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Selective Conditions Are Required for the Induction of Invariant NKT Cell Hyporesponsiveness by Antigenic Stimulation

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Activation of invariant (i)NKT cells with the model Ag α-galactosylceramide induces rapid production of multiple cytokines, impacting a wide variety of different immune reactions. In contrast, following secondary activation with α-galactosylceramide, the behavior of iNKT cells is altered for months, with the production of most cytokines being strongly reduced. The requirements for the induction of this hyporesponsive state, however, remain poorly defined. In this study, we show that Th1-biasing iNKT cell Ags could induce iNKT cell hyporesponsiveness, as long as a minimum antigenic affinity was reached. In contrast, the Th2-biasing Ag OCH did not induce a hyporesponsive state, nor did cytokine-driven iNKT cell activation by LPS or infections. Furthermore, although dendritic cells and B cells have been reported to be essential for iNKT cell stimulation, neither dendritic cells nor B cells were required to induce iNKT cell hyporesponsiveness. Therefore, our data indicate that whereas some bone marrow–derived cells could induce iNKT cell hyporesponsiveness, selective conditions, dependent on the structure and potency of the Ag, were required to induce hyporesponsiveness. The Journal of Immunology, 2015, 195: 3838–3848.

Invariant (i)NKT cells are characterized by the expression of an identical TCRαβ rearrangement, Vα14-Jα18 in mice and Vα24-Jα18 in humans, and their recognition of lipid Ags presented by CD1d. iNKT cells rapidly produce copious amounts of various cytokines following activation with Ags or with cytokines, and they participate in innate immune responses, as well as bridging the innate and adaptive immune responses (1–5). Many studies of NKT cells have used the model Ag α-galactosylceramide (αGalCer), which has an extraordinarily high affinity for the iNKT cell TCR when bound to CD1d. This response is characterized by the production of both Th1 cytokines such as IFN-γ and Th2 cytokines such as IL-4 (1–5). However, it has been reported that iNKT cells become unresponsive to a secondary challenge following a primary activation with αGalCer in vivo, which has been compared with anergy in conventional T cells (6–8). We recently demonstrated that some αGalCer-pretreated iNKT cells secrete IL-10 when activated and display an IL-10–dependent regulatory function (9). Furthermore, smaller proportions of iNKT cells with a similar surface phenotype and the ability to produce IL-10 can be found in untreated mice and humans, indicating that IL-10+ iNKT cells are a new subset of iNKT cells that we termed NKT10 cells (9). Because not all the Ag-experienced iNKT cells produce IL-10, we refer to “iNKT cell hyporesponsiveness” in this study when discussing the function of NKT cell Ags.

Materials and Methods
Mice and bacteria
All mice were housed under specific pathogen-free conditions at the vivarium of the La Jolla Institute for Allergy and Immunology in accordance with the Institutional Animal Care and Use Committee guidelines. C57BL/6J mice and B6.129S2-Igsb6m1Ccp1/J (μMT−/−), B6.129S7-Iftgr1m1Atg/J (Iftgr−/−), B6.129S1-Il12a1m1Bm1/J (Il12−/−), B6.129P2-Ii18m1Atg1/J (Il18−/−), and B6.129S6-Cd1d2m1Spb1/J (Cd1d−/−) on the C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.129-Tcra–m1Trp (Ja18−/−) mice (10) and CD11c-DOG mice (11) on the C57BL/6 background were a gift of Dr. M. Taniguchi (RIKEN Institute, Yokohama, Japan) and Dr. Günter Hammerling (German Cancer Research Center, Heidelberg, Germany), respectively. All mouse experiments were performed under the Public Health Service policy. Spingobium yanoikuyae was purchased from American Type Culture Collection (Manassas, VA).

