Distinct Roles of Dendritic Cells and B Cells in Va14Ja18 Natural T Cell Activation In Vivo

Jelena S. Bezbradica, Aleksandar K. Stanic, Naoto Matsuki, Helene Bour-Jordan, Jeffrey A. Bluestone, James W. Thomas, Derya Unutmaz, Luc Van Kaer and Sebastian Joyce

http://www.jimmunol.org/content/174/8/4696

References
This article cites 64 articles, 39 of which you can access for free at:
http://www.jimmunol.org/content/174/8/4696.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Distinct Roles of Dendritic Cells and B Cells in Va14Ja18 Natural T Cell Activation In Vivo

Jelena S. Bezbradica,* Aleksandar K. Stanić,* Naoto Matsuki,* Helene Bour-Jordan,‡ Jeffrey A. Bluestone,‡ James W. Thomas,‡ Derya Unutmaz,* Luc Van Kaer,* and Sebastian Joyce2*

Va14Ja18 natural T (iNKT) cells are innate, immunoregulatory lymphocytes that recognize CD1d-restricted lipid Ags such as α-galactosylceramide (αGalCer). The immunoregulatory functions of iNKT cells are dependent upon either IFN-γ or IL-4 production by these cells. We hypothesized that αGalCer presentation by different CD1d-positive cell types elicits distinct iNKT cell functions. In this study we report that dendritic cells (DC) play a critical role in αGalCer-mediated activation of iNKT cells and subsequent transactivation of NK cells. Remarkably, B lymphocytes suppress DC-mediated iNKT and NK cell activation. Nevertheless, αGalCer presentation by B cells elicits low IL-4 responses from iNKT cells. This finding is particularly interesting because we demonstrate that NOD DC are defective in eliciting iNKT cell function, but their B cells preferentially activate this T cell subset to secrete low levels of IL-4. Thus, the differential immune outcome based on the type of APC that displays glycolipid Ags in vivo has implications for the design of therapies that harness the immunoregulatory functions of iNKT cells. The Journal of Immunology, 2005, 174: 4696–4705.

The innate immune system is central to maintaining the integrity of an organism constantly challenged by pathogens. Communications between key cellular components by direct cell-to-cell contact and through soluble mediators initiate and regulate the innate immune response. Va14Ja18 natural T (iNKT) cells are innate lymphocytes (1) that have immunoregulatory properties (2, 3). When activated in vivo, they prevent autoimmune diseases, maintain immune privilege, and support engraftment of transplanted tissues (2, 3). Furthermore, iNKT cells mediate adjuvant activities and consequently enhance tumor immunity and immune responses to pathogens (4). Paradoxically, prevention of autoimmune diseases requires IL-4, whereas immunity to tumors and pathogens requires IFN-γ. How the in vivo activation of iNKT cells leads to differential immune outcomes remains to be established.

The iNKT cells express an invariant Va14Ja18 TCR α-chain predominantly paired with a Vβ8.2 β-chain.Remarkably, in vivo iNKT cell activation leads to rapid and robust IL-4 response and a spectrum of Th1 and Th2 cytokines that mediate the immunoregulatory role of iNKT cells. Current evidence suggests that iNKT cells recognize self (5–7) as well as foreign (8) lipid Ags presented by CD1d molecules. Of the several cellular lipids that activate iNKT cells in vitro, only a few do so in vivo (9–12). α-Galactosylceramide (αGalCer), a marine sponge-derived glycolipid recognized for its potent antitumor activity in vivo (13, 14), has been used extensively to probe the physiological role of iNKT cells (15–20). Although αGalCer-mediated and physiological activation of iNKT cells might differ (7), αGalCer is currently being tested in the clinic to enhance tumor rejection (21–23). Thus, in vivo administration of αGalCer either i.v. or i.p. leads to specific presentation of the glycolipid by CD1d and the rapid elicitation of immunoregulatory cytokines by iNKT cells.

CD1d is expressed by CD4+8+ thymocytes, hepatocytes, B lymphocytes, macrophages, and dendritic cells (DC) (24–27). Therefore, each of these cell types has the potential to present αGalCer to iNKT cells in vivo. Steinman et al. (18) have shown that αGalCer-pulsed DC, upon adoptive transfer into naive mice, result in selective and sustained activation of iNKT cells to produce IFN-γ. This activity is not conferred by non-DC leukocytes pulsed with αGalCer. Nevertheless, both αGalCer-pulsed DC and non-DC leukocytes induce IL-4 from iNKT cells (18). Curiously, prior exposure to free αGalCer or αGalCer-pulsed non-DC leukocytes rendered iNKT cells unresponsive to subsequent challenge with αGalCer-pulsed DC (18). Most interestingly, activated iNKT cells stimulate DC maturation, and the sustained IFN-γ production results in the rejection of tumor cells in vivo (19, 20, 28).

