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The Mechanism of Splenic Invariant NKT Cell Activation Dictates Localization In Vivo

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Invariant NKT (iNKT) cells are glycolipid-specific innate lymphocytes emerging as critical players in the immune response to diverse infections and disease. iNKT cells are activated through cognate interactions with lipid-loaded APCs, by Ag-independent cytokine-mediated signaling pathways, or a combination of both. Although each of these modes of iNKT cell activation plays an important role in directing the humoral and cell-mediated immune response, the spatio-temporal nature of these interactions and the cellular requirements for activation are largely undefined. Combining novel in situ confocal imaging of αGalactosylceramide-loaded CD1d tetramer labeling to localize the endogenous iNKT cell population with cytokine reporter mice, we reveal the choreography of early murine splenic iNKT cell activation across diverse settings of glycolipid immunization and systemic infection with Streptococcus pneumoniae. We find that iNKT cells consolidate in the marginal zone and require dendritic cells lining the splenic marginal zone for activation following administration of cognate glycolipids and during systemic infection but not following exogenous cytokine administration. Although further establishing the importance of cognate iNKT cell interaction with APCs, we also show that noncognate iNKT-dependent mechanisms are sufficient to mediate effector outcomes, such as STAT signaling and dendritic cell licensing throughout the splenic parenchyma. Collectively, these data provide new insight into how iNKT cells may serve as a natural adjuvant in facilitating adaptive immune responses, irrespective of their tissue localization.

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Invariant NKT (iNKT) cells are a distinct lineage of αβ TCR+ lymphocytes that rapidly exert effector functions, such as secretion of the cytokines IL-4 and/or IFN-γ following encounter with lipid Ags presented in the context of the MHC I–like molecule CD1 (1), through cytokine-regulated mechanisms, or both (2). In addition, iNKT cells have a low threshold of activation, thus providing a functional bridge between initial innate immune defense and subsequent adaptive responses. These properties, in combination with their emerging role in diverse settings, including infection, cancer, and metabolic syndrome (3, 4), indicate that iNKT cells serve a critical role in the early events of immune activation, as well as provide a motivation for exploiting this cell type clinically to act as a natural adjuvant for enhancing protective immunity and/or controlling immune homeostasis. To fully realize the function of iNKT cells, as well as their potential as a therapeutic target, it is critical to identify the earliest activation events of this cell type in situ.

The spleen is the primary site of systemic immune surveillance and activation in response to blood-borne Ags. In particular, the marginal zone (MZ) of the spleen, which lies just outside of the lymphocyte-residing white pulp, contains a wide vascular bed, termed the “marginal sinus,” where the arterial blood supply terminates. This site serves as the primary access point for both particulate and cellular entry into the spleen (5). The MZ contains a number of innate leukocytes specialized for rapid detection of blood-borne Ags. For example, SIGNR-1+MARCO+ macrophages lining the marginal sinus are critical for recognition and degradation of systemic pathogens and complement the function of innate-like MZ B cells that are well-established to provide early Ab production for control of systemic pathogens (5). Notably, subsets of dendritic cells (DCs) also reside in the MZ and bridging channels under steady-state conditions (6), yet their role at this site is not clear. Thus, the splenic MZ is a tightly organized network of cells working together to optimally mediate rapid immunity for host protection.

Given the accelerated effector responses of iNKT cells and their relative abundance in the spleen compared with other lymphoid organs (7), it stands to reason that these innate leukocytes may also communicate with resident MZ cell types to immediately and dramatically impact the outcome of immune responses directed toward systemic infections or other foreign Ags. Specifically, iNKT cells recognize numerous bacterial glycolipids in the context of CD1d (8, 9), including one recently identified in Streptococcus pneumoniae (10), and they are robustly activated during S. pneumoniae infection by DC-derived IL-12 plus self-glycolipid containing CD1d (11). We (12, 13) and other investigators (14) recently showed that iNKT cells can provide cognate help to B cells, resulting in extrafollicular plasmablast formation and IgM and IgG3 production reminiscent of MZ B cell activation. MZ B cell Ab production is a key component of immune defense against S. pneumoniae (15), so B cell help from iNKT cells during this infection might be highly relevant but remains largely unstudied. Furthermore, iNKT cells have been identified by us (11) and other investigators (16) as critical for survival following in-
fection with URF918, a serotype 3 clinical isolate of S. pneumoniae. Systemic S. pneumoniae infection, described by the World Health Organization as a significant public health threat, is responsible for the deaths of more than half a million children a year, despite the introduction of multiple vaccines in the last 10 years. S. pneumoniae causes pulmonary pneumonia, otitis media, meningitis, and invasive disease or sepsisemia. S. pneumoniae of the serotype 3 is also one of a select group of serotypes found to be associated with increased risk for death during invasive disease in humans (17), with the rate of in-hospital death for patients systemically infected with serotype 3 reaching 50% (18). As such, it is important to dissect the role of splenic iNKT cells during this systemic infection relevant to public health.