Reagents and mAbs
The glycolipid Ags α-galactosylceramide (αGalCer) and OCH were obtained from Kyowa Hakko Kirin (Tokyo Research Park, Tokyo, Japan). C-glycoside (C-Gly) and GalA-glycosphingolipid (GSL; GSL-1) were obtained from the National Institutes of Health tetramer core facility (Emory University, Atlanta, GA). EF77 and SMC124 were prepared as described.
previosely (12). LPS and diphtheria toxin (DTx) were purchased from Sigma-Aldrich (St. Louis, MO). mAbs against the following mouse Ags were used in this study: CD3ε (145.2C11, 17A2), CD4 (GK1.5, RM4-5), CD8α (53-6.7, SH10), CD11b (M1/70), CD11c (HL.3), CD19 (ID3, 6D5), CD25 (PC61.5), CD44 (IM7), CD45.1 (A20), CD45.2 (104), CD45R/B220 (RA3-6B2), CD69 (H1.2F3), CD279/PD-1 (J43, RMP1-30), IFN-γ (XMG1.2), IL-4 (11B11, BVD6-24G2), IL-10 (JES3-9D7), Ly6C/G (Gr1), NK1.1 (PK136), NRP1/CD304 (polyclonal), TCRb (H57-597), and TNF (MP6-XT22). Abs were purchased from BD Biosciences (San Diego, CA), BioLegend (San Diego, CA), eBioscience (San Diego, CA), Invitrogen (Carlsbad, CA), or R&D Systems (Minneapolis, MN). Abs were biotinylated or conjugated to Pacific Blue, eFluor 450, V450, Brilliant Violet 421, Pacific Orange, V500, Brilliant Violet 570, Quantum Dot 605, Quantum Dot 655, eFluor 650, Brilliant Violet 650, Brilliant Violet 711, Brilliant Violet 785, Brilliant Violet 786, FITC, Alexa Fluor 488, PerCP, PerCP-Cy5.5, PerCP-eFluor 710, PE, PE–Texas Red, PE–CF594, PE–Cy5.5, PE–Cy7, allophycocyanin, Alexa Fluor 647, eFluor 660, Alexa Fluor 700, allophycocyanin-Cy7, or allophycocyanin-eFluor 780. Anti-mouse CD16/32 Ab (2.4G2) used for Fc receptor blocking was purified in our laboratory. Unconjugated mouse and rat IgG Abs were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Dead cells were labeled with blue, aqua, or yellow dead cell stain kit (Invitrogen). Preparation of fluorochrome-conjugated αGalCer-loaded CD1d tetramers were performed as described previously (13).

ELISA and flow cytometry

IFN-γ and IL-4 levels in plasma were determined by ELISA using reagents from BD Biosciences, according to the manufacturer’s recommendations. Flow cytometry was performed as described previously (13). Invariant Vα14–Jα18 TCR rearrangement NKT cells were defined throughout as live CD8εε CD1d/CD1d/CD44+ TCR/CD3+ CD1d/CD1d/CD44+ TCR/CD3+ CD1d/CD1d/CD44+ TCR/CD3+ NKT1.1+ cells. NK cells were defined as live TCR/CD3+. In vivo challenge

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CD3 mAbs i.v., which is known to activate ε response on rechallenge with stimulation by the memory T cells preferentially (22). Despite the strong initial efficacy of C-Gly in the induction of mainstream CD4 and CD8 T cells (23). Given the intermediate Repetitive antigenic stimulation has been shown to induce anergy in mainstream CD4 and CD8 T cells (23). Given the intermediate efficacy of C-Gly in the induction of NKT cell hyporesponsiveness, we investigated whether repetitive challenge could augment hyporesponsiveness. Therefore, we injected either OCH or C-Gly three times and measured the NKT cell response 1 mo later. Similar to the results from a single injection (Fig. 2A, 2B), three injections of OCH did not significantly alter the phenotype or function of NKT cells when restimulated and analyzed 1 mo later (Fig. 3A, 3B). In contrast, three injections of C-Gly changed the phenotype and function of restimulated NKT cells in a manner largely indistinguishable from the one following a single αGalCer

Repetitive injection or increased dose can augment NKT cell hyporesponsiveness

Repetitive antigenic stimulation has been shown to induce anergy in mainstream CD4 and CD8 T cells (23). Given the intermediate efficacy of C-Gly in the induction of NKT cell hyporesponsiveness, we investigated whether repetitive challenge could augment hyporesponsiveness. Therefore, we injected either OCH or C-Gly three times and measured the NKT cell response 1 mo later. Similar to the results from a single injection (Fig. 2A, 2B), three injections of OCH did not significantly alter the phenotype or function of NKT cells when restimulated and analyzed 1 mo later (Fig. 3A, 3B). In contrast, three injections of C-Gly changed the phenotype and function of restimulated NKT cells in a manner largely indistinguishable from the one following a single αGalCer