DC-induced iNKT cell activation is also critical for initiating bacterial immunity. For example, using Salmonella typhimurium as the model pathogen, Brenner et al. (7) demonstrated that a bacterial product(s) activates myeloid DC. The DC so activated secrete IL-12, which then enhances the low levels of activation of iNKT cells induced by DC-iNKT cell interaction. These processes were observed using human and mouse cells in vitro as well as in the mouse system in vivo (7).

The reports described above underscore the importance of DC in iNKT cell activation in vivo. Nevertheless, whether DC are the sole mediators of iNKT cell activation in vivo and what roles, if any, CD1d-positive, non-DC leukocyte types such as macrophages

*Department of Microbiology and Immunology and †Division of Rheumatology, Department of Medicine, Vanderbilt University School of Medicine, Nashville, TN 37232; and ‡Diabetes Center, University of California, San Francisco, CA 94143

Received for publication October 29, 2004. Accepted for publication January 31, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by Grants AI050834 (to J.A.B.); AI049131 (to D.U.); AI050593, NS044044, and HL062744 (to L.V.K.); HL069542 (to J.J.H.); and AI042284 (to S.J.) from the National Institutes of Health, as well as Juvenile Diabetes Research Foundation and Human Frontiers in Science Program grants (to S.J.).

2 Address correspondence and reprint requests to Dr. Sebastian Joyce, Department of Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, TN 37232. E-mail address: sebastian.joyce@vanderbilt.edu

3 Abbreviations used in this paper: iNKT, Va14Ja18 natural T; DC, dendritic cell; DT, diphtheria toxin; αGalCer, α-galactosylceramide; hDTR, human DT receptor; tg, transgenic; TID, type I diabetes.
and marginal zone B lymphocytes, which express high levels of CD1d, play in this process remain to be established. Our data indicate that DC enhance glycolipid Ag-induced activation of iNKT cells and the production of Th1 and Th2 cytokines, whereas B cells poorly activate iNKT cells to produce only Th2 cytokines. Additionally, B cells appear to have a suppressive role in DC-mediated iNKT cell activation. Surprisingly, macrophages and hepatocytes do not appear to play a significant role in αGalCer-induced iNKT cell activation. These findings may be exploited for the design of immunotherapies that selectively elicit certain immunoregulatory functions of iNKT cells.

Materials and Methods

Mice

C57BL/6, B6.129-μMT (29), NOD, and NOD.129-Mtf (30) mice were purchased from The Jackson Laboratory. B6.129-CD1d<sup>+</sup> mice have been described previously (31), and NOD × B6.CD80<sup>+</sup>B will be described elsewhere (H. Bour-Jordan and J. A. Bluestone, manuscript in preparation). FVB/N-human diphtheria toxin receptor transgenic (hDTR<sup>+</sup>) mice (32) were backcrossed for six-to-eight generations onto the C57BL/6 background, and, heterozygotes for the transgene were used in the studies described here. The hDTR<sup>+</sup>μMT mice were generated by crossing B6.FVB-hDTR<sup>+</sup> mice with B6.129-μMT mice, then breeding the F<sub>1</sub> progenies with B6.129-μMT mice. B6.129-H2Ab<sup>+</sup> mice have been described previously (33) and were a gift from C. Benoist and D. Mathis (Harvard Medical School, Boston, MA) to L. Van Kaer. All mice were bred and maintained in compliance with Vanderbilt’s institutional animal care and use committee regulations.

Abs and reagents

All Abs and reagents for ELISA and cell surface and intracellular staining were purchased from BD Pharmingen. Anti-F4/80-allophycocyanin (RM 2905) Ab was purchased from CalTag Laboratories. αGalCer was provided by Kirin Brewery. The preparation and use of CD1d-tetramer (CD1-tetramer) have been described previously (34).