Furthermore, iNKT cells are susceptible to activation across a spectrum of stimuli, ranging from selectively TCR–CD1d–glycolipid–mediated activation to TCR-CD1d–self glycolipid stimulation in combination with cytokine exposure to exclusively cytokine-dominated activation (11). The ramifications of these different forms of activation for iNKT effector function and the localization of splenic iNKT cells following stimulation by these different activators have not been described. The focus of the current study was to compare the dynamics of, and cellular requirements for, iNKT cell activation in response to blood-borne cognate lipid Ags compared with systemic cytokine or complex pathogen-mediated activation. To this end, we combined various chemokine and cytokine reporter mice with a new method of mCD1d-specific tetramer staining of fresh spleen tissue to track the localization and activation of the endogenous iNKT cell population in situ. Using these techniques, we find that iNKT cells are widely distributed throughout the spleen during steady-state conditions, but they rapidly consolidate in the MZ following immunization with synthetic glycolipids and during systemic S. pneumoniae infection, where they produce cytokine in a highly compartmentalized fashion. In contrast, in vivo exposure of iNKT cells to systemic cytokine fails to organize them along the MZ, despite dramatic activation and production of IFN-γ.

Given this localization pattern, it is not surprising that MZ DCs are a critical contributor to iNKT cell activation in response to both glycolipids and infection but are dispensable for activation by systemic cytokines. iNKT cell cytokine production, in turn, mediates global effects on the splenic microenvironment by inducing cytokine-specific STAT phosphorylation across the splenic parenchyma and DC mobilization to the T cell zone (TZ) in a noncognate manner. Collectively, our results demonstrate that iNKT localizes to the TZ unit density standardized to the TZ value (i.e., a ratio of specific regional localization, pixel intensity and square area were determined by ImageJ software for three defined regions: TZ, B cell follicles/zone, and MZ. Unit density was determined to be the specified pixel intensity per unit area. Normalized unit density is an expression of the sepfaction of iNKT cells following stimulation by these different activators share a common, global effector outcome, further highlighting the function of iNKT cells as a natural adjuvant during immunity and infection.

Materials and Methods

Animals

Mice were injected i.p. with 100 ng/mouse diphtheria toxin from Corynebacterium diphtheriae (D0564; Sigma-Aldrich) 24 h prior to immunization with αGalCer. For lipidosome experiments, mice were injected i.v. with 0.2 ml clodronate (CL)-loaded or PBS liposomes either 24 h or 3 wk prior to immunization with αGalCer. C571CD (or CL) was a gift of Roche Diagnostics (Mannheim, Germany) and was encapsulated in liposomes, as previously described (20).

Immunizations and S. pneumoniae infection

Mice were imunized i.v. with 0.5 mg/mouse αGalactosylceramide (αGalCer) or 40 mg/mouse GSL-1 in PBS containing 0.1% BSA and <0.25% DMSO or PBS/BSA/DMSO alone. Alternatively, some mice received i.v. injection of IL-12 (0.5 µg) and IL-18 (1.0 µg) or 1 x 10^6 CFU S. pneumoniae (strain URF918; provided by K. Kawakami, University of the Ryukyus, Nishihara, Okinawa, Japan).

Flow cytometry

iNKT cell activation was performed by dissociating spleens through a 0.7-µm cell strainer with the end of a 5-ml syringe plunger, to generate single-cell suspensions, followed by RBC lysis. For DC analysis, spleens were injected with a solution of 0.1 mg/ml Liberase TM and 20 µg/ml DNase I (Roche) in RPMI 1640 (In vitrogene) and shaken for 20 min at 37°C. A final concentration of 5 mM EDTA diluted in FCS was added to each sample for an additional 5 min at 37°C. Spleenocytes were filtered through a 100-µm cell strainer, red cell lysed, and maintained on ice for Ab staining and flow cytometry analysis. Spleenocytes were stained with Abs against TCR-β (H57-597), CD11b (M1/70) from BioLegend, and huCD2 (RPA.2-10), CD11c (HL3), MHC II (M5/114.15.2), CD8α (53-67), and B220 (RA3-68B2) from BD Biosciences. CD1d tetramers loaded with PBS57 (an αGalCer homolog) or left unloaded were obtained from the National Institutes of Health Tetramer Core Facility and incubated with splenocytes at room temperature for 20 min prior to surface Ab staining on ice. Glycolipid-loaded CD1d-tetramer was used for all FACS and confocal analyses, unless otherwise noted. Intracellular IFN-γ (XM1G1.2; BD Biosciences) was detected by following instructions in the eBioscience Foxp3 staining kit. Phosphorylation of STAT6 at tyrosine 641 (J71-773.58.11) and STAT1 at tyrosine 701 (4a) was detected as described previously (19). All flow cytometric data were acquired on a FACSCan II and analyzed with FlowJo software (TreeStar).