\[ \text{Th2-biasing Ag} \] It has a sphingosine base reduced in length and exhibits a decreased antigenic potency and a weaker TCR affinity than does αGalCer (16). C-Gly has a carbon–carbon bond substituting for the O-glycosidic linkage of the galactose sugar to the sphingosine (17). In terms of TCR affinity, C-Gly is weaker still compared with OCH (18–21), but it induces a systemic Th1 response (17). The Th1-biasing effect of C-Gly is predominantly a consequence of increased IFN-γ production by NK cells activated downstream of NKT cell stimulation, as the ratio of IFN-γ/IL-4 cytokines immediately produced by the NKT cells themselves is comparable irrespective of the Ag injected (20). To investigate the long-term effects of stimulation with OCH or C-Gly on NKT cells, we injected each compound once and measured the NKT cell response 1 mo later by rechallenge of the mice with αGalCer. We and others described previously that a single pretreatment with αGalCer reduced the frequency of peripheral NKT cells and led to wide range of phenotypic and functional changes in these cells (7–9). Markers such as CD25, CD69, CD122, CD127, CD154 (CD40L), and NK1.1 were expressed at lower levels, whereas markers associated with regulatory T cells, such as CD152 (CTLA4), CD279 (PD-1), CD304 (NRP1), and FR4 were strongly upregulated (9). Additionally, the expression of proinflammatory cytokines was reduced, whereas the production of IL-10 was increased in αGalCer-pretreated NKT cells (7–9).

Similar changes were observed in this study 1 mo after αGalCer injection (Fig. 2A, 2B), including decreased CD69 expression and intracellular cytokine staining for IFN-γ, TNF, and IL-4, together with increased expression of CD279 and CD304 and staining for IL-10 (Fig. 2A, 2B). Therefore, these data demonstrate the expected NKT cell hyporesponsiveness. In contrast, a single pretreatment with OCH did not lead to significant alterations in the NKT cell phenotype or effector function compared with control animals (Fig. 2A, 2B). Interestingly, a single pretreatment with C-Gly resulted in a phenotype intermediate between the αGalCer- and OCH-treated mice (Fig. 2A, 2B). Similar changes were observed when IFN-γ and IL-4 levels in plasma 90 min after αGalCer rechallenge were analyzed (Supplemental Fig. 2). To address the question whether any type of strong TCR triggering would lead to NKT cell hyporesponsiveness, we injected anti-CD3ε mAbs i.v., which is known to activate NKT cells and memory T cells preferentially (22). Despite the strong initial stimulation by the αCD3ε Ab, the NKT cell phenotype and response on rechallenge with αGalCer 1 mo later was comparable to control animals (Fig. 2C, 2D and data not shown). Taken together, these data indicate that the potential of a TCR-mediated stimulus to induce NKT cell hyporesponsiveness depends on Ag structure but does not directly correlate with its antigenic strength.

![Graph showing cytokine production](image)

**FIGURE 2.** NKT cell hyporesponsiveness does not solely depend on strong TCR-mediated activation. (A and B) C57BL/6 (B6) mice were either left untreated or injected i.v. with 4 μg OCH, C-Gly, or αGalCer as indicated. One month later mice were injected i.v. with 1 μg αGalCer, and 90 min later expression surface markers (A) and the production of indicated cytokines (B) by splenic NKT cells were analyzed. For (A), representative data (right panel) and summary graphs (left panel) are shown. The used gating strategy for NKT cells is depicted in Supplemental Fig. 1. (C and D) C57BL/6 (B6) mice were either left untreated or i.v. injected with 4 μg αGalCer or 1 μg anti-CD3ε (145.2C11) Abs as indicated. One month later mice were injected i.v. with 1 μg αGalCer, and 90 min later expression of CD69 (C) and of indicated cytokines (D) by splenic NKT cells was analyzed. Statistically significant differences of treated groups versus the control group are indicated. Representative data from one of two independent experiments are shown. ***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05.
injection (Fig. 3A, 3B). To discriminate whether the observed effect of repetitive C-Gly challenge is due to the increased dose applied or due to the timing of the injections, we directly compared the injection of the same 12 μg amount of C-Gly either given once or in a total of three separate aliquots of 4 μg. Both treatments induced iNKT cell hyporesponsiveness to a similar degree that was comparable to a single αGalCer injection (Fig. 3C, 3D). We also tested three injections of anti-CD3ε mAbs and did not observe any changes in iNKT cells in regard to phenotype or cytokine production (Fig. 3E, 3F), confirming that the changes we observed with αGalCer and C-Gly were not simply the result of repeated strong TCR triggering. Collectively, these data indicate that the efficiency of some Ags to induce iNKT cell hyporesponsiveness requires a minimum antigenic potency that can be achieved by repetitive challenge or by increasing the amount in a single dose.