Flow cytometry

Splenocytes of individual, age-matched (4- to 8-wk-old) mice treated with αGalCer or vehicle, as the control, were stained for four-color flow cytometric analysis using the following Abs: anti-B220-FITC, anti-CD8<sub>/H9251</sub>, anti-CD3, and a sandwich ELISA was performed as previously described (34). Intracellular cytokine staining

Splenocytes from mice treated with αGalCer or vehicle control were blocked with anti-CD16/CD32 (FcγRIII/IIIR). Cells were first stained for CD3e and DX5 (for NK cells) or with CD3e and CD1 tetramer (for iNKT cells), then for intracellular IFN-γ after fixing and permeabilizing with Cytofix/Cytoperm solution (BD Pharmingen) according to the manufacturer’s protocol. Flow cytometry and data analysis were performed as described above.

DC depletion

For DC depletion, mice were injected i.p with 4 ng of diphtheria toxin (DT; Sigma-Aldrich)/g body weight (~100 ng/mouse). Controls were injected with corresponding volume of PBS, which served as the vehicle to deliver DT.

Cell sorting and adoptive transfer

Cells were dispersed by collagenase D (Roche) treatment, washed, reacted with anti-CD11c-coated magnetic beads (Miltenyi Biotec), and separated using an autoMACS sorter (Miltenyi Biotec). Sorted DC were pulsed with αGalCer (0.1–1 μg/ml) in overnight culture. After extensive washes with PBS, 6 × 10<sup>5</sup> αGalCer-pulsed DC were adoptively transferred i.v. into hDTR<sup>+</sup> mice treated with DT — 20–24 h previously. Control mice received the same number of DC pulsed with PBS. For B cell transfer experiments, splenocytes were reacted with anti-B220-coated magnetic beads (Miltenyi Biotec) and separated using autoMACS sorter. Approximately 25–30 × 10<sup>6</sup> sorted B cells were transferred i.v. into recipient μMT or μMT:hDTR<sup>+</sup> mice 24 h (the time required for B cells to home to the spleen; Ref. 35) before αGalCer treatment. In the experiments with μMT:hDTR<sup>+</sup> recipients, DT was delivered i.p. at the time of B cell transfer or, in some experiments, 12 h before transfer. Both methods yielded similar results (data not shown).

In vitro stimulation assay

For in vitro iNKT cell activation studies, C57BL/6-derived DC, macrophages, and B cells were sorted using anti-CD11c-, anti-CD11b-, and anti-B220-coated magnetic beads, respectively. Sorted cells (>90% pure) were used as stimulators in the assay. B6.129-H2Ab<sup>+</sup>-derived iNKT cells were obtained by depletion of CD11c<sup>+</sup>, CD11b<sup>+</sup>, and B220<sup>+</sup> cells. The iNKT cell fraction, enriched ~20-fold, was stimulated with 50 ng/ml αGalCer in the presence of C57BL/6-derived DC (10:1 T cell to DC ratio), macrophages (10:1 T cell to macrophages ratio), or B cells (5:1 T to B cell ratio) for 3 days in triplicate. Culture supernatants were collected, and ELISA was performed as previously described (34).

Results

In the spleen, DC, B lymphocytes and macrophages make up the CD1d<sup>+</sup>positive cell types (25–27). Because iNKT cells appear to localize to the same sites where DC, B cells, and macrophages are known to reside in the spleen (36) (data not shown), we hypothesized that upon αGalCer administration, the different CD1d<sup>+</sup>positive cell types have the potential to present Ag and elicit distinct iNKT cell functions. Thus, we decided to systematically address the role of DC, B cells, and macrophages in the induction of iNKT cell immune response in vivo using three genetically altered mouse models: 1) B6.FVB-hDTR<sup>+</sup> (hDTR<sup>+</sup>) mice that can be conditionally rendered DC deficient within ~12 h of DT administration for ~48 h (32); 2) μMT mice, which are congenitally B cell deficient (29); and 3) hDTR<sup>+</sup>:μMT mice (this study), a hybrid cross between the previous two strains, that can be rendered DC deficient in addition to B cell deficiency.

The hDTR<sup>+</sup> mice, which lack a functional DTR, express human DTR-GFP under control of the murine CD11c enhancer promoter cassette, which restricts CD11c expression to DC (32). DT administration to hDTR<sup>+</sup> mice selectively depletes DC within 12 h and maintains a DC-deficient state until 36 h (32). DT was neither toxic to mice lacking the hDTR transgene (data not shown), nor did it affect macrophages (see below) or B and T lymphocytes in hDTR<sup>+</sup> and nontransgenic animals (Ref. 32; data not shown). Most importantly, DT treatment did not alter iNKT cell number in hDTR<sup>+</sup> mice (data not shown). Note that the described specificity of DT is achieved only if animals are maintained as hemizygotes for the transgene. When homozygotes were treated with DT, it was partially toxic for CD8<sup>+</sup> T cells (Ref. 32; our unpublished observations) and in some experiments for iNKT cells as well (our unpublished observations). Thus, hemizygous hDTR<sup>+</sup> mice are a tractable model to dissect the role of DC and other CD1d<sup>+</sup> cell types in Ag presentation to iNKT cells in vivo.