Generation of mixed bone marrow chimeras

Bone marrow from B cell–deficient µMT mice heterozygous for the Kn2 allele was mixed with either (wild-type) WT or CD1d−/− bone marrow at a 3:1 ratio and injected into the tail veins of lethally irradiated (1000 rad split into two doses) WT C57BL/6 hosts to generate mice selectively lacking CD1d expression by B cells. To evaluate the role of CD1d on DCs, some irradiated mice received a 50:50 mix of CD1d+ and CD1d−/− bone marrow. All chimeras were used for experiments 6–8 wk following donor cell transplantation. Specific deletion of CD1d on splenic B220+ cells or CD11c+ MHCII+ DCs from test chimeras was confirmed by flow cytometry.

Diphtheria toxin and liposome administration

Confocal/fluorescent microscopy

For detection of GFP, YFP, and PBS57-CD1d tetramer staining, spleens were harvested and immediately placed in 5% agarose blocks for sectioning. Spleen tissue was cut into 200-µm-thick sections using a vibrating microtome. Tissue Ab staining was performed on ice for 60 min, with the exception of PBS57-CD1d or unloaded tetramer staining, which was performed at room temperature for 30 min prior to staining with other Abs on ice and analysis by confocal microscopy (Leica TCS SP5). Glycolipid-loaded CD1d-tetramer was used for all FACS and confocal analyses, unless otherwise noted. Surface Ab staining included B220, CD11c, μCD2, CD169 (3D6.112; AbD Serotec), and SIGN-R1 (22D1; eBioscience). For in situ evaluation of splenic macrophage and DC subsets, spleens were immediately frozen in optimal cutting temperature embedding compound over liquid nitrogen. Frozen spleens were cut into 6-µm sections on a cryostat (Leica) and fixed in a mixture of ice-cold 75% acetone/25% ethanol for 5 min. Sections were blocked in PBS plus 2% BSA and 5% normal mouse serum for 60 min prior to specific Ab staining. To quantitate localization, pixel intensity and square area were determined by ImageJ software for three defined regions: TZ, B cell follicles/zone, and MZ. Regions were defined based on labeling with CD4/8 (TZ), B220 (B cell zone), and SIGN-R1 (MZ). Unit density was determined to be the specified pixel intensity per unit area. Normalized unit density is an expression of unit density standardized to the TZ value (i.e., a ratio of specific regional unit density/TZ unit density).

IL-12 ELISA

For detection of serum IL-12, ELISA plates were coated with anti-mouse IL-12p40 (C15.6; BD) and then blocked with 1% BSA/PBS. Serum samples were diluted in 0.1% BSA/PBS. Purified murine IL-12p40 (BD) was used...
for a standard curve. Bound IL-12 was detected with rat anti-mouse IL-12p40/e70-hiotia (C17.8; BD), followed by streptavidin-alkaline phosphatase (BD) using pNpp (Sigma-Aldrich) diluted to 1 mg/ml in alkaline buffer (Sigma-Aldrich). Plates were read on a Molecular Devices SpectraMax Plus384 ELISA reader at 405 nm.

Statistical analysis
Nonparametric t tests and one-way ANOVA tests were performed using GraphPad PRISM 5 software.

Results
Splenic iNKT cell localization differs according to the stimulus
To determine the location of the endogenous iNKT cell population in WT mice under steady-state and various activating conditions, we developed an in situ technique by which we detected glycolipid-loaded CD1d tetramer labeling of iNKT cells in fresh splenic tissue by confocal microscopy (Fig. 1). Abs against B220, CD4, and SIGN-R1 (a marker of MZ macrophages) were used to delineate the B cell follicles, T cell area, and MZ, respectively. Consistent with a recent study that used confocal imaging in combination with indirect labeling techniques or transfer of purified iNKT cells into congenic recipients to follow splenic iNKT cell migration (21), we consistently found CD1d tetramer+ cells dispersed throughout the splenic white and red pulp during their endogenous resting state (Fig. 1A, 1B, data not shown). Tetramer labeling of spleens from naïve NKT-deficient CD1d−/− mice demonstrated negligible background binding (Fig. 1C, 1D), as did labeling with unloaded CD1d tetramer on WT spleen sections from un.injected mice (Supplemental Fig. 1A, 1B). Kinetic experiments using upregulation of the high-affinity α-chain of IL-2R subunit (CD25) as an early marker of activation demonstrated that iNKT cells were activated within 4 h after i.v. immunization with αGalCer (Fig. 1E). Similar activation kinetics were observed using intracellular IFN-γ production as an outcome measure (Fig. 1F, 1G). We next determined the location of CD1d-tetramer+ iNKT cells at this time of peak activation. Four hours after αGalCer immunization, there was no significant change in the number of splenic CD1d-tetramer+ iNKT cells (Fig. 1H). However, confocal images and fluorescent quantification revealed that αGalCer stimulates iNKT cells to accumulate at the MZ and bridging channels of the spleen (Fig. 1I) compared with vehicle-injected controls (Fig. 1K). This is consistent with redistribution, but not enhanced recruitment, of this cell type from the peripheral circulation. To determine whether the iNKT cell response that we observed with a synthetic lipid agonist is similar to encounter with bacterial glycolipids, BALB/c mice were immunized with a glycosphingolipid (GSL-1) expressed by the Sphingomonadaceae family of bacteria (9). Intravenous administration of GSL-1 resulted in rapid accumulation of iNKT cells within the MZ and bridging channels of the spleen at the same 4-h time point as for αGalCer administration compared with vehicle-injected controls (Fig. 1I, 1K). The observations of iNKT cell location under steady-state and glycolipid-mediated activation conditions were confirmed using heterozygous CXCR6-eGFP knock-in mice in which iNKT cells highly express eGFP (Supplemental Fig. 1C, 1D) (22). Collectively, these results indicate that iNKT cells rapidly localize to the vascular-rich region of the spleen and cluster at the MZ and bridging channels following immunization with diverse cognate lipid Ags.

