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Activation of Invariant NKT Cells by the Helminth Parasite Schistosoma mansoni

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Mouse CD1d-restricted NKT cells, including invariant (i)NKT cells, are innate cells activated by glycolipid Ags and play important roles in the initiation and regulation of immune responses. Through their ability to promptly produce large amounts of Th1 and/or Th2 cytokines upon TCR engagement, iNKT cells exert crucial functions in the immune/inflammatory system during bacterial, protozoan, fungal, and viral infections. However, their roles during metazoan parasite infection, which are generally associated with strong Th2 responses, still remain elusive. In this study, we show that during the course of murine schistosomiasis, iNKT cells exhibit an activated phenotype and that following schistosome egg encounter in the liver, hepatic iNKT cells produce both IFN-γ and IL-4 in vivo. We also report that schistosome egg-sensitized dendritic cells (DCs) activate, in a CD1d-dependent manner, iNKT cells to secrete IFN-γ and IL-4 in vitro. Interestingly, transfer of egg-sensitized DCs promotes a strong Th2 response in recipient wild-type mice, but not in mice that lack iNKT cells. Engagement of TLRs in DCs is not necessary for iNKT cell stimulation in response to egg-sensitized DCs, suggesting an alternative pathway of activation. Finally, we propose that self, rather than parasite-derived, CD1d-restricted ligands are implicated in iNKT cell stimulation. Taken together, our data show for the first time that helminths can activate iNKT cells to produce immunoregulatory cytokines in vivo, enabling them to influence the adaptive immune response. The Journal of Immunology, 2006, 176: 2476–2485.

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mature DCs is involved in iNKT cell activation during some infections (11, 21). As mentioned above, in some cases, foreign microbial Ags presented by CD1d can also engage the TCR expressed by some iNKT subpopulations (8, 10–12). Albeit widely studied during viral, bacterial, fungal, and protozoan parasite infection (for reviews, see Refs. 19 and 20), the role of iNKT cells has not been investigated during metazoan parasite infection.

Schistosomiasis is a chronic parasitic disease caused by the extracellular parasite Schistosoma. A key feature of the immune response in Schistosoma mansoni-infected mice is the occurrence of a strong Th2 response triggered by parasite eggs that are gradually deposited in host tissues, particularly in the liver, as early as wk 5 postinfection (for review, see Ref. 22). We have shown recently that CD1d plays an important role in the induction of Th2 responses during murine schistosomiasis (23). This suggested the involvement of CD1d-restricted cells in the early immunological events leading to the generation of the Th2 response during schistosomiasis. In agreement with this hypothesis, Zaccone et al. (24) recently reported an expansion of iNKT cells in nonobese diabetic mice following treatment with egg Ags and demonstrated that the Th2 response induced after immunization prevented the onset of type 1 diabetes in these mice. In the present study, we demonstrate that hepatic iNKT cells are phenotypically activated during the course of murine S. mansoni infection and that, rapidly after schistosome egg encounter in the liver, iNKT cells produce both IFN-γ and IL-4. We also present evidence that egg-sensitized DCs activate iNKT cells to secrete both IFN-γ and IL-4 in vitro and to promote a Th2 response in vivo. Activation of iNKT cells, in response to egg-sensitized DCs, does not require TLR-2 and TLR-3 expression on DCs, two TLR members recently described to be involved in DC maturation in response to parasite eggs. Finally, we suggest that iNKT cell activation in response to schistosome eggs is dependent on the presentation of self, rather than parasite-derived, CD1d-restricted ligands by DCs.

Materials and Methods

Animals

Six- to 8-wk-old female wild-type (WT) C57BL/6 mice were purchased from Janvier. The generation of CD1d-deficient (CD1d<sup>−/−</sup>) and Jol18<sup>−/−</sup> mice (backcrossed at least 10 times in C57BL/6) has been described already (25, 26). Mice that lack the Jol18 segment are devoid of iNKT cells, but the other lymphoid cell lineages are intact. Mice that lack CD1d are devoid of CD1d-restricted T cells, including iNKT cells. Both CD1d<sup>−/−</sup> and Jol18<sup>−/−</sup> mice were bred in our own facility in pathogen-free conditions. The generation of TLR2<sup>−/−</sup>, TLR3<sup>−/−</sup>, and MyD88-deficient C57BL/6 mice has been described earlier (27–30). Mice deficient in both TLR2 and TLR3 were generated at the Laboratoire de Généétique Expérimentale et Moléculaire. β Hexosaminidase B (Hexb)<sup>−/−</sup> (31) and IL-12p40<sup>−/−</sup> mice were provided by R. Roia (National Institutes of Health, Bethesda, MD) and The Jackson Laboratory, respectively.