Efficient in vivo elicitation of iNKT cell functions requires DC

In vivo stimulation of iNKT cells with αGalCer results in rapid and robust cytokine response. Therefore, iNKT cells from C57BL/6 and hDTR<sup>+</sup> mice treated with either PBS or DT for 20–24 h were stimulated in vivo with αGalCer. Serum IL-2 and IL-4 were measured after 2 h, and IFN-γ was measured after 6 h of in vivo αGalCer stimulation. To analyze the results quantitatively, the amount of cytokine response secreted was plotted against splenic iNKT cell number. The data revealed consistently decreased (<50%)
IL-2 and IL-4 responses by mice depleted of CD11c+ DC compared with normal mice (Fig. 1A). Interestingly, in the absence of DC, the IFN-γ response was completely abolished (Fig. 1A). To confirm these results, CD1 tetramer-positive cells were stained for intracellular IL-2 and IFN-γ 2 h after αGalCer administration and analyzed by flow cytometry. The serum cytokine response shown in Fig. 1A was consistent with the flow cytometric data in that iNKT cells did not express intracellular IFN-γ, although they expressed decreased levels of IL-4 in the absence of DC (Fig. 1B). Because of the concordance between our serum and intracellular cytokine response data (Fig. 1, A–C), we have chosen to analyze and describe the serum cytokine response in the ensuing experiments. From these data, we predict that the weak Th2 response to αGalCer in the absence of DC may be due to inefficient Ag recognition when B cells and/or macrophages present the glycolipid in vivo.

TCR down-modulation is a hallmark of Ag recognition by T lymphocytes including iNKT cells (37–39). To determine whether iNKT cells efficiently recognize Ag when presented by B cells and macrophages, the level of Va14Ja18 TCR expression was determined 6 h after αGalCer administration in vivo. The data revealed that iNKT cells down-modulate their TCR regardless of whether DC or other CD1d+/– cells present αGalCer (Fig. 1D).

In vivo, αGalCer-activated iNKT cells transactivate NK cells to secrete IFN-γ (17). In mice made genetically deficient of iNKT cells (e.g., Ja180/0 or Cd1d0/0), αGalCer administration does not have such an effect on NK cells (data not shown), suggesting that
αGalCer-mediated NK cell activation is iNKT cell dependent (17). Thus, NK cell-derived IFN-γ measurement could be used as an indirect assay of iNKT cell function. To determine the role of DC in the iNKT-NK cell cross-talk, hDTRtg mice treated with either DT or PBS 24 h previously were injected with αGalCer i.p. Six hours later, intracellular IFN-γ expression in NK cells was determined. The data revealed that NK cell (B220negCD3eGDX50m splenocytes) transactivation by αGalCer-activated iNKT cells requires DC (Fig. 1E).

To confirm that the loss of iNKT cell functions in DT-treated hDTRtg animals is due to the absence of DC, we adoptively transferred 6 × 10⁵ unpulsed or αGalCer-pulsed DC into DC-depleted hDTRtg mice (Fig. 2A). An i.v. injection of DC-enriched splenocyte preparation successfully restored DC in deficient recipients (Fig. 2B, left panels). To test whether transferred αGalCer-pulsed DC reconstituted function, we monitored serum IFN-γ and intracellular IFN-γ in NK cells in the recipients 6 h after adoptive transfer. DC reconstitution and αGalCer presentation rescued NK cell transactivation (Fig. 2B, right panels) and IFN-γ production (Fig. 2C). Furthermore, DC reconstitution successfully restored full serum and intracellular iNKT cell-specific IL-4 responses to αGalCer (Fig. 2, D and E). Thus, complete (Th1 and Th2) in vivo activation of iNKT cells requires αGalCer presentation by DC. In addition to their role in Ag presentation, DC are major mediators of the cross-talk between iNKT lymphocytes and NK cells in vivo.