In addition to cognate lipid immunization, two other forms of activation for iNKT cells have been characterized: one driven exclusively by high levels of cytokines, such as IL-12 and IL-18 (23), and the other induced by a combination of self-ligand–CD1d recognition plus IL-12 costimulation (2). Indeed, we recently showed that the cytokine-driven “indirect” form of iNKT cell activation dominates the immune response during infection, even if the pathogens contain known CD1d cognate ligands (11). To compare these two other methods of iNKT cell activation to encounter of cognate Ag-mediated activation, WT mice were administered rIL-12 and rIL-18 i.v. or were infected with S. pneumoniae. We determined that IFN-γ production by iNKT cells peaked at 2 h after cytokine administration and 8 h after S. pneumoniae infection (Supplemental Fig. 2A, 2B). As previously shown, IL-4 production was not detectable under either condition (data not shown) (11). Interestingly, iNKT cells also accumulated in the splenic MZ during infection (Fig. 1L). This localization pattern is the same as we detected after cognate glycolipid immunization and suggests that the requirement for CD1d engagement dictates the localization of iNKT cells in the spleen. In contrast, i.v. IL-12 and IL-18 administration robustly activates iNKT cells, but they remain widely distributed throughout the spleen (Fig. 1M), reflecting the same distribution observed in PBS vehicle–injected mice (Fig. 1N).

Consolidation of iNKT cells yields compartmentalization of effector cytokine production
As demonstrated in our in situ tetramer-labeling studies, some iNKT cells remained in the TZ following lipid administration. To delineate the exact location of iNKT cell activation and effector cytokine production, we used IL-4 dual reporter (4get/KN2) (24) and IFN-γ reporter (Great) (25) mice. iNKT cells from 4get/KN2 mice are constitutively GFP+ as a result of the persistent expression of IL-4 mRNA (26) and, upon activation, they rapidly express huCD2 on their cell surface, indicating IL-4 protein production (24, 27). Following i.v. αGalCer administration, splenic CD1d-tetramer+ iNKT cells began to express huCD2 at 2 h, with peak production occurring at 4 h postimmunization (Fig. 2A, 2B), a kinetic similar to IFN-γ production (Fig. 1E–G). Importantly, Fig. 2A confirms that production of IL-4 (i.e., huCD2 expression) was limited to CD1d+ tetramer+ iNKT cells following in vivo administration of αGalCer. This observation allowed the use of huCD2 as a marker for localizing the endogenous IL-4–producing iNKT cell population in the spleen of mice in situ. At 4 h postimmunization of 4get/KN2 mice, we found IL-4–producing huCD2+ cells almost exclusively within the MZ and bridging channels of the spleen (Fig. 2C, left panel, Fig. 2D). Mice homozygous for the 4get allele were used as a huCD2 negative–staining control (Fig. 2C, middle panel). To corroborate our flow cytometry data and confirm that huCD2+producing cells detected within the MZ were iNKT cells, we combined our in situ tetramer-labeling technique with huCD2 detection. Consistent with the results described above, CD1d-tetramer+ iNKT cells that coexpressed with huCD2 were found outside of the white pulp clustered around the bridging channels and MZ (Fig. 2C, right panel). To confirm that our characterization of effector cytokine-secreting iNKT cells as consolidating in the MZ is not unique to IL-4–secreting iNKT cells, we evaluated the localization of iNKT cells producing IFN-γ using mice engineered to report IFN-γ production via YFP expression (Great) (25). αGalCer immunization showed that, at the time of iNKT consolidation in the MZ, the IFN-γ–producing cells were also consolidated in the MZ (Supplemental Fig. 2C) compared with the DMSO vehicle controls (Supplemental Fig. 2D). At this time point following αGalCer administration, IFN-γ production is largely limited to iNKT cells (data not shown); therefore, this result is consistent with MZ consolidation of IFN-γ–secreting iNKT cells. We next determined that i.v. administration of GSL-1 resulted in rapid production of IL-4 and huCD2 expression by iNKT cells with a kinetic similar to αGalCer immunization (Fig. 2E). Similar to our experiments using αGalCer, IL-4–producing huCD2+ iNKT cells accumulated within the MZ and bridging channels after GSL-1 administration (Fig. 2F, 2G). In summary, these results indicate that iNKT cells rapidly localize to the
vascular-rich region of the spleen and produce the canonical cytokines IL-4 and IFN-γ in a highly compartmentalized fashion following immunization with cognate glycolipid Ags. These data clearly inform us as to the localization of specific IL-4– and IFN–γ-producing iNKT cells in response to cognate Ag.