Reagents

mAbs against mouse CD3ε (FITC conjugated), CD4 (PerCP-Cy5.5 or FITC conjugated), NK1.1 (PE, FITC, or biotin conjugated), CD69 (biotin conjugated), allophycocyanin-conjugated streptavidin, IFN-γ (PE conjugated), and IL-4 (PE conjugated) were purchased from BD Pharmingen. allophycocyanin-conjugated CD1d<sub>α-GC</sub> tetramer (termed tetramer in this work) was prepared from murine CD1d/murine β<sub>2</sub>-microglobulin expression vector (33). The purified anti-CD3 mAb was purchased from BD Biosciences. α-GC was obtained from Kirin Brewery.

Preparation of schistosome eggs

_S. mansoni_ (Puerto Rican strain) eggs were obtained from the liver of infected golden hamsters after portal vein perfusion. The absence of contaminating hamster tissue fragments in the egg preparation was checked by microscopic analysis. The absence of endotoxin in the parasite preparations (10<sup>5</sup> parasites/ml) was checked by a Limulus test (Sigma-Aldrich).

Preparation of schistosome egg lipids and analysis

Total lipids from _S. mansoni_ eggs were extracted by successive treatment with chloroform/methanol mixtures, followed by phase partitioning according to Folch (34). Constituents from the organic phase were separated onto a Kieselgel column (Merck) equilibrated in chloroform and eluted sequentially with 2 bed volumes of increasing methanol concentrations (from 5 to 100%) in chloroform. This allowed the isolation of different compounds as shown by Silicagel (Merck) TLC (35). The different fractions were analyzed as heptanonic acid derivatives of the constituents liberated using acid-catalyzed methanolation by gas chromatography/mass spectrometry (GC/MS) in the electron impact mode of ionization on a Carlo Erba GC 8000 gas chromatograph coupled to a Finnigan Automa II mass spectrometer (36, 37). These procedures allowed us to identify fractions containing neutral lipids, phospholipids, and GSLs with low and higher monosaccharide contents. Fractions containing GSLs with low monosaccharide content were further analyzed using nuclear magnetic resonance (NMR) spectroscopy using Bruker AMX 400NB spectrometer 9.4T, 400MHz, and its pulse program bank (Centre Commun de Mesures NMR). Chemical shifts were referenced relative to the residual methyl peak at 3.31 ppm for <sup>1</sup>H at 300 K. For NMR analysis, dried glycolipids were dissolved in 450 μl of a mixture of 2:1, v/v of d<sub>4</sub>-methanol and d<sub>6</sub>-chloroform. For biological activity assays, dried lipid pellets were dissolved in a 0.5% polysorbate-20 (Sigma-Aldrich), 0.9% NaCl solution at a concentration of 1 mg/ml (as determined by GC/MS), sonicated for 15 min in a water bath, and diluted in PBS just before use.

Analysis of iNKT cell activation

Mice were infected with 50 _S. mansoni_ cercariae or were injected with 15 × 10<sup>4</sup> freshly isolated _S. mansoni_ eggs in 200 μl of PBS or PBS alone into the caecal vein. Perfused livers were harvested at different time points postinfection or postinjection and homogenized using a 90-μm porosity filter. After extensive washes, liver homogenates were resuspended in a 33% Percoll gradient, and, after centrifugation, the cells in the pellet were recovered. RBC were removed by lysis in 155 mM NH<sub>4</sub>Cl (pH 7.4) containing 10 mM NaHCO<sub>3</sub> and 0.1 mM EDTA. Cell suspensions were stained either with anti-CD3ε, anti-NK1.1, and anti-CD69 mAbs, or with anti-CD3ε and CD1d<sub>α-DC</sub> tetramer. Briefly, cells were incubated for 30 min with the appropriate dilutions of FITC- or PE-conjugated mAbs, allophycocyanin-conjugated CD1d<sub>α-DC</sub> tetramer, or biotin-conjugated mAb, followed by incubating with allopurinol-conjugated streptavidin in PBS containing 2% FCS and 0.01% NaN<sub>3</sub>. The presence of cytoxins was assessed by intracellular staining. Briefly, cells were fixed in PBS 2% paraformaldehyde for 10 min, then resuspended in PBS plus 2% FCS and 0.1% saponin (permeabilization buffer) and incubated with PE-conjugated mAb against mouse CD1d<sub>α</sub> or against anti-iNKT mAb, or with permeabilization buffer. Cells were acquired and analyzed on a FACS Calibur (BD Biosciences) cytometer using the CellQuest software.

