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

Gerhard Wingender,*† Alysia M. Birkholz,* Duygu Sag,†‡ Elisa Farber,§ Sampada Chitale,‡ Amy R. Howell,§ and Mitchell Kronenberg*‡

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: 000–000.

Materials and Methods
Mice and bacteria

All mice were housed under specific pathogen-free conditions at the vi varium 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-Igh-b6m1mt1Jm1Jm1Jm (Igh-b6m1 mt1Jm1Jm1Jm), B6.129S2-Igh-b6m1mt1Jm1Jm1Jm mouse (10) and CD11c-DOG mice (11) on the C57BL/6J background were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.129-Tcra-JJ/J (Ja18/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 in an American Association for the Accreditation of Laboratory Animal Care–accredited facility with prior approval of the La Jolla Institute for Allergy and Immunology Animal Care Committee in accordance with the Public Health Service policy. Sphingobium 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.
previously (12). LPS and diphtheria toxin (DTxs) were purchased from Sigma-Aldrich (St. Louis, MO). mAbs against the following mouse Ags were used in this study: CD3 (145.2C11, 17A2), CD4 (GK1.5, RM4-5), CD8α (53-6.7, 5H10), CD11b (M1/70), CD11c (HL3), CD19 (1D3, 6D5), CD25 (PC61.5), CD44 (IM7), CD45.1 (A20), CD45.2 (104), CD45RB/B220 (RA3-6B2), CD69 (H1.2F3), CD279/PD-1 (J43, RMP1-30), IFN-γ (XMG1.2), IL-4 (11B11, BVD6-24G2), IL-10 (JES5-9D7), Ly6C/G (Gr1), NK1.1 (PK136), NRP1/CD304 (polyclonal), TCRβ (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, V500, 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 878, 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 CD3+ CD19/CD45R2− TCR/CD3+ CD1d/CD1d/αGalCer-tetramer+ cells. NK cells were defined as live TCR/CD3+ NK1.1+ cells.

In vivo challenge
NKT cells were αGalCer pretreated by injection of 4 µg αGalCer i.v. and analyzed 4–6 wk later or as otherwise indicated. Acute activation in vivo was induced by injection of 1 µg αGalCer i.v. followed by analysis 90 min later or as otherwise indicated. For the depletion of NK cells, mice were i.p. injected with 50 µg/mouse anti-asialo-GM1 Ab (rabbit IgG, IgM, IgA) (Wako Pure Chemical Industries, Richmond, VA) 24 h in advance. For viral or bacterial infection 5 × 10⁵ PFU murine CMV (MCMV) Smith strain (provided by Chris Benedict, La Jolla Institute, La Jolla, CA) or 1 × 10⁸ S. yanoikuyae bacteria were injected i.p. For depletion of dendritic cells (DCs), CD11c-DOG mice were i.p. injected with 8 ng/g body weight of DTxs as described previously (11), resulting in a ~95% loss of CD4+ and CD8+ CD11c+ DCs in the spleen within 24 h (Supplemental Fig. 4). One day after DTx treatment mice were challenged with αGalCer as indicated.

Sample preparation
Single-cell suspensions from spleen were prepared as described previously (14). Heparinized whole blood was centrifuged at 2000 × g for 10 min at room temperature to obtain plasma.

Bone marrow chimera
Bone marrow transplantations were performed as described previously (15). Lethal irradiations were performed in a [137Cs] irradiator (600 rad twice, 3 h apart) and C57BL/6J or Cd1d−/− mice were reconstituted with unfractioned bone marrow from wild-type (wt) C57BL/6J mice as indicated. Mice were treated with trimethoprim/sulfamethoxazole in drinking water for 2 wk after transplantation. Experiments were performed 3–4 mo after bone marrow transplantation.

Statistical analysis
Results are expressed as mean ± SEM. Statistical comparisons were drawn using a two-tailed Student t test (Excel, Microsoft, Redmond, WA; GraphPad Prism, GraphPad Software, San Diego, CA) for all paired samples or otherwise using an ANOVA test (GraphPad Prism). A p value <0.05 was considered statistically significant. Each experiment was repeated at least twice with 2–4 mice per group, and background values were subtracted. Graphs were generated with GraphPad Prism (GraphPad Software).

Results
NKT cell hyporesponsiveness does not solely depend on strong TCR-mediated activation
αGalCer is characterized by an exceptional antigenic potency, and therefore we addressed whether other NKT cell Ags, with differing degrees of antigenic strength, also would cause NKT cell hyporesponsiveness. To this end, we compared the secondary NKT cell response to αGalCer, so that each mouse received the same secondary stimulus, after an initial stimulation either with αGalCer or related compounds that differ with regard to relatively subtle chemical changes (Fig. 1). OCH is the prototypical

![FIGURE 1. Chemical structures of the GSL Ags](http://www.jimmunol.org/ download/fig1.jpg)