However, following recombinant cytokine administration or during *S. pneumoniae* infection, the production of IFN-γ by other cells, including NK1.1^+^ TCRβ^−^ NK cells, precludes the ability to detect iNKT-specific IFN-γ production in situ during those stimulating conditions (I.L. King, M. Mohrs, and E.A. Leadbetter, unpublished observations). Instead, under those conditions, the localization pattern of iNKT cells, as determined by confocal imaging (Fig. 1L, 1M), at the times of peak IFN-γ production by iNKT cells (Supplemental Fig. 2A, 2B) gives the clearest suggestion of localization of cytokine-secreting iNKT cells.

**MZ DCs are critical for iNKT cell activation under diverse inflammatory conditions**

The distinct localization patterns of iNKT cells following cognate and, to a lesser extent, pathogen activation versus cytokine-driven
stimulation prompted us to investigate which splenic APC(s) are required for elaboration of the iNKT cell effector program under these conditions. Given that iNKT cells accumulate and produce IL-4 and IFN-γ at the splenic MZ in response to cognate lipid Ag administration, we first determined the cell type resident in the MZ niche responsible for cognate activation of iNKT cells following systemic glycolipid administration, an event dependent on CD1d-restricted Ag presentation (3). Although the splenic MZ contains distinct subsets of macrophages and DCs, MZ B cells express particularly high levels of CD1d (28) and are potent activators of iNKT cells in vitro (13, 29). However, analysis of mixed bone marrow chimeras, in which B cells were selectively deficient in CD1d, did not result in any loss of iNKT cell IL-4 production following αGalCer administration in vivo (Fig. 3A). A study by Schmieg et al. (30) found that DCs were indispensable in early iNKT activation in vivo by depleting CD11c⁺ cells from mice expressing the simian diphtheria toxin receptor under control of the CD11c promoter (CD11c-DTR mice) prior to αGalCer administration. Crossing CD11c-DTR mice to the KN2 IL-4 reporter strain, we obtained similar results using IL-4 (huCD2 expression) as an outcome measure (Fig. 3B). However, a recent report indicated that diphtheria toxin treatment to these mice depletes DCs, as well as CD169⁺ and SIGNR-1⁺ macrophage subsets lining the MZ (31). To investigate the potential contribution of macrophages in this system, we next depleted both macrophage subsets by i.v. administration of liposome-encapsulated CL to WT mice, a well-established method of splenic macrophage depletion (Fig. 3C) (32). In contrast to previous observations (30), however, CL treatment also abrogated splenic iNKT cell production of IL-4 (Fig. 3D), despite having no impact on the frequency of total splenic iNKT cells (Fig. 3E). Thus,
further investigation into the relevant APC(s) for iNKT cell activation was required.

In the course of our studies, we consistently observed an ∼50% reduction in total splenic CD11c+MHCII+ DCs 24 h after CL treatment (Fig. 3F), with a more profound loss in the CD8+ subset than in the CD8− subset (Fig. 3G). When we examined the spleens of CL-treated mice histologically, we found selective depletion of CD11c+ cells lining the MZ, whereas the CD11c+ cells in the TZ appeared to be relatively unaffected (Fig. 3H), a result consistent with a previous report (33). To more closely test a role for MZ DCs versus macrophages in early iNKT cell activation, we took advantage of the distinct reconstitution kinetics of splenic macrophages compared with DCs (32). Consistent with previous observations, both SIGN-R1+ MZ and CD169+ metallophilic splenic macrophage populations remained depleted for ≥3 wk after a single injection of CL, whereas DC numbers were restored in frequency and location within 1 wk (Fig. 4A–C) (31, 32). Using this strategy, we immunized mice with αGalCer at either 24 h or 3 wk post-CL treatment to assess iNKT cell canonical cytokine production. iNKT cell IL-4 (Fig. 4D, 4E) and IFN-γ (Fig. 4F) production was lost in αGalCer-immunized animals 24 h after CL injection, a time point at which both MZ DCs and macrophages were depleted (Fig. 4A, 4B). In contrast, iNKT cell production of IL-4 in response to αGalCer immunization was completely restored in mice 3 wk after CL treatment when macrophages were still absent but MZ DCs had been restored. The numbers of IFN-γ–producing iNKT cells generated in response to αGalCer was also restored (Fig. 4F) but not quite to the extent achieved in intact mice, which suggests that the DCs may not be completely reconstituted yet or there could be different cellular requirements for iNKT production of IFN-γ than for IL-4. Collectively, these results indicate that MZ DCs are a critical APC for iNKT cell activation following cognate lipid immunization.