RNA extraction, cDNA synthesis, and real-time PCR

Total RNA from perfused livers was extracted by guanidine isothiocyanate and was isolated by ultracentrifugation over a cesium chloride cushion (36). The different fractions were separated according to Folch (34). Constituents from the organic phase were separated according to Folch (34). Constituents from the organic phase were separated according to Folch (34). Constituents from the organic phase were separated according to Folch (34). Constituents from the organic phase were separated according to Folch (34).

DCs/Liver mononuclear cell coculture system

DCs were generated from the bone marrow of mice, as described previously (38). DCs were sensitized with live eggs (1:200 cells), α-GC (100 μg/ml), or egg-derived lipid fractions (10 μg/ml) for 12 h, extensively washed (and, in some cases, filtered to remove remaining parasite eggs), and cultured in the presence of liver mononuclear cells (LMCs) at a ratio of 1:5 (10<sup>5</sup> DCs + 5 × 10<sup>5</sup> LMCs/well) in round-bottom 96-well plates in
RPMI 1640 supplemented with 5% FCS. In some cases, liver CD4+ NK1.1+ and CD4+ NK1.1- cells were sorted using a FACSVantage (BD Pharmingen), and purified cells were cocultured with egg-sensitized DCs (10^8 DCs + 2.5 x 10^4 CD4+ NK1.1+ or CD4+ NK1.1- cells/well). Ninety-nine percent of sorted CD4+ NK1.1+ cells were tetramer positive after reanalysis (data not shown). After 48 h, IFN-γ, IL-4, IL-5, and IL-10 production was measured in the culture supernatants by ELISA (BD Pharmingen and R&D Systems).

Immunization protocol and analysis of the immune response

Egg-sensitized or nonsensitized DCs (1.5 x 10^6/animal) were injected i.v. into recipient mice. Seven days later, spleen cells (5 x 10^6 cells/well in flat-bottom 96-well plate) were stimulated with anti-CD3 mAb (5 μg/ml) for 2 days at 37°C. During the last 18 h, 0.5 Ci of [3H]thymidine/well was added. IFN-γ, IL-4, IL-13, IL-5, and IL-10 production was measured in the culture supernatants by ELISA.

Statistical analysis

Results are expressed as the mean ± SD. The statistical significance of differences between means was calculated using Student’s t test. A value of p < 0.05 was considered significant.

Results

iNKT cells become activated during S. mansoni infection

In mice, in vivo activation of iNKT cells by the canonical ligand α-GC leads to a rapid de novo synthesis of Th1 and Th2 cytokines and to dramatic changes in the expression of cell surface receptors (2). In concert with an enhanced CD69 up-regulation, iNKT cells down-regulate the expression of the Vα14 receptor and NK1.1 (B6 background), thus leading to an apparent decreased frequency of iNKT cells, as judged by FACS staining (39, 40).

We first investigated whether, during murine schistosomiasis, iNKT cells in the liver (the main site of egg deposition) exhibit an activated phenotype. Because almost all hepatic iNKT cells from C57BL/6 mice coexpress CD3 and NK1.1 (41), we first determined, in a kinetic manner, the frequency of CD3+ NK1.1+ cells in the liver of S. mansoni-infected mice. As depicted in Fig. 1A, upper panel, the percentage of detectable hepatic CD3+ NK1.1+ cells slightly increased, although not significantly, at wk 2 postinfection to dramatically diminish and stabilize between the third and the eighth week of infection, a time at which the experiment was stopped. In contrast, the absolute number of detectable hepatic CD3+ NK1.1+ cells increased during infection, with a maximal elevation at wk 3 postinfection (~3-fold) (Fig. 1A, lower panel). Importantly, the reduced proportion of CD3+ NK1.1+ cells was confirmed by using anti-CD3 Ab plus CD1d/α-GC tetramer (tetramer), a probe that exclusively stains iNKT cells (Fig. 1B, shown is at wk 7 postinfection). Next, we monitored the expression of CD69, an early activation marker of iNKT cells. We found that from wk 2–8 (Fig. 1C), the level of CD69 expression on liver iNKT cells was significantly enhanced. In contrast, no cytokine within liver iNKT cells was observed during infection, as judged by intracellular FACS staining (data not shown).

Taken together, these results show that, during the course of murine schistosomiasis, the iNKT cell population in the liver exhibits an activated phenotype as early as 2–3 wk postinfection.

iNKT cells are activated following S. mansoni egg encounter in vivo

Although iNKT cell activation during infection precedes with egg production by matured worms (wk 5), we postulated, in light of our recent observations (23), that the egg stage of the parasite may activate iNKT cells in the liver. During S. mansoni infection, egg production is asynchronous, and this may explain the lack of cytokine detection in iNKT cells in our kinetic study.