**iNKT cell hyporesponsiveness is induced by Th1-biasing compounds**

Although αGalCer may be classified as a Th0 Ag, because of the large amounts of IFN-γ and IL-4 it stimulates, C-Gly elicits a higher ratio of IFN-γ to IL-4 (1–5, 17). Therefore, we addressed whether other Th1-biasing Ags also could induce iNKT cell hyporesponsiveness. To this end, we analyzed the responses to EF77 or SMC124 (Fig. 1), two GSL Ags based on the structure of the plakoside A GSL isolated from the marine sponge *Plakortis simplex*, which induce a Th1-biased pattern of cytokine secretion (1–5, 12). We injected these Ags i.v. and analyzed the iNKT cell

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**FIGURE 3.** Repetitive injection or increased dose can augment iNKT cell hyporesponsiveness. (A and B) C57BL/6 (B6) mice were either left untreated or injected i.v. once with 4 μg αGalCer (1×αGC) or three times every other day with 4 μg OCH (3×OCH) or C-Gly (3×C-Gly) as indicated. One month later mice were injected i.v. with 1 μg αGalCer and 90 min later splenic iNKT cells were analyzed for the expression of surface makers (A) and of intracellular cytokines (B). (C and D) C57BL/6 (B6) mice were either left untreated or injected i.v. once with 4 μg αGalCer (αGC), once with 12 μg C-Gly (1×C-Gly), or three times every other day with 4 μg C-Gly (3×C-Gly, i.e., 12 μg in total) as indicated. One month later mice were injected i.v. with 1 μg αGalCer and 90 min later splenic iNKT cells were analyzed for the expression of surface makers (C) and of intracellular cytokines (D). (E and F) C57BL/6 (B6) mice were either left untreated or injected i.v. once with 4 μg αGalCer (1×αGC) or three times every other day with 1 μg anti-CD3ε (145.2C11) Abs (3×αCD3εAb) as indicated. One month later mice were injected i.v. with 1 μg αGalCer, and 90 min later splenic iNKT cells were analyzed for the expression of CD69 (D) and of intracellular cytokines (E). Differences in the amount of IFN-γ+ iNKT cells detectable in different experiments depended largely on the fluorochrome conjugated to the used Ab in the particular experiment [e.g., for IFN-γ Alexa Fluor 700 (B and F) versus PE-C594 (D)]. Regardless, within an experiment, consistent differences were observed between groups, and statistically significant differences are indicated. Representative data from one of at least two independent experiments are shown. *p < 0.05, **p ≤ 0.01, ***p ≤ 0.001.
response 1 mo later after rechallenge with αGalCer. As shown in Fig. 4A and 4B, the phenotype and function of iNKT cells pretreated with αGalCer or SMC124 were comparable. Furthermore, pretreatment with EF77 also induced iNKT cell hyporesponsiveness, albeit to a lower degree (Fig. 4A, 4B). One of the key differences between the Th1 cytokine– versus Th2 cytokine–biasing iNKT cell Ags is the ability of the Th1-biasing Ags to trans-activate NK cells, downstream of iNKT cell activation, to produce large amounts of IFN-γ (6–8, 24, 25). To investigate whether NK cells play a role in the induction of iNKT cell hyporesponsiveness, we repeated the experiments after depletion of NK cells. However, NK cell depletion at the time of the initial αGalCer pretreatment did not reduce iNKT cell hyporesponsiveness upon rechallenge (Fig. 4C, 4D). Similarly, in mice deficient for the IFN-γ receptor (Ifngr−/−) the αGalCer-induced iNKT cell hyporesponsiveness was unaffected (Fig. 4E, 4F). Therefore, the large amounts of IFN-γ produced by NK cells, or any other function induced by these cells, is not required for the induction of iNKT cell hyporesponsiveness.