To further investigate the APC requirements for iNKT cell activation during systemic infection, we repeated these CL-depletion studies with S. pneumoniae–infected mice. As expected, iNKT cells failed to become activated during S. pneumoniae infection in mice treated with CL 24 h prior to infection (Fig. 5A). Given our previous work describing the importance of both CD1d and IL-12 from APCs in mediating iNKT activation during infection (2), these data suggested that acute treatment of mice with CL prior to
pneumococcal infection eliminated self-lipid–presenting CD1d, the source of IL-12 critical for iNKT cell activation, or both. Indeed, the increase in serum IL-12p40 detected 8 h after systemic *S. pneumoniae* infection was not observed in infected, CL-treated animals (Fig. 5B). Importantly, both iNKT cell activation and serum IL-12p40 levels were completely restored in mice infected 3 wk after CL treatment (Fig. 5A, 5B), a time point at which MZ DCs, but not SIGN-R1+ macrophages, were allowed to repopulate the splenic MZ. To corroborate our depletion studies and to confirm the importance of DC-derived IL-12 in combination with CD1d to mediate iNKT cell activation in vivo during infection, we assessed iNKT cell activation following pneumococcal infection of *batf3*−/− mice, which have a specific deletion of splenic CD8+ DCs (34). Importantly, this subset was shown to reside in the MZ during

FIGURE 4. MZ DCs mediate cytokine production by iNKT cells. (A and B) Immunofluorescent images of spleen sections from 4get/KN2 mice 24 h or 3 wk after CL treatment. Mice treated with PBS liposomes are shown for comparison. Scale bar, 200 μm. (C) Contour plots showing the frequency of total splenic CD11c+MHCI+ DCs at each time point described in (A). (D) Percentages of CD1d-tetramer+ splenocytes producing IL-4 (huCD2+) from representative individuals. A summary of the percentages of IL-4–producing (huCD2+) (E) and IFN-γ–producing (F) iNKT cells after DMSO vehicle or αGalCer immunization from all 4get/KN2 mice treated with PBS or CL. Results shown are representative of two individual experiments with three to five mice/group. **p < 0.001, ***p < 0.0001.
homeostasis (6) and to be the dominant source of IL-12 in a number of adjuvant- and infection-based models of immunity (35–37). Indeed, iNKT cells from batf3−/− mice produced significantly less IFN-γ in response to S. pneumoniae infection than did WT controls (Fig. 5C), which correlated with reduced serum levels of IL-12p40 from the same animals (Fig. 5D). The residual IFN-γ production in S. pneumoniae–infected batf3−/− mice could be a result of activation through cytokines and/or CD1d on other DC subsets or partial contribution of an entirely different APC (2).

One concern with the above studies was that acute treatment of CL may have off-target effects on iNKT cells and inhibit their activation, irrespective of phagocyte depletion. To address this concern, we again treated animals with CL and, 24 h later, administered rIL-12 and rIL-18, as described above. As shown in Fig. 5E, cytokine-driven iNKT activation was similar in both PBS- and CL-treated immunized mice, indicating that iNKT cells are not intrinsically defective under these conditions. These results also confirmed that cytokines are sufficient to activate iNKT cells, and MZ DCs are no longer required if the cytokine is extrinsically provided. Collectively, these results indicate that MZ-resident DCs are the critical APC responsible for presenting foreign or self-Ags and providing cytokines for iNKT cell activation during both immunization and infection.

iNKT cells exert their effector program beyond their cognate partners

Given the restricted localization of iNKT cells under cognate-activating conditions, we next considered whether iNKT cells direct their effector functions to their local site of activation. In the course of our studies, we observed that CD11c+MHCI+ DCs dramatically upregulated CCR7 expression (Fig. 6A) and relocated from the MZ and bridging channels to the TZ following αGalCer administration (Fig. 6B), a response previously observed following activation with TLR agonists (38, 39) or CD40–CD40L interactions (39). Importantly, DC relocation occurred in a CD1d-dependent manner (Fig. 6B). To consider whether this effector outcome required cognate interactions, we generated mixed bone marrow chimeras, in which 50% of DCs lacked CD1d expression but 50% of DCs expressed normal levels of CD1d (Fig. 6C, 6D). Unexpectedly, we found that cognate iNKT–DC interactions were dispensable for the upregulation of CCR7 by splenic DCs, because both CD1d+ and CD1d− DCs in these chimeras upregulated CCR7 expression equally after αGalCer immunization (Fig. 6E). We also demonstrated previously that, during infection, despite localized production, cytokine signaling can saturate the entire immune compartment of lymphoid organs (19). Given that iNKT cell activation can take place, depending on the stimulus, at distinct loci within the splenic parenchyma, we further postulated that the effector range of their cytokines extends beyond their immediate microenvironment. To investigate this question, we measured the levels of phospho-STAT6 and phospho-STAT1 in the total splenic B cell compartment as readouts of IL-4 and IFN-γ signaling, respectively, by intracellular flow cytometry (19). Although we found iNKT-derived IL-4 and IFN-γ to be produced in a tightly restricted manner in proximity to the MZ of the spleen (Figs. 1, 2), FACS analysis detected CD1d-dependent induction of high levels of both phospho-STAT6 and phospho-STAT1 in the entire splenic B220+ B cell population within 4 h after αGalCer administration (Fig. 6F–H). Because all splenic B cells show evidence of having received IL-4 signals, these results suggest that, irrespective of the location in which iNKT cells are activated, they contain the potential to condition the entire lymphoid organ environment to either regulate or potentiate the immune response.