Therefore, we developed a more synchronous model of egg deposition in the liver by injecting freshly isolated live eggs (or vehicle) into the coecal vein of naive mice; afterward, the phenotypic activation of hepatic iNKT cells was investigated by...
flow cytometry. As depicted in Fig. 2A, 24 and 48 h (data not shown) after injection, the percentage of detectable CD3⁺ NK1.1⁺ cells decreased by 30% in the liver of egg-injected mice, compared with controls. This decreased frequency was accompanied by an up-regulation of CD69 expression on most of the iNKT cells 24 h (Fig. 2A, right panel) and 48 h (data not shown) postinjection. In contrast, no CD69 up-regulation was observed in CD4⁺ NK1.1⁺ cells (NK cells) nor in CD4⁺ NK1.1⁻ (conventional T cells) (data not shown). These data show that liver iNKT cells are activated following parasite egg injection.

To substantiate the above findings, we examined whether iNKT cells produce cytokines following egg encounter in the liver. First, real-time RT-PCR analysis of liver mRNAs showed an increase in IFN-γ 24 h (~5-fold), but not 48 h, after egg injection (Fig. 2B). In sharp contrast, compared with vehicle-injected mice, the levels of IL-4, IL-5, and IL-10 mRNAs in the liver of egg-injected mice were unchanged at these time points (data not shown). Interestingly, the enhanced IFN-γ mRNA level was not observed in the liver from egg-injected Jα18⁻/⁻ mice (Fig. 2B), suggesting that iNKT cells are mandatory for IFN-γ synthesis by liver cells. To investigate whether iNKT cells could be a source of cytokine production in this setting, the kinetics of cytokine synthesis by iNKT cells was determined by intracellular FACS analysis. As shown in Fig. 2C, 24 h, and to a lesser extent 48 h, after egg injection, there was an increased number of NK1.1⁺ tetramer⁺ cells stained positively for IFN-γ. Interestingly, we also detected an increased number of NK1.1⁺ tetramer⁺ cells positive for IL-4, particularly 48 h after egg injection. As shown in Fig. 2C, both CD4⁺ and CD4⁻ iNKT cell subsets stained positively for IFN-γ and IL-4. In contrast, the number of liver NK1.1⁺ tetramer⁺ cells positive for IL-5 and IL-10 was not significantly modified in egg-injected WT mice (data not shown). Of note, the number of CD4⁺ NK1.1⁺ cells (NK cells) as well as of CD4⁺ NK1.1⁻ (conventional T cells) positive for IFN-γ or IL-4 was not changed in egg-injected mice (data not shown).
Egg-sensitized DCs activate iNKT cells via CD1d

Previous data suggested that DCs have a strong ability to present CD1d-restricted glycolipids to iNKT cells, thus leading to primary iNKT cell response (16). Thus, we assessed whether eggs could activate iNKT cells via DCs in vitro and whether it occurs through a CD1d-dependent mode of Ag presentation. To this end, WT or CD1d<sup>−/−</sup> DCs were sensitized for 12 h with parasite eggs and, after extensive washing, were cocultured with LMCs collected from WT mice. After 48 h, cytokine concentration in culture supernatants was quantified by ELISA. As shown in Fig. 3A, egg-sensitized WT DCs induced IFN-γ, IL-4, and IL-5 (but not IL-10; data not shown) production by LMCs. In contrast, egg-sensitized CD1d<sup>−/−</sup> DCs had a strongly reduced ability to promote IFN-γ (85% reduction) and IL-4 (total abrogation) production by liver cells, whereas IL-5 production was unaffected.

We next evaluated the contribution of CD1d-restricted liver cells, including iNKT cells, in this phenomenon. As represented in Fig. 3B, the production of IFN-γ was strongly impeded when egg-sensitized WT DCs were cocultured with LMCs prepared from either Jα<sub>18</sub>−/− (60% reduction) or CD1d<sup>−/−</sup> (50% reduction) mice, whereas no IL-4 production was detected with liver cells from either Jα<sub>18</sub>−/− or CD1d<sup>−/−</sup> mice. Of note, in response to egg-sensitized WT DCs, an enhanced IL-5 secretion by liver cells lacking CD1d-restricted cells, including iNKT cells, was observed. Thus, in this model, iNKT cells are fully responsible for the production of IL-4 by liver cells, whereas they are only in part involved in IFN-γ and totally dispensable for IL-5 synthesis.

To investigate whether iNKT cells could be a source of cytokine release, egg-sensitized DCs were cocultured with purified CD4<sup>+</sup>NK1.1<sup>+</sup> hepatic cells. As represented in Fig. 3C, egg-sensitized DCs induced substantial amounts of both IFN-γ and IL-4, but not IL-5 nor IL-10 (data not shown). In contrast, non-iNKT liver cells (CD4<sup>+</sup>NK1.1<sup>+</sup> cells) failed to produce cytokines.