Macrophages play no role in stimulating iNKT cell function in vivo

Macrophages express CD1d and low levels of CD11c. To test whether DT treatment of hDTRtg mice results in the loss of macrophages as well, untreated and treated mice were stained for macrophage-specific (F4/80), DC-specific (CD11c), and granulocyte-specific (Ly6G) markers and were analyzed by flow cytometry. DT-treated hDTRtg mice specifically lose CD11c⁺ DC, but not F4/80⁺ macrophages (Fig. 2F) as previously reported (32) using the regimen described in this study (see Materials and Methods). Interestingly, DT induced DC death results in an increase in granulocyte numbers (Fig. 2F). This increase in granulocytes did not stimulate iNKT cell function (Fig. 1, A and B).

To determine whether macrophages play a role in iNKT cell function, the hDTR transgene was introgressed into B6.129-μMT B cell-deficient mice. The resulting hDTRtg,μMT mice were treated with DT for 24 h and stimulated with αGalCer, injected i.p. Two hours later, serum IL-4 was measured. Macrophages in the absence of DC and B cells do not significantly activate iNKT cells in vivo (Fig. 2G) despite the fact that they down-modulate TCR expression (data not shown). Thus, DC and B cells, but not F4/80⁺-positive macrophages, appear to stimulate iNKT cell function in vivo.

Presentation of αGalCer by B lymphocytes results in reduced and IL-4-biased iNKT cell function

B cells express CD1d and hence have the potential to present glycolipid Ags to iNKT cells. Under certain circumstances, B cells can directly anergize/tolerize conventional CD8⁺ T cells against peptide Ags (40, 41). Although iNKT cell-mediated B cell transactivation has been studied (17, 42), the role of B cells in glycolipid Ag presentation remains less explored (43, 44). DC-depleted hDTRtg animals showed poor functional response to αGalCer, suggesting that other CD1d⁺ APC insufficiently activate iNKT cells in vivo. That notwithstanding, B cells up-regulated CD69, an early activation marker, 6 h after αGalCer injection even in mice made deficient in DC (Fig. 3A).

To define the role of B lymphocytes in iNKT cell activation in vivo, we determined the early cytokine response of B6.129-μMT

FIGURE 2. Adoptive transfer of C57BL/6-derived DC into hDTRtg animals restores iNKT cell function. A, Schematic rendition of the experimental plan. DC were depleted from hDTRtg mice with a single dose of DT. DC-enriched C57BL/6 splenocytes were pulsed with vehicle or αGalCer. After 24 h, 6 × 10⁵ cells were adoptively transferred into DC-depleted hDTRtg mice, and iNKT cell function was measured six hours later. B, Freshly purified and αGalCer-pulsed DC were transferred into DT-treated hDTRtg mice. Six hours after adoptive transfer, reconstitution of splenic DC in recipients was monitored using CD11c and CD8α markers (left panels). The numbers indicate the percentage of CD11c⁺ cells, which includes CD8α⁺ and CD8α⁻ subsets within electronically gated B22₀low splenocytes. The IFN-γ response to αGalCer-pulsed DC in vivo was measured by monitoring intracellular IFN-γ as described in Fig. 1E. Note that control, DC-depleted hDTRtg mice did not respond to αGalCer stimulation (top panel). C, After 24 h of DT treatment, hDTRtg mice were injected with unpulsed or αGalCer-pulsed DC. Serum IFN-γ was measured 6 h after αGalCer administration in vivo. The data shown are representative of two experiments, which yielded very similar results. D and E, Freshly purified and αGalCer-pulsed DC were transferred into DT-treated hDTRtg mice. Three hours after adoptive transfer, serum (D) and iNKT cell intracellular (E) IL-4 responses to αGalCer-pulsed DC in vivo were measured and presented as described in Fig. 1B. Note that the low IL-4 response in mice that did not receive αGalCer but were given DC corresponds to the serum cytokine data presented in Fig. 1A. F, hDTRtg,μMT mice were injected with a single dose of DT. After 24 h, DC (CD11c⁺ cells), macrophages (F4/80⁺ cells), and granulocytes (Ly6G⁺ cells) were stained and analyzed by flow cytometry. Numbers indicate the percentage of a particular cell subset among total splenocytes. The data shown are representative of two experiments (n = 20). G, IL-4 was measured in DC-depleted hDTRtg and hDTRtg,μMT sera 2 h after αGalCer injection i.p. and compared with the IL-4 level elicited by DT-treated control hDTRtg animals. The data shown are representative of two experiments.
(μMT) mice to αGalCer. In the absence of B cells, αGalCer elicited up to 3- to 5-fold higher amounts of IL-2 and IL-4 in serum (Fig. 3B) compared with wild-type iNKT cells 2 h after in vivo activation. Most Interestingly, μMT mice also secrete detectable IFN-γ, which is barely detectable in the serum of wild-type mice, 2 h after αGalCer stimulation in vivo (Fig. 3B), probably due to high intracellular IFN-γ production (Fig. 3C). Furthermore, consistent with the serum IFN-γ response, μMT iNKT cells more efficiently transactivated NK cells compared with wild-type iNKT cells (Fig. 3D). The rapid and robust cytokine response in μMT mice was due to neither high μMT DC numbers (data not shown) nor differences in TCR down-regulation (data not shown). Thus, B cells appear to suppress DC-mediated αGalCer-induced iNKT cell function in vivo.

