iNKT cells in response to S. typhimurium are difficult to reconcile with our data. In vivo studies used CD1d-blocking Abs to demonstrate TCR-dependent IFN-γ production. It is possible that Abs to CD1d could have had a direct effect on APCs, independent of iNKT cells, that led to a reduction in inflammatory cytokine production and a consequent reduction in IFN-γ production by iNKT cells (49).

Similarly, we did not find that TLR activation of APCs induced a detectable TCR signal in iNKT cells, although it was previously reported to increase the synthesis of enzymes responsible for the synthesis of stimulatory self-Ags (13, 16, 17, 50). Although again, other in vivo experiments showed that iNKT cells with and without a TCR produced similar levels of IFN-γ in response to LPS (48). One possible explanation is that a stronger stimulation of the innate immune system achieved in vivo may somehow inhibit TCR-dependent activation of iNKT cells. TLR stimulation was also reported to inhibit the synthesis of an enzyme, α-Gal A, that would degrade a putative lipid self-Ag (14). Regarding the role of α-Gal A, using the Nur77gfpmouse reporter mouse, we did not find evidence that APCs in mice deficient for this enzyme induced a TCR signal in iNKT cells in vivo. In the α-Gal A-deficient model, the previous data supporting TCR-dependent activation of iNKT cells relied on loss of the αGalCer-loaded CD1d-tetramer^ TCRβ^ population and on the increased expression of the surface marker CD69 (14). In our hands, however, population loss was not a consistent and reliable measure, and CD69 expression is not strictly TCR dependent, because it is also influenced by inflammatory stimuli.

In the cases of infection with S. pneumoniae and S. paucimobilis, in which there was a detectable TCR signal in the Nur77gfpmouse reporter mouse, the question remains as to whether that signal was due to the presence of a foreign Ag or the induction of the synthesis of a self-Ag, although these alternatives are not mutually exclusive, and both types of Ags could be present (17). The evidence provided here is only correlative with the need for a foreign Ag to induce a TCR-mediated signal in the infections studied, although if self-Ag were dominant during each of the three bacterial infections, it is puzzling that a signal from the reporter mouse could only be obtained using bacteria with a foreign Ag. There is no evidence that the iNKT cells from the S. typhimurium infected mice were less activated; in fact, by intracellular cytokine staining they were at least as activated as the populations from S. pneumoniae or S. paucimobilis infected mice. It has not been possible to resolve the issue of foreign versus self-Ag recognition so far, however, as it has not been feasible to remove either the microbial Ag or the self-Ag by genetic deletion, without causing unacceptable secondary effects. For example, S. pneumoniae mutants that cannot synthesize the glycosylated diacylglycerol Ag have proved not to be viable (M.K., unpublished observations).

Regarding the self-Ag, problems include the diversity of self-Ags reported, with both GSLs and phospholipids, and secondary effects on the health of the mice and the functioning of the endosomal system in APCs when basic components of glycolipid synthesis pathways are eliminated (51, 52).

Although S. typhimurium infection did not stimulate the iNKT TCR as a result of endogenous lipid Ag presentation in our experiments, we are not proposing that this pathway for iNKT cell activation is unimportant. It is known that iNKT cells do receive TCR stimulation from endogenous lipid during iNKT cell thymic selection (53, 54). For example, in the Nur77gfpmouse, intrathymic stage 0 iNKT cell precursors expressed a high level of GFP (30). Furthermore, thymic NKT2 cells from BALB/c mice, which produce IL-4 at steady-state, showed an increased expression of GFP compared with NKT1 or NKT17 cells (55). In addition, other types of infections remain to be tested for iNKT cell TCR stimulation, as was done in this report. For example, infection with the helminth parasite Schistosoma mansoni, in which iNKT cell activation has been shown to be dependent on CD1d after schistosome egg encounter, remains one of a number of possible examples of iNKT cell TCR stimulation mediated by an endogenous lipid, as a foreign lipid from S. mansoni has not been reported (56).

In conclusion, we have demonstrated that the Nur77gfpmouse provides a sensitive tool for directly measuring iNKT cell TCR stimulation that detects endogenous and foreign lipid agonists even when they vary greatly in potency. In addition, our data provided insight into the role of iNKT cell TCR stimulation in iNKT cell activation during infections, differentiating between infections that are dependent on TCR stimulation and infections that are not. We showed that TCR-independent activation applies to bacterial and viral infections and, furthermore, that the ability to detect TCR-mediated activation in vivo correlated with the presence of foreign Ag. This approach may be useful to identify those situations in which a self-Ag-mediated activation of iNKT cells is most relevant, for example, in the context of sterile inflammatory conditions such as ischemia-reperfusion injury (57).
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Disclosures

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