Collectively, these data strongly suggest that in response to parasite eggs, DCs instigate a process capable of activating, via CD1d, liver iNKT cells to produce in vitro both IFN-γ and IL-4.

iNKT cells from mice immunized with egg-sensitized DCs are important to mount Th2 responses in vivo

It is now established that cytokine production by in vivo activated iNKT cells can provide help to naive T cells during their priming in lymphoid organs, and can therefore modulate the nature and/or the intensity of the adaptive immune responses that occur at latter time points (2). Therefore, having established that egg-sensitized DCs activate iNKT cells in vitro to produce immunoregulatory cytokines, we aimed to determine the in vivo contribution of iNKT cells in the promotion and/or the polarization of conventional T cells following immunization with egg-sensitized DCs. First, we evaluated the role of CD1d, expressed by DCs, in these settings. For this purpose, WT or CD1d<sup>−/−</sup> DCs were sensitized with live eggs and then injected i.v. into WT mice. Seven days later, the acquired immune response was studied by restimulating spleen cells with anti-CD3 mAb. As represented in Fig. 4A, stimulation of spleen cells from WT mice previously injected with unpulsed WT or CD1d<sup>−/−</sup> DCs, with anti-CD3, resulted in a moderate cellular proliferation and IFN-γ secretion. In contrast, upon CD3 restimulation, spleen cells from WT mice immunized with egg-pulsed DCs (WT or CD1d<sup>−/−</sup>) proliferate vigorously and produce high amounts of both IFN-γ and Th2-type cytokines. Of note, intracellular FACS staining indicated that CD4<sup>+</sup>tetramer<sup>−</sup> cells were positive for IFN-γ and IL-4, whereas no labeling was detected in CD4<sup>+</sup>tetramer<sup>+</sup> cells (data not shown). This indicates that, in this setting, immunization of mice with egg-pulsed DCs induces the production of cytokines by conventional T cells, but not by iNKT cells. Interestingly, cytokine released into the supernatants of these cultures differed dramatically because CD1d<sup>−/−</sup> egg-pulsed DCs induced the activation of cells that secrete IFN-γ, but little IL-4, IL-13, IL-5, and IL-10 (83, 65, 86, and 45% reduction compared with WT egg-pulsed DCs, respectively) (Fig. 4A). In agreement with our previous finding in BALB/c (23), these results suggest that, in the C57BL/6 system, and upon schistosome egg/DC contact, the CD1d mode of Ag presentation is crucial in the priming of Th2 lymphocytes.

To confirm the above finding, WT egg-pulsed DCs were transferred into WT, CD1d<sup>−/−</sup>, or Jα<sub>18</sub>−/− mice. As seen in Fig. 4B, whatever the mouse strain used to generate spleen cells, injection of unpulsed DCs had a similar effect on cellular proliferation and IFN-γ production, upon CD3 stimulation. Of note, although the transfer of egg-sensitized DCs into CD1d<sup>−/−</sup> mice resulted in comparable T cell priming, relative to that induced in WT recipient mice, the transfer of egg-sensitized DCs into Jα<sub>18</sub>−/− mice resulted in decreased proliferation, although not statistically significant. In contrast, the levels of released cytokines were dramatically different. Thus, although the production of IFN-γ was unaffected, spleen cells from CD1d<sup>−/−</sup> or Jα<sub>18</sub>−/− mice produce diminished levels of IL-4, IL-13, IL-5, and IL-10, compared with WT animals (50–62, 62–35, 70–65, and 65–58% reduction, respectively).
These results clearly show that, in this model of immunization, iNKT cells from immunized mice contribute to the promotion of the Th2-biased immune response triggered by egg-sensitized DCs.

**iNKT cell activation does not require TLR engagement in DCs in response to parasite eggs**

We next sought to determine how iNKT cells become activated in response to egg-sensitized DCs. An increasing body of evidence suggests that DC/pathogen interactions lead to a DC activation/maturation process that may culminate in iNKT cell activation (11, 21). In this process, IL-12 production by mature DCs strongly cooperates with the CD1d/TCR pathway to activate iNKT cells (21). Activation of TLRs by pathogens is the main pathway by which DCs become activated (42–44). We have shown recently that schistosome eggs activate DCs in vitro to mature and to produce immunostimulatory factors, including IL-12, via TLR2 and TLR3 (38, 45). To check the potential involvement of these TLRs, WT, TLR2, TLR3, or double TLR2/TLR3-deficient DCs were sensitized with live eggs, and their ability to activate LMCs in vitro was then assessed. Furthermore, the role of MyD88, a crucial adapter protein involved in TLR activation (except TLR3) (46), was also investigated. To demonstrate that these deficiencies did not alter the ability of DCs to present CD1d-restricted ligands, the glycolipid α-GC was used as a positive control. Irrespective of the mouse strain used to generate DCs, incubation of DCs with α-GC resulted in a comparable release of both IFN-γ and IL-4 by LMCs (Fig. 5A). Similarly, compared with egg-sensitized WT DCs, TLR2, TLR3, double TLR2/TLR3, or MyD88 deficiencies had no effect on liver cell stimulation.