Cytokine-driven stimulation does not induce iNKT cell hyporesponsiveness
iNKT cells can be activated directly via the TCR or via cytokines, most prominently by IL-12 in concert with IL-18 for the majority population of Th1 cytokine–biased iNKT cells (NKT1 cells) in C57BL/6 mice (9, 26, 27). Therefore, we addressed whether such cytokine-driven activation would also lead to iNKT cell hyporesponsiveness. To this end, we injected 40 μg LPS either once or

**FIGURE 4.** iNKT cell hyporesponsiveness is induced by Th1-biasing compounds. (A and B) C57BL/6 (B6) mice were either left untreated or injected i.v. with 4 μg EF77, SMC124 (SMC), or αGalCer (αGC) as indicated. One month later mice were injected i.v. with 1 μg αGalCer, and 90 min later splenic iNKT cells were analyzed for the expression of surface markers (A) and intracellular cytokines (B). Statistically significant differences of treated groups versus the control group are indicated. (C and D) Control C57BL/6 (control) mice or mice NK cell depleted 1 d earlier with anti-asialo GM1 Ab (asGMAb) were either left untreated or injected i.v. with 4 μg αGalCer (ctr/αGC). One month later 1 μg αGalCer was injected i.v., and splenic iNKT cells were analyzed 90 min later for the expression of surface markers (C) and of intracellular cytokines (D). Statistically significant differences (ANOVA) of B6 (control versus αGC) versus the NK-depleted asialo GM1 (control versus asGMAb) groups are indicated. (E and F) Control C57BL/6 (B6) mice or mice deficient for the IFN-γ receptor (Ifngr−/−) were either left untreated or injected i.v. with 4 μg αGalCer (αGC). One month later 1 μg αGalCer was injected i.v., and splenic iNKT cells were analyzed 90 min later for the expression of surface markers (E) and of intracellular cytokines (F). Statistically significant differences of treated groups versus the control group are indicated. Representative data from one of two independent experiments are shown. *p < 0.05, **p ≤ 0.01, ***p ≤ 0.001.
three times i.v. and analyzed the iNKT cell response 1 mo later. As shown in Fig. 5A and 5B, LPS, even when given three times, did not induce iNKT cell hyporesponsiveness, as indicated by the unaltered phenotype and cytokine response compared with the control mice. To directly address the role of IL-12 and IL-18 in the induction of iNKT cell hyporesponsiveness, we measured the long-term effects of αGalCer challenge in mice deficient for either cytokine. However, the lack of either the p35 subunit of IL-12 or IL-18 did not change the outcome of αGalCer-induced iNKT cell hyporesponsiveness (Fig. 5C, 5D). We also tested the secondary response of iNKT cells following exposure to infectious agents. MCMV can stimulate iNKT cells via IL-12 and IL-18 (9, 27) or type I IFN (6–9, 28) and does not induce a TCR-mediated signal (10, 29, 30). In contrast, S. yanoikuyae provides both TCR- and cytokine-dependent activation of iNKT cells (11, 31, 32). However, neither viral infection with MCMV nor bacterial infection with S. yanoikuyae induced any signs of hyporesponsiveness in the iNKT cells, based on the phenotype and cytokine response (Supplemental Fig. 3). Taken together, these data suggest that cytokine-driven activation of iNKT cells does not lead to hyporesponsiveness.

iNKT cell hyporesponsiveness can be induced by bone marrow–derived cells

CD1d is widely expressed on hematopoietic, but also on non-hematopoietic, cells (12, 33, 34). To investigate the cellular requirements for the αGalCer-induced iNKT cell hyporesponsiveness, we addressed the role of hematopoietic cells. To this end, we generated bone marrow chimeras by transferring C57BL/6 wt bone marrow into irradiated wt control (wt→wt) or CD1d-deficient hosts (wt→KO). As iNKT cells are selected in the thymus by double-positive thymocytes, they develop in wt→KO chimeras despite the absence of CD1d on nonhematopoietic cells (1, 4, 13, 35). After reconstitution, αGalCer was injected into wt→KO and control wt→wt chimeras and 4 wk later the iNKT cell response was analyzed after rechallenge with αGalCer in vivo. The response of iNKT cells from wt→KO and control wt→wt chimeric mice was comparable, irrespective of the αGalCer pretreatment, as indicated by the expression of surface markers and the production of cytokines (Fig. 6). These data indicate that presentation of αGalCer by CD1d on hematopoietic cells is sufficient to cause αGalCer-induced iNKT cell hyporesponsiveness. The reciprocal KO→wt chimeras could not be analyzed because these mice would not have iNKT cells, but our data do not rule out a redundant role for expression of CD1d by nonhematopoietic cells in the induction of hyporesponsiveness.

