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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 *i*NKT 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 *i*NKT cell Ags could induce *i*NKT 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 *i*NKT cell activation by LPS or infections. Furthermore, although dendritic cells and B cells have been reported to be essential for *i*NKT cell stimulation, neither dendritic cells nor B cells were required to induce *i*NKT cell hyporesponsiveness. Therefore, our data indicate that whereas some bone marrow–derived cells could induce *i*NKT 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 TCRα rearrangement, Vα14–Jα18 in mice and Vα24–Jα18 in humans, and their recognition of lipid Ags presented by CD1d. *i*NKT 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 *i*NKT cells have used the model Ag α-galactosylceramide (αGalCer), which has an extraordinarily high affinity for the *i*NKT 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 *i*NKT 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 *i*NKT cells secrete IL-10 when activated and display an IL-10–dependent regulatory function (9). Furthermore, smaller proportions of *i*NKT 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$^+$ *i*NKT cells are a new subset of *i*NKT cells that we termed NKT10 cells (9). Because not all the Ag-experienced *i*NKT cells produce IL-10, we refer to "*i*NKT cell hyporesponsiveness" in this study when discussing the function of populations of αGalCer-pretreated *i*NKT cells. The profound changes in *i*NKT cells that can be induced by αGalCer stimulation are long lasting, for at least 3–4 mo (6–9). A better understanding of the underlying mechanisms, however, is essential to allow successful manipulation of *i*NKT cells for therapy. In this study, we have explored the requirements, in terms of the type of stimulation and the relevant APCs, for inducing this hyporesponsive *i*NKT cell phenotype.

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-*Igh-6*^{tm1Cgn}/J (*μMT*$^{-/-}$), B6.129S7-*Ifngr1*^{tm1Agt}/J (*Ifngr*$^{-/-}$), B6.129S1-*Il12a*^{tm1Jm}/J (*Il12*$^{-/-}$), B6.129P2-*Il18*^{tm1Aki}/J (*Il18*$^{-/-}$), and B6.129S6-*Cd1d1/Cd1d2*^{tm1Spb}/J (*Cd1d*$^{-/-}$) on the C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.129-*Tcra-J*^{tm1Tgi} (*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 Hämmerling (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

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The online version of this article contains supplemental material.

Abbreviations used in this article: C-Gly, C-glycoside; DC, dendritic cell; DTx, diphtheria toxin; αGalCer, α-galactosylceramide; GSL, glycosphingolipid; *i*, invariant; MCMV, murine CMV; wt, wild-type.

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previously (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, 5H10), CD11b (M1/70), CD11c (HL3), CD19 (1D3, 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), 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, 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 CD8α$^-$ CD19/CD45R$^-$ CD44$^+$ TCR/CD3$^+$ CD1d/αGalCer-tetramer$^+$ cells. NK cells were defined as live TCR/CD3$^-$ NK1.1$^+$ cells.

In vivo challenge

*i*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 μl/mouse anti–asialo-GM1 Ab (rabbit IgG, IgM, IgA) (Wako Pure Chemical Industries, Richmond, VA) 24 h in advance. For viral or bacterial infection 5×10^4 PFU murine CMV (MCMV) Smith strain (provided by Chris Benedict, La Jolla Institute, La Jolla, CA) or 1×10^8 *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 DTx 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 $2000 \times g$ for 10 min at room temperature to obtain plasma.

Bone marrow chimeras

Bone marrow transplantations were performed as described previously (15). Lethal irradiations were performed in a [^{137}Cs] 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

iNKT 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 *i*NKT cell Ags, with differing degrees of antigenic strength, also would cause *i*NKT cell hyporesponsiveness. To this end, we compared the secondary *i*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

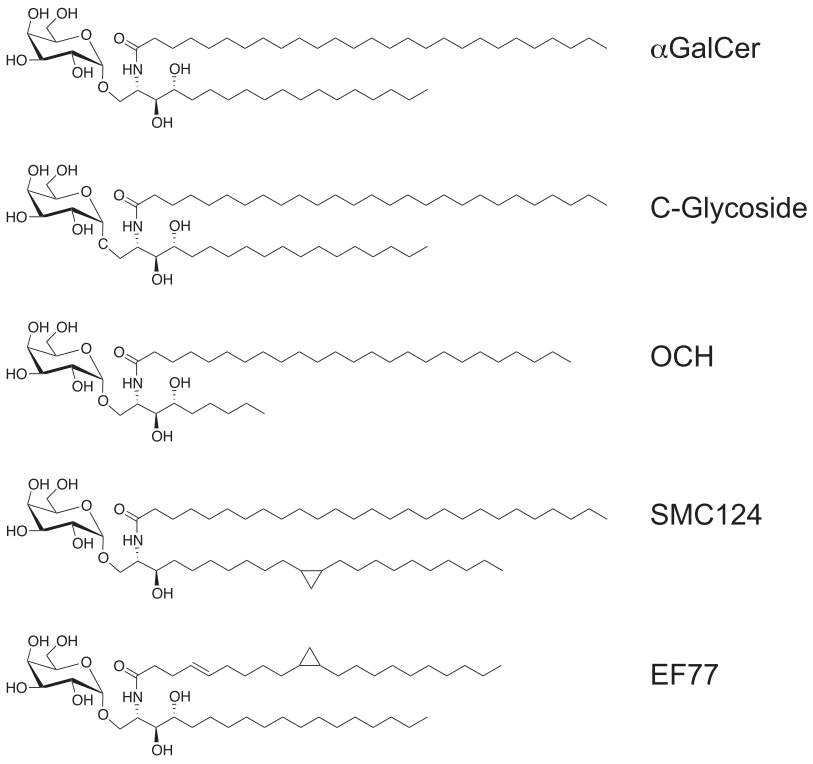


FIGURE 1. Chemical structures of the GSL Ags used.