To confirm this finding, IL-12 deficient DCs were used to stimulate LMCs. In agreement with a recent report (21), IL-12 deficiency did not modify the ability of α-GC-pulsed DCs to activate LMCs (Fig. 5B). Similarly, the production of IL-12 by egg-sensitized DCs was not mandatory to activate liver cells to produce IFN-γ or IL-4.

These data collectively suggest that, in response to schistosome eggs, TLR engagement in DCs is dispensable for iNKT cell activation.

**iNKT cell activation requires self, rather than parasite-derived, CD1d-restricted ligands**

We next sought to determine whether iNKT activation by egg-pulsed DCs requires self and/or parasite-derived/CD1d complexes. To this end, total lipids from schistosome eggs were extracted by the Folch procedure, and fractionated compounds of the organic phase were tested for their ability to activate LMCs in our experimental setting. Analysis of the fractions by GC/MS (data not shown) and by TLC (Fig. 6A, upper panel) indicated the presence of two fractions containing neutral lipids (essentially cholesterol and triglycerides) (fractions 5 and 7.5), three fractions migrating as monohexosylceramides (fractions 12.5, 15, and 17.5) (for detailed composition, Fig. 7), several fractions migrating as polyhexosylceramides (fractions 30, 35, 40, 45, 50, 80, 90, and 100), and two fractions containing phospholipids (fractions 60 and 70). As seen in Fig. 6A, bottom panel, total lipids from schistosome eggs failed to induce IFN-γ or IL-4 (data not shown) production by liver cells. Similarly, when tested individually (or in some case as pools), fractions obtained from total lipids were also devoid of activating properties in this setting. This suggests that schistosome eggs do not appear to express (glyco)lipids capable of activating iNKT cells via DCs.

Recently, Mattner et al. (11) reported that, in response to some pathogens that are devoid of CD1d-restricted ligands, DCs deficient in hexB fail to activate iNKT cells, a phenomenon ascribed to a defect in catabolizing isoglobotetrahexosylceramide to iGb3 in the lysosome. In marked contrast, pathogen-expressing CD1d-restricted ligands maintain their ability to activate iNKT cells, via hexB<sup>−/−</sup> DCs. Thus, after checking that hexB<sup>−/−</sup> DCs do not display any phenotypic alteration and mature normally, relative to

**FIGURE 4. iNKT cells from immunized mice are important in the Th2 response elicited by egg-sensitized DCs. A, Unpulsed or egg-pulsed WT or CD1d<sup>−/−</sup> DCs were injected i.v. into WT mice. B, Unpulsed or egg-pulsed WT DCs were injected into WT, 3a18<sup>−/−</sup>, or CD1d<sup>−/−</sup> mice.**

Seven days after injection, spleens were removed and cells were restimulated with plate-bound anti-CD3 mAb. Cytokine production and proliferation were measured after 2 days and after 4 days of culture, respectively. IFN-γ, IL-4, IL-13, IL-5, and IL-10 concentrations in the supernatants were assayed by ELISA. Results represent the mean of triplicate cultures ± SD (n = 6). *p < 0.01 (compared with cytokine production by WT splenic cells injected with egg-sensitized WT DCs). One representative experiment of three is shown (same experiment for A and B).
During infection, we observed a sustained decreased proportion of hepatic (and splenic; T. Mallevaey, manuscript in preparation) iNKT cell population within mononuclear cells, starting 3 wk after infection. This result may be explained by a preferential recruitment of iNKT cell population within mononuclear cells, starting 3 wk after hepatic (and splenic; T. Mallevaey, manuscript in preparation) derived, CD1d-restricted ligands.

iNKT cells to produce immunoregulatory cytokines in vitro, enabling them to bias in vivo the immune response toward a Th2 profile. Finally, we show that the egg stage of the parasite instigates an activating, TLR-independent pathway in DCs that culminates in iNKT cell activation, probably through self, not parasite-derived, CD1d-restricted ligands.