Restoration of endogenous DC in hDTRtg mice restores iNKT cell function

To determine whether the effect of DC depletion by DT treatment of hDTRtg mice was reversible, mice unstimulated or stimulated with αGalCer were restimulated with the glycolipid 7 days later (Fig. 4A). The ability of in vivo αGalCer-stimulated iNKT cells to transactivate NK cells was determined. Restoration of endogenous DC in hDTRtg mice depleted of DC 7 days earlier restored αGalCer-induced iNKT cell function and, hence, transactivation of NK cells (Fig. 4B). Interestingly, however, DC-depleted hDTRtg mice stimulated with αGalCer 7 days previously were resistant to αGalCer-induced iNKT cell reactivation by glycolipid (Fig. 4B) despite re-expression of Vα14Jα18 TCR to normal levels (data not shown) and restoration of endogenous DC (Fig. 4C). Consistent with resistance to iNKT cell reactivation by αGalCer, DC did not up-regulate CD86 (Fig. 4C). Interestingly, iNKT cells failed to down-modulate their invariant TCR after a second αGalCer administration (Fig. 4D). Our data suggest that CD11c+ DC are not required or responsible for inducing iNKT cell resistance to restimulation in vivo.

To determine whether B cells were responsible for iNKT cell resistance to reactivation by αGalCer, μMT mice were restimulated with αGalCer 7 days after first stimulation (Fig. 4E). As observed with hDTRtg iNKT cells, μMT iNKT cells were also resistant to reactivation by αGalCer and hence did not transactivate NK cells to express intracellular IFN-γ (Fig. 4F). Resistance to reactivation was consistent with poor DC activation, because μMT DC did not up-regulate CD86 in response to restimulation of iNKT cells with αGalCer (Fig. 4G). Thus, iNKT cells can be rendered resistant to restimulation even in the absence of B cells. Together, our data suggest that the observed unresponsiveness is manifested directly within iNKT cells as a memory of a previous activation event.

NOD B cells, but not DC, stimulate iNKT cell function in vivo

Repeated αGalCer administration to young, prediabetic NOD female mice prevents the onset of type 1 diabetes (TID) in an IL-4− and IL-10-dependent manner (45). Interestingly, NOD DC were shown to be dysfunctional (46–49), whereas their B cells appear to play an important Ag-presenting role (50, 51). Based on these findings and because we have found that DC depletion does not completely abolish IL-4 secretion by iNKT cells, we reasoned that NOD B cells, and not their DC, were responsible for αGalCer presentation, activation of iNKT cells, and subsequent IL-4 production in NOD mice. To test this hypothesis, the serum IL-4 response was determined in NOD, NOD.129-μMT, and C57BL/6 mice 2 h after αGalCer injection i.p. αGalCer elicited low levels of serum IL-4 from NOD mice that develop B cells compared with C57BL/6 animals (Fig. 5A). Surprisingly, unlike B6.129-μMT mice whose DC in the absence of B cell inhibition elicited a stronger cytokine response by iNKT cells (Fig. 3B), NOD.129-μMT animals (Fig. 5A) elicited low levels of IL-4, similar to those in NOD mice (Fig. 5A). This suggests that NOD DC are unable to activate iNKT cells, NOD iNKT cells are unable to respond, or both.