Previously (12), chemical structures of the GSL Ags were used.
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 iNKT cell stimulation, as the ratio of IFN-γ/IL-4 cytokines immediately produced by the iNKT cells themselves is comparable irrespective of the Ag injected (20). To investigate the long-term effects of stimulation with OCH or C-Gly on iNKT cells, we injected each compound once and measured the iNKT 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 iNKT 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 iNKT 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 iNKT cell hyporesponsiveness. In contrast, a single pretreatment with OCH did not lead to significant alterations in the iNKT 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 iNKT cell hyporesponsiveness, we injected anti-CD3ε mAbs i.v., which is known to activate iNKT cells in the induction of mainstream CD4 and CD8 T cells (23). Given the intermediate efficacy of C-Gly in the induction of iNKT cell hyporesponsiveness, we investigated whether repetitive challenge could augment hyporesponsiveness. Therefore, we injected either OCH or C-Gly three times and measured the iNKT 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 iNKT 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 iNKT cells in a manner largely indistinguishable from the one following a single αGalCer

| FIGURE 2. | iNKT 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 iNKT cells were analyzed. For (A), representative data (right panel) and summary graphs (left panel) are shown. The used gating strategy for iNKT 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 iNKT 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 < 0.05, **p ≤ 0.01, ***p ≤ 0.001.
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), 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

**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 pre-treated 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<sup>−/−</sup>) 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
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 (Il12−/−) or IL-18 (Il18−/−) did not change the outcome of αGalCer injection (Fig. 5C, 5D). This conclusion was based on the observation that a 3-d in vitro GalCer-pretreated culture of splenocytes from such mice did not induce NKT cell hyporesponsiveness in vivo (7, 13).

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 i.v. and splenic iNKT cells were analyzed 90 min later for the expression of surface makers (C) and of intracellular cytokines (D). Statistically significant differences of treated groups versus the control group are indicated. Representative data from one of at least two independent experiments are shown.

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 was sufficient to induce iNKT cell hyporesponsiveness, 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.

FIGURE 5. Cytokine-driven stimulation does not induce 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 with 40 μg LPS, either once (1 × LPS) or three times every other day (3 × LPS) 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) Control C57BL/6 (B6) mice or mice deficient for the p35 chain of IL-12 (Il12−/−) or IL-18 (Il18−/−) 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 (C) and of intracellular cytokines (D). Statistically significant differences of treated groups versus the control group are indicated. Representative data from one of at least two independent experiments are shown. *p < 0.05, **p ≤ 0.01, ***p ≤ 0.001.
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 pretreated 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 C11d−/− 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−/− mice (17, 36) and analyzed the iNKT cells 1 mo later. iNKT cells from both μMT−/− 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. 7E, 7F and data not shown). These data demonstrate that B cells 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.

**FIGURE 6.** iNKT cell hyporesponsiveness can be induced by bone marrow–derived cells. (A and B) Lethally irradiated C57BL/6 (wt) or C11d−/− (KO) mice were reconstituted with C57BL/6 bone marrow (wt→wt or wt→KO), rested for 12 wk, and then either left untreated or injected i.v. with 4 μg αGalCer (αGC). 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 (ANOVA) of wt→wt (control versus αGC) versus the wt→KO (control versus αGC) groups are indicated. Representative data from one of two independent experiments are shown.
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...
intracellular cytokines (Bm the spleen as described. C57BL/6 (B6) or DC-depleted CD11c-DOG (DOG) mice were either left untreated or injected i.v. with 4 indicated. Representative data from one of three independent experiments are shown.

Il12 IL-18 or IFN-γ can amplify the functional changes in threshold is reached, repetitive/chronic exposure or increased dose provided that they surpass a minimal antigenic strength. Once this induction of signaling by IFN-γ (24, 25). However, this transferential ability to induce the of one month we investigated in this study (42).

FIGURE 8. iNKT cell hyporesponsiveness does not require DCs. (A and B) CD11c-DOG mice were depleted <95% of CD4⁺ and CD8⁺ CD11c⁺ DCs in the spleen as described. C57BL/6 (B6) or DC-depleted CD11c-DOG (DOG) mice were either left untreated or injected i.v. with 4 μg αGalCer (αGC). 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 of intracellular cytokines (B). Statistically significant differences (ANOVA) of B6 (control versus αGC) versus the DC-depleted DOG (control versus αGC) groups are indicated. Representative data from one of three independent experiments are shown.

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 Th1/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
ther B cells nor DCs are essential for these changes. However, we noticed a tendency for a reduced NK 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 NK cell hyporesponsiveness in vivo (Fig. 7E, 7F). 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 NK cell hyporesponsiveness. However, we consider this unlikely, in light of the systemic nature of anergy induction (7, 9), the fact that NK 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 NK 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 CD1c1c-DOG mice (11) indicated that CD1c1c1c DCs are not required to induce αGalCer-induced NK cell hyporesponsiveness in vivo (Fig. 8). Collectively, our data demonstrate that although presentation of αGalCer by hematopoietic cells is sufficient to cause NK 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 NK 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 NK cell hyporesponsiveness in vivo. It is likely, however, that hyporesponsiveness by NK cells requires specific properties of the cell presenting αGalCer. It has been suggested that Th1-biasing Ags preferentially load onto CD1d in lysosomes and localize on the cell surface in lipid rafts (56, 57). It also was reported that Th1-biasing Ags also cause changes in DCs, such as increased CD70 and CD86 expression, that support Th1 responses (37).

In summary, we demonstrate in this study that Th0- and Th1-biasing Ags, but not a Th2-biasing Ags, can induce long-term NK cell hyporesponsiveness once a minimal threshold of antigenic strength is reached. This can be achieved by a sufficient Ag dose or repetitive/chronic exposure. Furthermore, although hematopoietic cells can induce NK cell hyporesponsiveness, neither B cells nor DCs are essential for these changes.

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Disclosures

The authors have no financial conflicts of interest.

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