B cells are not required to induce iNKT cell hyporesponsiveness

It has been reported that injection of αGalCer-loaded B cells is sufficient to induce iNKT cell hyporesponsiveness in vivo (7, 13). This conclusion was based on the observation that a 3-d in vitro culture of splenocytes from such mice in the presence of αGalCer
led to reduced proliferation, as measured by thymidine incorporation, and reduced levels of IFN-γ in the culture supernatant (7, 11). However, we noticed a tendency for a reduced frequency of splenic iNKT cells in mice treated with αGalCer (Refs. 9, 14 and data not shown), which in some, but not all, experiments was statistically significant. Nonetheless, this reduced number of responding cells could offer an alternative explanation for the previously reported in vitro findings (7, 16). To avoid this potential caveat, we restimulated iNKT cells with αGalCer in vivo and analyzed the iNKT cell response directly ex vivo on the single cell level. By this approach, the response of iNKT cells from control mice or mice injected 1 mo earlier with αGalCer-loaded B cells did not differ in the expression of surface markers or in the production of cytokines (Fig. 7A–C and data not shown). However, we observed that B cells loaded with αGalCer in vitro and injected i.v. led to an activation of iNKT cells in the host even when the transferred B cells were derived from Cd1d<sup>−/−</sup> mice (Fig. 7D, 7E). Furthermore, the trans-activation of NK cells was indistinguishable after the injection of B cells from either background (Fig. 7E). This indicated that αGalCer could efficiently be cross-presented by host cells in vivo after uptake of the injected B cells, and it reveals a cautionary note for defining the relevant APC type for iNKT cells in any experiment in which Ag-pulsed APCs are injected into recipients.

Although these data indicate that cross-presentation of αGalCer does not lead to Ag-induced iNKT cell hyporesponsiveness, they do not settle the question as to whether B cells are required for this induction. To definitely address the requirement for B cells in the induction of iNKT cell hyporesponsiveness in vivo, we injected αGalCer into B cell–deficient μMT<sup>−/−</sup> mice (17, 36) and analyzed the iNKT cells 1 mo later. iNKT cells from both μMT<sup>−/−</sup> and wt control mice were similarly altered by the αGalCer pretreatment and did not differ in the expression of surface markers or in the degree of reduction in the production of cytokines (Fig. 7F, 7G and data not shown). These data demonstrate that B cells are not necessary to induce αGalCer-induced iNKT cell hyporesponsiveness.

**iNKT cell hyporesponsiveness does not require DCs**

A recent study reported that CD8α<sup>+</sup> DCs are the dominant APC type for activating iNKT cells with injected Ags (18–21, 37). Therefore, we addressed whether DCs also are necessary for the αGalCer-induced iNKT cell hyporesponsiveness. To eliminate DCs in vivo we used transgenic mice expressing the DTx receptor under the control of the CD11c promoter (CD11c-DOG mice) (11, 17). Injection of DTx into CD11c-DOG mice led to depletion of <95% of CD4<sup>+</sup> and CD8<sup>+</sup> DC<sup>+</sup> DCs in the spleen within 24 h (Refs. 11, 20 and Supp. Fig. 4). αGalCer was injected into control and DC-depleted CD11c-DOG mice and 4 wk later the iNKT cell response was analyzed after rechallenge with αGalCer in vivo. However, iNKT cells from control and from DC-depleted CD11c-DOG mice were similarly altered by the αGalCer pretreatment and did not differ in the expression of surface markers or in the production of cytokines (Fig. 8A, 8B and data not shown). These data demonstrate that CD11c<sup>high</sup> DCs are not necessary to induce αGalCer-induced iNKT cell hyporesponsiveness.

**Discussion**

Initial activation of iNKT cells with αGalCer induces a rapid production of multiple cytokines; however, following secondary activation the production of most proinflammatory cytokines is blunted. In this study, we report on two aspects of this iNKT cell hyporesponsiveness. First, our data demonstrate that Th0- and Th1-biasing GSL Ags can induce iNKT cell hyporesponsiveness, but not a Th2-biasing Ag or cytokine-driven iNKT cell activation owing to TLR engagement as a result of LPS exposure or infections. Second, although presentation of αGalCer by hematopoietic cells can induce iNKT cell hyporesponsiveness, we did not find a nonredundant function either for B cells or DCs for these changes.