Discussion

The inability to localize and track the endogenous iNKT population as it initiates the effector program in vivo has been a long-standing roadblock to advancing our understanding of this innate T lymphocyte subset. Other groups (40, 41) tried unsuccessfully to identify endogenous individual splenic iNKT cells by tetramer in WT mice and instead used TCRβ NK1.1 labeling of endogenous cells, adoptive transfer of sorted iNKT cells, or fluorescent-diffusion strategies (21) to probe the localization of iNKT cells in a posttransfer state. These approaches found the majority of resting iNKT cells to be localized in the TZ, or parainterstitial lymphoid sheath (PALS) (41), or in the MZ, PALS, and red pulp (21). However, using a novel method of in situ mCD1d tetramer staining, in combination with relevant cytokine reporter mice, we can now unambiguously identify a restricted localization and cytokine-production profile for endogenous splenic iNKT cells. In agreement with previously published reports, we find that endogenous iNKT cells are widely
FIGURE 6. Noncognate effector functions of iNKT cells in vivo. (A) Graphs showing CCR7 expression of CD11b−CD8− (left panel) or CD11b−CD8+ (right panel) DCs from WT or CD1d-deficient mice 12 h after immunization with αGalCer or DMSO vehicle. (B) Confocal microscopy analysis of spleen sections from WT or CD1d-deficient mice 12 h after immunization with αGalCer or DMSO vehicle. Scale bar, 200 μm. (C) Frequency of CD45.1+ WT or CD45.2+ CD1d−/− DCs from unimmunized mixed chimeric mice, as described in Materials and Methods. (D) CD1d expression by WT or CD1d−/− CD8α+ DCs from mixed chimeras. (E) CCR7 geometric mean fluorescence intensity (MFI) of WT or CD1d−/− CD8α+ DCs in mixed chimera from DMSO vehicle- or αGalCer-immunized mice at the 12-h time point. (F) Phosphorylation of STAT6 in the total B220+ splenocyte population was assessed by flow cytometry in WT, CD1d−/−, and STAT6−/− mice 4 h after αGalCer immunization. Geometric mean fluorescence intensity (MFI) of pSTAT6 (G) and pSTAT1 (H) after DMSO vehicle or αGalCer immunization. All data shown are representative of at least two individual experiments with three or four mice/group. **p < 0.001.
and evenly distributed throughout the parenchyma of the spleen, including B and T cell follicles in the PALS, the MZ, and red pulp (data not shown). Importantly, we find that this distribution only changes to a restricted MZ localization following cognate activation by model and bacterially derived glycolipids or S. pneumoniae infection, presumably as a result of arrest in proximity to Ag-loaded APCs. Barral et al. (42) showed that iNKT cells arrest near lymph node subcapsular sinus macrophages in response to particulate glycolipid Ag and similarly described how iNKT cells arrest in the MZ in close proximity to Ag-rich regions within 2 h of administration of particulate αGalCer (21). This is consistent with live-imaging studies from the Kubes laboratory (43) characterizing iNKT arrest in the liver following Kupffer cell capture and presentation of Borrelia burgdorferi Ags. Michael Dustin and colleagues (22, 44) provided the earliest description of iNKT cell arrest in the liver in response to αGalCer and GSL-1. Interestingly, the Dustin group (44) also found that iNKT cells arrest in the liver in response to systemic IL-12 plus IL-18. This contrasts with our data showing that iNKT cells remain widely and evenly distributed throughout the spleen following activation by systemic cytokines. The difference between our findings and those of the Dustin group could be explained by the different subsets of iNKT cells or APCs that populate the spleen versus the liver, or it could be a consequence of different integrin- or chemokine-mediating requirements or a combination of both. The MZ is often invoked as the likely point of interaction for iNKT cells and activating APCs bearing pathogens and pathogen-derived Ags collected from the blood flowing into the spleen through the marginal sinus (13). Our data showing consolidation by activated iNKT cells and the requirement for MZ DCs after glycolipid immunization and during systemic infection are also consistent with a key activation mechanism of iNKT cells being via engagement of CD1d-presented foreign or self-Ag in the MZ (11). iNKT cells can be activated by a combination of TCR-mediated recognition of CD1d and TCR-independent activation by cytokines on a sliding scale of relative ratios (45). Specifically, iNKT cells are activated to produce IFN-γ and IL-4 by TCR recognition of the glycolipids tested in this study in the context of CD1d. They can also produce IFN-γ following TCR engagement of CD1d-presenting self-glycolipids when receiving costimulation from IL-12. Finally, a robust mixture of systemic IL-12 plus IL-18 in the absence of CD1d can also drive iNKT cells to produce copious IFN-γ (45). Despite these diverse activation approaches, we found MZ DCs to be a critical bottleneck for most forms of iNKT cell activation in vivo. In the case of glycolipid administration, MZ DCs are likely capturing and presenting cognate glycolipid Ag directly (21), thus requiring redistribution of splenic iNKT cells. During infection, recognition of S. pneumoniae by MZ DCs via pattern recognition receptors stimulates production and release of soluble IL-12, which combines with self-glycolipid presentation by CD1d to activate iNKT cells (2, 11). At the other end of the spectrum, iNKT cells can be activated in place throughout the parenchyma of the spleen by systemic administration of IL-12 plus IL-18. MZ DCs are also a very likely source of systemic IL-12 and IL-18 that would be generated by physiologic conditions, but we eliminated this step for MZ DCs in our system by providing the cytokines ex vivo. In summary, MZ DCs are critical partners for iNKT cells in the spleen during their response to myriad forms of activating stimuli, from cognate glycolipids to systemic infection.