To distinguish among these possibilities, C57BL/6 and NOD DC were purified (data not shown), pulsed with αGalCer, and transferred into DC-depleted hDTRtg recipients (data not shown). The results indicate that αGalCer-pulsed NOD DC very poorly, if at all, activate hDTRtg iNKT cells (Fig. 5B, top panel). Additional data revealed that NOD iNKT cells poorly responded to αGalCer-pulsed C57BL/6 DC transferred in vivo (Fig. 5B, bottom panel). Therefore, the deficiency lies within both NOD iNKT cell responders as well as NOD DC stimulators. In contrast, when C57BL/6 or NOD B cells were transferred into DC-depleted and B cell-deficient hDTRtg,μMT mice (data not shown), αGalCer elicited a similar IL-4 response from C57BL/6 iNKT cells (Fig. 5C). These data suggest that NOD B cells, and not DC, are responsible for the αGalCer-induced, iNKT cell-generated IL-4 response in vivo.

Dampening of iNKT cell-derived cytokine response by B cells is cell-cell contact dependent

B cells, compared with DC, poorly stimulate iNKT cell function in vivo (Fig. 1). Additionally, the absence of B cells results in a 3- to 5-fold higher cytokine response by iNKT cells (Fig. 3B). Two plausible mechanisms may explain these findings. One possibility is that B cells, being the most numerous CD1d+ cell type in the spleen, bind αGalCer and interact with iNKT cells. Nevertheless,
because only a small subset (5–10%) of B cells (marginal zone) constitutively express costimulatory CD80/CD86 molecules (52). B cells deliver signal 1 (Ag presentation and recognition), but the majority are unable to deliver signal 2 (costimulation through CD28). In this process, B cells occupy iNKT cells due to high avidity Ag/TCR interactions, as evidenced by Va14Ja18 TCR down-modulation (Fig. 1D), inefficiently activating iNKT cells. Alternatively, the interaction of iNKT cells with B cells induces inhibitory signals, which dampen iNKT cell function.

To determine whether cell-cell contact is essential for the B cell-mediated suppressive effect on iNKT cell function, B6.129-MT mice were reconstituted with CD1d<sup>+</sup> or CD1d<sup>−/−</sup> B cells. Twenty-four hours after B cell transfer, iNKT cells were stimulated in vivo by αGalCer injection i.p., and serum IL-2 and IL-4 responses were monitored (Fig. 6A, left panel). We found that CD1d<sup>+</sup> expression and hence B cell-iNKT cell interaction were essential for dampening iNKT cell function (Fig. 6A, right panels).

To determine the role of signal 2 in efficient iNKT cell activation, the above experiment was repeated using B cells that constitutively express CD80 (NOD<sup>+</sup>B6-CD80<sup>tg</sup>B). The data revealed that enforced CD80 expression on B cells showed the same level of serum IL-4 as that on nontransgenic B cells (data not shown). Thus, the lack of costimulation (i.e., signal 2) when B cells present αGalCer does not explain the low IL-4 response.

The role of DC, B cells, and macrophages in iNKT cell activation was re-examined under more controlled, in vitro conditions. Purified DC, B cells, and macrophages were used as αGalCer-presenting cells, and iNKT cells enriched from B6.129-H2IAb<sup>−/−</sup> splenocytes were used as responders in an in vitro stimulation assay. The data obtained (Fig. 6B) supported the in vivo results and
revealed that DC are the most potent APC in inducing IFN-γ and IL-4 responses from iNKT cells in vivo and in vitro. The addition of B cells to the culture suppressed DC-mediated iNKT cell activation (Fig. 6B). Nevertheless, B cells, in the absence of DC, induced lower levels of IL-4, but not IFN-γ, from iNKT cells (Fig. 6B). In contrast, macrophages appeared to play a minor role, if any, in eliciting cytokine secretion by iNKT cells. Macrophage-dependent αGalCer presentation stimulated a minor IFN-γ, but not IL-4, response from iNKT cells in vivo (Fig. 6B). Thus, the in vitro responses of iNKT cells elicited by different APC recapitulated the in vivo responses.

**Discussion**

Almost all studies focus on the in vitro and in vivo roles of DC in Ag presentation to and activation of iNKT cells (7, 18–20, 28, 53, 54). However, the role(s), if any, played by CD1d1-positive B cells, macrophages, and hepatocytes in Ag presentation to iNKT cells remains undefined. Because iNKT cells are considered to be in an activated/memory state (55, 56), any CD1d1-positive cell that is capable of binding exogenously administered Ag should theoretically elicit function from them. In this report, therefore, we have analyzed the roles of the different CD1d1-positive cells in αGalCer presentation in vivo and in vitro using three genetically altered strains of mice, viz, conditional DC-deficient hDTRtg mice, B cell-deficient μMT mice, and both conditional DC- and congenital B cell-deficient hDTRtg;μMT mice.