Induction of iNKT cell hyporesponsiveness has previously largely been investigated with the Th0 Ag αGalCer (6–9). In the present study, we demonstrate that this feature is shared with several Th1-biasing Ags (Fig. 4A, 4B), in particular with C-Gly...
and the plakoside A analogs EF77 and SMC124 (7–9, 12). In contrast, the Th2-biasing compound OCH (16, 22) did not induce iNKT cell hyporesponsiveness (Figs. 2A, 2B, 3A, 3B). It has been suggested that the ability of an Ag to induce iNKT cell hyporesponsiveness correlates with its antigenic strength (23, 38). However, our data do not support this model. First, C-Gly was able to induce long-term iNKT cell hyporesponsiveness, whereas OCH, which has more avid binding to the iTCR when complexed to CD1d than C-Gly, was not able to induce hyporesponsiveness (Figs. 2A, 2B, 3A, 3B). Second, a strong activation of iNKT cells with an agonistic anti-CD3ε Ab, either once or repetitively, did not lead to iNKT cell hyporesponsiveness (Fig. 2C, 2D, 3D, 3F). Rather, our data indicated that the induction of long-term iNKT cell hyporesponsiveness is a particular feature of Th0- and Th1-biasing iNKT cell Ags, which is not shared with a Th2-biasing Ag. This interpretation is in line with some previous data suggesting that some Th1-biasing iNKT cell Ags (39–41), but not a Th2-biasing Ag (39), may induce long-term iNKT cell hyporesponsiveness. The reason for the opposite results described in the present study and previously (38) is unknown. However, as Huang et al. (38) analyzed the secondary iNKT cell response only seven days after the initial challenge, the timing of the analysis...
could explain the differences between our studies. Indeed, it has been shown that the Th2-biasing iNKT cell Ag C20:2 can induce a short-lived hyporesponsiveness in iNKT cells that lasts for about one week; however, that is not sustained for the longer time frame of one month we investigated in this study (42).

One difference between Th1- and Th2-biasing Ags is their differential ability to induce the trans-activation of NK cells in vivo (24, 25). However, this trans-activation of NK cells (Fig. 4C, 4D) or signaling by IFN-γ (Fig. 4E, 4F) was not a requirement for the induction of iNKT cell hyporesponsiveness. Therefore, at this time the reason for the lack of iNKT cell hyporesponsiveness induced by the Th2-biasing Ag OCH is not known. It has been suggested that Th1-biasing Ags are characterized by prolonged iNKT cell stimulation in vivo, which could be due either to increased TCR affinity, stability of the Ag/CD1d-complexes or unknown pharmacokinetic properties of the Ags. For example, the synthesis and testing of C-Gly was stimulated by the supposition that the C-glycosidic bond would provide for a more stable compound resistant to catabolism (17). In line with this prolonged stimulation hypothesis, we previously reported that the CD1d complexes on the surface of APCs for several Th1-biasing Ags had an increased half-life in vivo (12, 20, 40). Furthermore, structural data suggest that some Th1-biasing compounds have increased molecular contacts with CD1d that may promote prolonged binding to CD1d in vivo, and therefore prolonged stimulation of iNKT cells (12, 40, 43). Taken together, our data support a model whereby only Th0/1-biasing Ags have the capability to induce long-term iNKT cell hyporesponsiveness, provided that they surpass a minimal antigenic strength. Once this threshold is reached, repetitive/chronic exposure or increased dose can amplify the functional changes in iNKT cells, as shown in the present study for C-Gly.

Besides Ag-driven activation via the TCR, iNKT cells can also be activated by cytokines, most prominently IL-12 in concert with IL-18 or IFN-α/β (26–28). Data presented in the present study with Il12−/− and Il18−/− mice, LPS injection, and MCMV infection indicate that cytokine-driven activation of iNKT cells does not lead to or influence hyporesponsiveness. Additionally, in preliminary experiments with Il15−/−, Il12rb−/− mice and with wt mice infected with Escherichia coli we also did not observe any influence on iNKT cells hyporesponsiveness (data not shown). Taken together, these data support the conclusion that proinflammatory cytokines are not involved in the induction of αGalCer-induced iNKT cell hyporesponsiveness. In contrast, other reports suggested that some, but not all, bacterial infections could induce iNKT cell hyporesponsiveness (44). The reason for this discrepancy is not known. However, the timing could be important in the present study as well, as following the i.v. injection of LPS a short-lived (2–3 d) lack of iNKT cell responsiveness toward TCR triggering was reported that waned within 1 wk (45).