Much of the literature describing iNKT cell responses to Ag, interaction with other cells, and receiving of helper signals from DCs, MZ B cells, B helper neutrophils, macrophages, eosinophils, and basophils, considers iNKT localization to be oriented around the MZ during activation (46, 47). Successful use of our new in situ iNKT cell–labeling technique using lineage-specific tetramers suggests that the conditions that produce MZ localization of iNKT cells are relevant for iNKT responses to both highly pure glycolipid Ags and systemic infections. Furthermore, early production of IFN-γ by this population of iNKT cells throughout the spleen conditions the entire environment for a rapid coordinated immune defense, including enhancing activation of innate responders like NK cells, macrophages, DCs, and neutrophils, as well as driving relevant activation of adaptive defenders, such as CD4 T cells and B cells. Perhaps as one example of this, we also noted iNKT-dependent upregulation of CCR7 and relocation by MZ DCs from the MZ and bridging channels into the TZ in the spleens of mice immunized with αGalCer. Furthermore, we demonstrate that these changes, evidence of DC licensing, occur independently of cognate interactions with iNKT cells (i.e., the DCs themselves do not need to specifically engage an iNKT cell via CD1d to receive this licensing signal), suggesting that iNKT cells mediate this aspect of DC licensing via a soluble mediator. The specific soluble factor(s) mediating DC relocation to the TZ requires further investigation.

In summary, our data suggest that iNKT cells use diverse methods to exert their effector functions, the results of which can have a profound effect on the entire lymphoid tissue landscape, further exemplifying their critical role as a bridge between innate and adaptive immune responses. In the future, combining tetramer labeling of fresh tissue sections for confocal imaging with genetically engineered reporter- or gene-deficient mice will provide a unique approach to dissecting the migration requirements for splenic iNKT cells under various conditions and to answer many future questions about the kinetics and localization of iNKT cells in other primary and secondary lymphoid organs.

Disclosures

The authors have no financial conflicts of interest.

References

dependent follicular helper NKT cells that provide cognate help for B cell responses. *Nat. Immunol.* 13: 35–43.


**Supplemental Figure 1.** (A) Confocal images of unloaded-CD1d tetramer labeling of spleen sections from unimmunized WT mice. Unloaded tetramer labeled in red, SignR1 in blue, B220 in white. (B) shows the yellow inset in (A) at higher magnification. (C) Flow cytometric analysis of whole splenocytes from DMSO vehicle or αGalCer-immunized CXCR6-GFP+/- mice. (D) Confocal microscopy images from CXCR6-GFP+/- mice 4 hours after immunization with DMSO vehicle or αGalCer. Merged images are preceded by single color depictions from the same image. All data shown is representative of three experiments with two mice per group. For all images shown, scale bar equals 100μm (A,B) or 200μm (D).

**Supplemental Figure 2.** (A,B) Graph depicts the frequency of IFNγ-producing cells from the total splenic CD1d-tetramer+ population at the time points indicated following i.v. administration of (A) IL-12 + IL-18 or PBS vehicle or (B) αGalCer or S. pneumoniae infection. Uninfected (uninf.) mice are shown in (B) for comparison. (C,D) Confocal image and quantitation of YFP normalized unit density of fluorescence in distinct splenic regions 4 hours after immunization of IFNγ-YFP Great reporter mice with αGalCer (C) or DMSO vehicle (D) using a method similar to that described in Figure 1. All data shown is representative of three (A,B) or two (C,D) separate experiments with 3-4 mice per group.
Supplemental Figure 1.
Supplemental Figure 2.

A

% IFNγ+ tetramer+

0.5hr  1hr  2hr  4hr

PBS  IL-12 + IL-18

B

% IFNγ+ tetramer+

uninf.  4hr  8hr  12hr  4hr

S. pneumoniae  αGalCer

C

αGalCer

B220

tetramer

IFNγ (YFP)

SIGN-R1

D

DMSO

Normalized Unit Density

Normalized Unit Density

TZ  MZ  BZ

TZ  MZ  BZ

ns

ns

ns

ns

****  ****

ns