During infection, we observed a sustained decreased proportion of hepatic (and splenic; T. Mallevaey, manuscript in preparation) iNKT cell population within mononuclear cells, starting 3 wk after infection. This result may be explained by a preferential recruit-
cytokine synthesis in iNKT cells is transient, at least after α-GC administration (33, 47). Therefore, the asynchronous egg deposition in the liver during infection may explain the above finding.

To circumvent this, we used a more synchronous model of activation by transferring live eggs into the coecal vein of mice. The percentage of liver iNKT cells decreased at 24 and 48 h postadministration (with an average of 30%). Of note, this was not due to differences in the potency of the stimuli used to activate iNKT cells. Also, considering that iNKT cells only transiently produce cytokines, this result may be explained by the fact that iNKT cell activation following intracoecal injection of eggs is not a phenomenon as synchronous as following i.v. injection of α-GC or live bacteria. Whether or not the early cytokine production by iNKT cells also occurs during infection and whether it can influence the ensuing immune response are still open questions that necessitate further studies. Recent evidence suggests that early activation of iNKT cells in the liver is important in the development of the acquired immune response (48) and that the immediate cytokine responses of iNKT cells are difficult to polarize (33, 49). Therefore, the fact that, after egg encounter, iNKT cells immediately produce both IL-4 and IFN-γ is not reminiscent with their potential role in the polarization of the acquired immune response toward a Th2 direction.

Having established that iNKT cells are activated in vivo upon egg encounter, we next determined the contribution of DCs in iNKT cell stimulation in vitro. LMCs incubated with egg-sensitized DCs produced, in a CD1d-dependent manner, substantial amounts of both IFN-γ and IL-4. Of note, in this system, LMCs failed to produce IL-5 and IL-10, in contrast to the sum of fatty acid methyl-esters (FAMEs) or as percentage of long-chain bases (LCBs). phyt, phytosphingosine; sphe, sphingosine; sphe6oh, 6-hydroxysphingenine.

To circumvent this, we used a more synchronous model of activation by transferring live eggs into the coecal vein of mice. The percentage of liver iNKT cells decreased at 24 and 48 h postadministration (with an average of 30%). Of note, this was not due to an increased number of non-iNKT cells in the liver (data not shown), thus suggesting that the apparent decreased frequency of iNKT cells is due to TCR and NK1.1 down-regulation. Furthermore, the remaining detectable iNKT cells up-regulate CD69. More importantly, FACS analysis revealed in vivo IFN-γ protein accumulation in iNKT cells 24 and 48 h after egg injection, a finding in agreement with the quantitative RT-PCR analysis (Fig. 2B). Although no increased IL-4 transcript was detected in the liver of egg-injected mice compared with controls, a result that may be explained by the relatively high constitutive expression of IL-4 transcript in iNKT cells (33, 47), the percentage of IL-4-positive liver iNKT cells was enhanced 24 h, and particularly 48 h, after egg injection. In contrast, no increase of IL-5 and IL-10 protein synthesis within iNKT cells could be detected (data not shown). Interestingly, stimulation of iNKT cells does not appear to cause downstream activation of NK cells (CD69 induction and IFN-γ production), a phenomenon known to occur after α-GC administration (13). This suggests that, in this model, secondary signals necessary to stimulate NK cells, such as those provided by DC-derived IL-12 production or iNKT cell-derived IFN-γ, are not sufficient. Of note, the percentages of iNKT cells producing IFN-γ or IL-4 are relatively low (~2%), even at earlier time points postinjection (data not shown), compared with those induced by the potent agonist α-GC (generally >50%; our observation and Refs. 4 and 8) or by live bacteria (10–30% for IFN-γ; 2% for IL-4) (21). This may be due to differences in the potency of the stimuli used to activate iNKT cells. Also, considering that iNKT cells only transiently produce cytokines, this result may be explained by the fact that iNKT cell activation following intracoecal injection of eggs is not a phenomenon as synchronous as following i.v. injection of α-GC or live bacteria. Whether or not the early cytokine production by iNKT cells also occurs during infection and whether it can influence the ensuing immune response are still open questions that necessitate further studies. Recent evidence suggests that early activation of iNKT cells in the liver is important in the development of the acquired immune response (48) and that the immediate cytokine responses of iNKT cells are difficult to polarize (33, 49). Therefore, the fact that, after egg encounter, iNKT cells immediately produce both IL-4 and IFN-γ is not reminiscent with their potential role in the polarization of the acquired immune response toward a Th2 direction.