It has been shown that αGalCer derived from αGalCer-loaded B16 melanoma cells can be cross-presented by DCs in vivo (46); however, this has not been shown for hematopoietic cells. In the present study, we demonstrate that αGalCer associated with Cd1d+/− B cells is efficiently cross-presented after i.v. injection, leading to an iNKT cell activation that is indistinguishable from the stimulation achieved with Ag-loaded wt B cells (Fig. 7D, 7E). Similar preliminary results were obtained after injection of αGalCer-loaded Cd1d−/− bone marrow-derived DCs (data not shown). Our finding that αGalCer is efficiently cross-presented in vivo provides a cautionary note for the interpretation of experiments involving transfer of αGalCer-loaded cells. Such experiments cannot discriminate between stimulation of iNKT cells by αGalCer presented by the injected cells and cross-presented by host cells.

Ag presentation by different APC populations has been suggested to be critical for iNKT cell stimulation with particular Ags and in particular organs (37, 47–49). For example, it was reported that the presentation of Th1-biasing Ags is largely dependent on presentation by DCs/macrophages, whereas Th2-biasing compounds are more promiscuous with regard to the APC type (48). In contrast, there is evidence indicating that CD8α+ DCs are the critical APC for
the initial presentation of all Ags in vivo, irrespective of their Th1- or Th2-biasing properties (37). Nonetheless, the requirements for the immediate NK T cell activation are not necessarily identical with the requirements for long-term effects leading to iNK T cell hyporesponsiveness. Therefore, we investigated in the present study the role of two APC populations, B cells, and DCs. Based on in vitro data generated after the transfer of αGalCer-loaded B cells, it had been suggested that B cells could induce the hyporesponsive state in iNK T cells (7). In contrast, when we analyzed the iNK T cell response from similarly treated mice on a single-cell level rather than on a population level, we could not detect any long-term changes in the iNK T cell phenotype and cytokine response (Fig. 7B, 7C). However, we noticed a tendency for a reduced iNK T cell frequency in splenocytes from mice pretreated with αGalCer-loaded B cells (Fig. 7A), which could potentially explain the previous in vitro findings (7). Importantly, our data with B cell–deficient μMT−/− mice directly demonstrated that B cells are not required for the induction of iNK T cell hyporesponsiveness in vivo (Fig. 7F, 7G). We cannot exclude the possibility that the few B-1 cells remaining in μMT−/− mice (50, 51) could be responsible for the observed induction of iNK T cell hyporesponsiveness. However, we consider this unlikely, in light of the systemic nature of anergy induction (7, 9), the fact that iNK T cells in many organs do not circulate extensively (52, 53), and the paucity of B-1 B cells in some sites (54).

It has been reported that i.v. injection of αGalCer-loaded bone marrow–derived DCs (6) or primary splenic DCs (7) does not induce iNK T cell hyporesponsiveness, and we could reproduce this finding with bone marrow–derived DCs (data not shown). However, as noted above, because of extensive Ag cross-presentation, no conclusion could be drawn about the role of DCs in the induction of hyporesponsiveness (Fig. 7D, 7E). Importantly, our data with CD11c−DCG mice (11) indicated that CD11cIgG DCs are not required to induce αGalCer-induced iNK T cell hyporesponsiveness in vivo (Fig. 8). Collectively, our data demonstrate that although presentation of αGalCer by hematopoietic cells is sufficient to cause iNK T cell hyporesponsiveness (Fig. 6), neither presentation by B cells or DCs is required. It has been reported that the depletion of macrophages via clodronate liposome treatment also does not induce iNK T cell hyporesponsiveness (55). Taken together, these data suggest that none of the classical bone marrow–derived APCs, DCs, B cells, and macrophages are essential for the presentation of αGalCer in the induction of iNK T cell hyporesponsiveness in vivo. It is likely, however, that hyporesponsiveness by iNK T cells requires specific properties of the cell presenting αGalCer. It has been suggested that Th1-biasing Ags preferentially load onto CD1d in lipid rafts (56, 57). It also was reported that Th1-biasing Ags also cause changes in DCs, such as increased GalCer. It has been suggested that although presentation of αGalCer by hematopoietic cells is sufficient to cause iNK T cell hyporesponsiveness, neither presentation by B cells nor DCs are essential for these changes.

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Disclosures

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