A detailed analysis using these strains revealed that DC, which are mainly dispersed in the T cell area and scattered in the B cell follicles (57), are the most efficient αGalCer-presenting cells in vivo. Of the remaining CD1d1-positive cells, neither macrophages, which are clustered in the marginal zone of the spleen, nor hepatocytes were capable of presenting αGalCer to iNKT cells in vivo. B cells,
including the marginal zone B cells that express CD80/CD86 (52) and high levels of CD1d1 (27) around which most iNKT cells congregate (Ref. 36; data not shown), are not effective NKT cells activators in this system. Additionally, their presence severely dampens iNKT cell activation by DC. Our findings are surprising from the standpoint that DC are critical for eliciting primary T cell responses, but not for recall responses. In this regard, therefore, iNKT cells perhaps behave like naive, conventional T lymphocytes despite the fact that they are thought to be in an activated/memory state.

Cellular basis for differential roles for iNKT cells in vivo

The mechanism by which iNKT cells impart Th1 (enhance tumor immunity, adjuvant function of αGalCer) and Th2 (down-modulation of several autoimmune diseases) functions remains unclear. Previous reports have shown that αGalCer-pulsed DC, but not non-DC leukocytes, effectively activate iNKT cells in vivo (18). Only 5–10% of the adoptively transferred B cells repopulate the spleen, while the remaining are lost to an unknown mechanism. Furthermore, it has been observed in MT mice that the maximal reconstitution occurs by 24 h (35). Therefore, it remains unclear whether the inability of the transferred B cells to present αGalCer in vivo was due to inefficient reconstitution or poor Ag presentation function. Conditional in vivo depletion of DC provides a good model to address which cells actually present the Ag in vivo. Our findings indicate that only DC efficiently activate iNKT cells when αGalCer is administered in vivo. This finding is consistent with those reported by...
Implications for therapeutic use of αGalCer

The therapeutic regimen that uses αGalCer in mouse models and in clinical trials to enhance antitumor immunity or to down-regulate autoimmune responses was established arbitrarily (2, 37). Because of its potent immune modulatory effect (2, 3) and hepatotoxicity (62), a thorough understanding of the cellular and molecular bases of αGalCer function is critical. Our results indicate that iNKT cell Ag delivery by DC and B cells would be efficacious when requiring Th1 and Th2 responses, respectively. Previous reports have demonstrated that αGalCer administration to prediabetic NOD mice results in a polarized Th2 response, even to autoantigens otherwise thought to precipitate TID (45, 63). In this study we demonstrated that NOD DC, despite constitutive, high level expression of CD80, CD86, and CD40L as well as their ability to secrete large amounts of IL-12 upon activation (48), are defective in αGalCer presentation to iNKT cells. In contrast, NOD B cells present the administered αGalCer, which elicits low levels of IL-4. The resulting IL-4 appears sufficient to prevent the onset of TID in the NOD model (45, 63).

The data presented in this study also indicated that repeated administration of αGalCer may be superfluous, because once activated, iNKT cells poorly, if at all, respond to a second administration of the glycolipid. This unresponsiveness lasts at least 1 wk, at which time they are known to recover in numbers and character after the first bout of αGalCer-mediated activation (37–39). This finding suggests that the toxic effects of αGalCer can be controlled by prudent in vivo administration at intervals when iNKT cells are optimally functional.

In conclusion, our findings have important implications for appropriate Ag delivery for specific therapeutic intervention whose basis depends on differential activation of Th1 or Th2 responses. First, the ability of B cells to selectively elicit IL-4 from iNKT cells suggests that glycolipid-pulsed B cells could serve as a means of Ag delivery to prevent the onset of autoimmune responses where iNKT cells play this role. This mode of presentation would yield a Th2-biased immune response. Second, a combination of B cell depletion using rituximab, a chimeric CD20-specific mAb (64), followed by Ag delivery by DC can be exploited when the IFN-γ response and, hence, Th1 immunity underlie the therapeutic basis. Such circumstances include the induction of adaptive immunity to pathogens and tumors.

Acknowledgments
We thank Kirin Brewery for synthetic αGalCer, A. J. Joyce for technical assistance, and J. J. Hawiger for sharing breeding pairs of hDTR* mice.

Disclosures
The authors have no financial conflict of interest.

References