Having established that iNKT cells are activated in vivo upon egg encounter, we next determined the contribution of DCs in iNKT cell stimulation in vitro. LMCs incubated with egg-sensitized DCs produced, in a CD1d-dependent manner, substantial amounts of both IFN-γ and IL-4. Of note, in this system, LMCs failed to produce IL-10, an important regulatory cytokine involved in the control of the immune response during schistosomiasis (22, 50). Interestingly, liver cells from CD1d−/− and Jre18−/− mice failed to produce IL-4, but maintained their ability to synthesize IFN-γ (~50% reduction compared with WT littermates). This indicates that the contribution of iNKT cells in IL-4 production by LMCs is total, whereas that in IFN-γ production is partial. The cellular source(s) for IFN-γ synthesis is under investigation. Of note, although IL-5 was detected in this model of activation, CD1d-dependent cells (including iNKT cells) were dispensable for, and even inhibited, its production. Of importance, the use of sorted liver cells indicates that, in this setting, iNKT cells can produce both IL-4 and IFN-γ (Fig. 3C), but not IL-5 nor IL-10, in response to egg-sensitized DCs. In marked contrast, schistosomula- or adult worm-sensitized DCs were unable to activate liver cells, including iNKT cells, to produce cytokines (T. Mallevaey, manuscript in preparation). Thus, schistosome eggs selectively activate iNKT cells in vitro to produce immunoregulatory cytokines, via DCs.

In response to some microbial pathogens, TLR activation in DCs appears to be important in iNKT cell stimulation. In this mechanism, IL-12 production by DCs cooperates with signaling
pathways triggered by self Ag/CD1d complexes to activate iNKT cells both in vitro and in vivo (11, 21). Thus, as a next step, we investigated whether LMC activation, in response to egg-sensitized DCs, requires the recruitment of TLR2 and/or TLR3, two TLR members that participate in DC activation in response to schistosome eggs (38, 51). In our experimental systems, TLR2 and/or TLR3 (as well as MyD88) deficiencies had no impact on LMC activation (Fig. 5). Moreover, DCs lacking IL-12p40 (and thus IL-12 and IL-23) still promoted LMC activation. In aggregate, these data argue that, in response to schistosome eggs, activating pathways triggered in DCs by both TLR2 and TLR3 are dispensable for iNKT cell activation. The mechanisms by which eggs induce in DCs a TLR-independent pathway(s) that culminates in iNKT cell activation are presently unclear. It is likely that other pattern recognition receptors, which function in a MyD88-independent manner, are involved.

Next, we investigated whether self and/or parasite-derived CD1d-restricted ligands are involved in iNKT cell activation. We first hypothesized that putative CD1d-restricted ligands may exist in schistosome eggs. Indeed, recent evidence suggests that GSLs with α-linked sugars related to α-GC are more widely distributed than previously thought (10, 11). Analysis of the monohexosylceramide-containing fractions, however, revealed that they only contain β-glucosylceramide (Fig. 7). Similarly, in the different parasite lipid fractions, no β-anomeric GSL was detected. Although these types of compounds are generally good iNKT cell activators (4), their apparent absence in Schistosoma did not firmly preclude the presence of active CD1d-restricted ligands in this parasite. However, within the detection limit of our assay, our data suggest that parasite eggs are probably devoid of such activating compounds. This inferred that self ligands might be involved in iNKT cell activation in this experimental setting. Recently, Mattner et al. (11) showed that DCs deficient in hexB, a lysosomal enzyme involved in the catabolism of several GSL substrates, including potentially isoglobotetrahexosylceramide to iGb3, fail to activate iNKT cells, except if exogenous CD1d-restricted ligands are provided to DCs. In our setting, upon egg contact, hexB−/− DCs had a markedly diminished capacity to stimulate LMCs, a result that supports the hypothesis that iGb3 and/or related products are implicated in their activation. Whether the CD1d/TLR pathway is sufficient to trigger iNKT cell stimulation or whether it requires additional costulatory factors is under investigation.

Irrespective of the mechanisms of iNKT cell activation, we investigated whether iNKT cells could modulate the nature of the adaptive immune response triggered by egg-sensitized DCs (used in this study as a vector of immunization). Our data show that parasite eggs from immunized mice provide help for the induction of Th2 responses, at least in this model of DC transfer experiments. This is in agreement with our previous report suggesting that CD1d-restricted cells are important in the Th2 response in this experimental setting, as well as during infection (23). Although likely, whether this effect is due to in vivo cytokine production by iNKT cells is still unknown. As previously discussed, the use of Jα18−/− mice should allow us to more fully decipher the role of iNKT cells in the outcome of the immune response during infection. In a more general manner, because our data suggest that DC/ schistosome interactions play a role in iNKT cell functions, it seems important in the future to investigate the influence of this cell population (as well as of other CD1d-restricted cell populations) in the outcome of both innate and adaptive immune responses not only during schistosomiasis, but also during other helminthic infections.

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Disclosures
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