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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 *i*NKT cell stimulation, as the ratio of IFN-γ/IL-4 cytokines immediately produced by the *i*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 *i*NKT cells, we injected each compound once and measured the *i*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 *i*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 *i*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 *i*NKT cell hyporesponsiveness. In contrast, a single pretreatment with OCH did not lead to significant alterations in the *i*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 *i*NKT cell hyporesponsiveness, we injected anti-CD3ε mAbs i.v., which is known to activate *i*NKT cells and memory T cells preferentially (22). Despite the strong initial stimulation by the αCD3ε Ab, the *i*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 *i*NKT cell hyporesponsiveness depends on Ag structure but does not directly correlate with its antigenic strength.

Repetitive injection or increased dose can augment iNKT 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 *i*NKT cell hyporesponsiveness, we investigated whether repetitive challenge could augment hyporesponsiveness. Therefore, we injected either OCH or C-Gly three times and measured the *i*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 *i*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 *i*NKT cells in a manner largely indistinguishable from the one following a single αGalCer

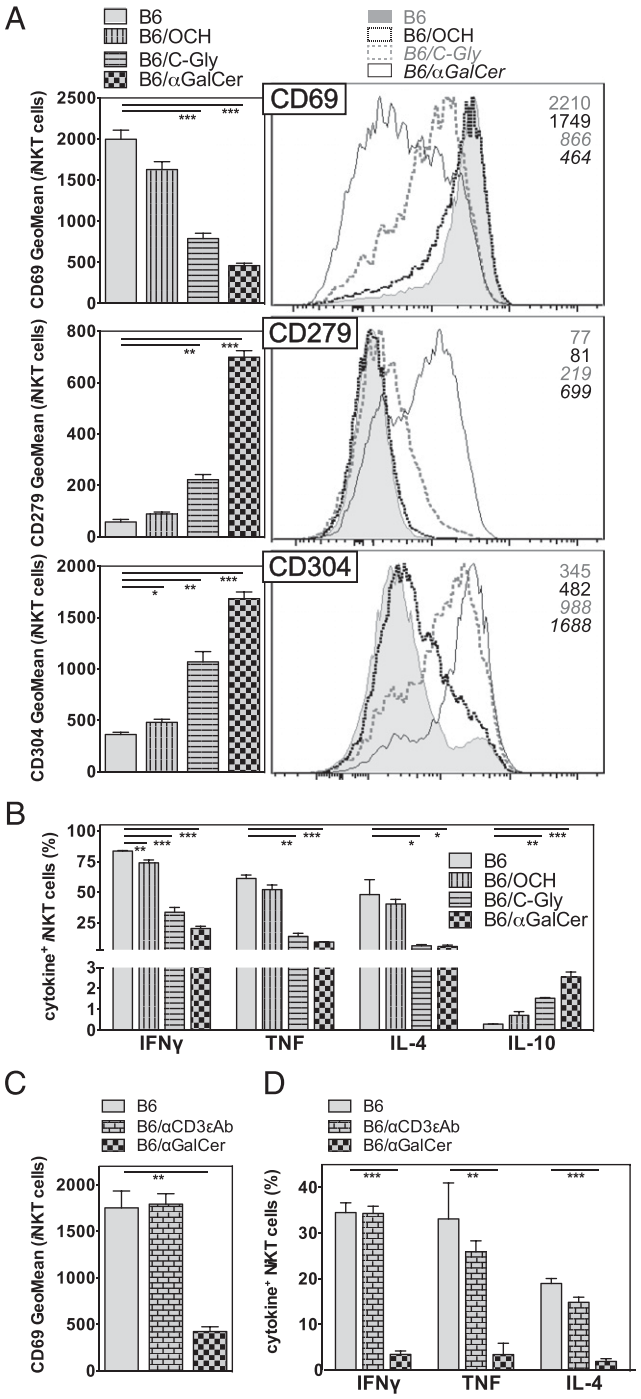


FIGURE 2. *i*NKT cell hyporesponsiveness does not solely depend on strong TCR-mediated activation. (**A** and **B**) C57BL/6 (B6) mice were either left untreated or injected i.v. with 4 μg OCH, C-Gly, or αGalCer as indicated. One month later mice were injected i.v. with 1 μg αGalCer, and 90 min later expression surface markers (A) and the production of indicated cytokines (B) by splenic *i*NKT cells were analyzed. For (A), representative data (*right panel*) and summary graphs (*left panel*) are shown. The used gating strategy for *i*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 *i*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. $*p < 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

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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 *i*NKT 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 *i*NKT 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 *i*NKT 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 *i*NKT 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 *i*NKT cell

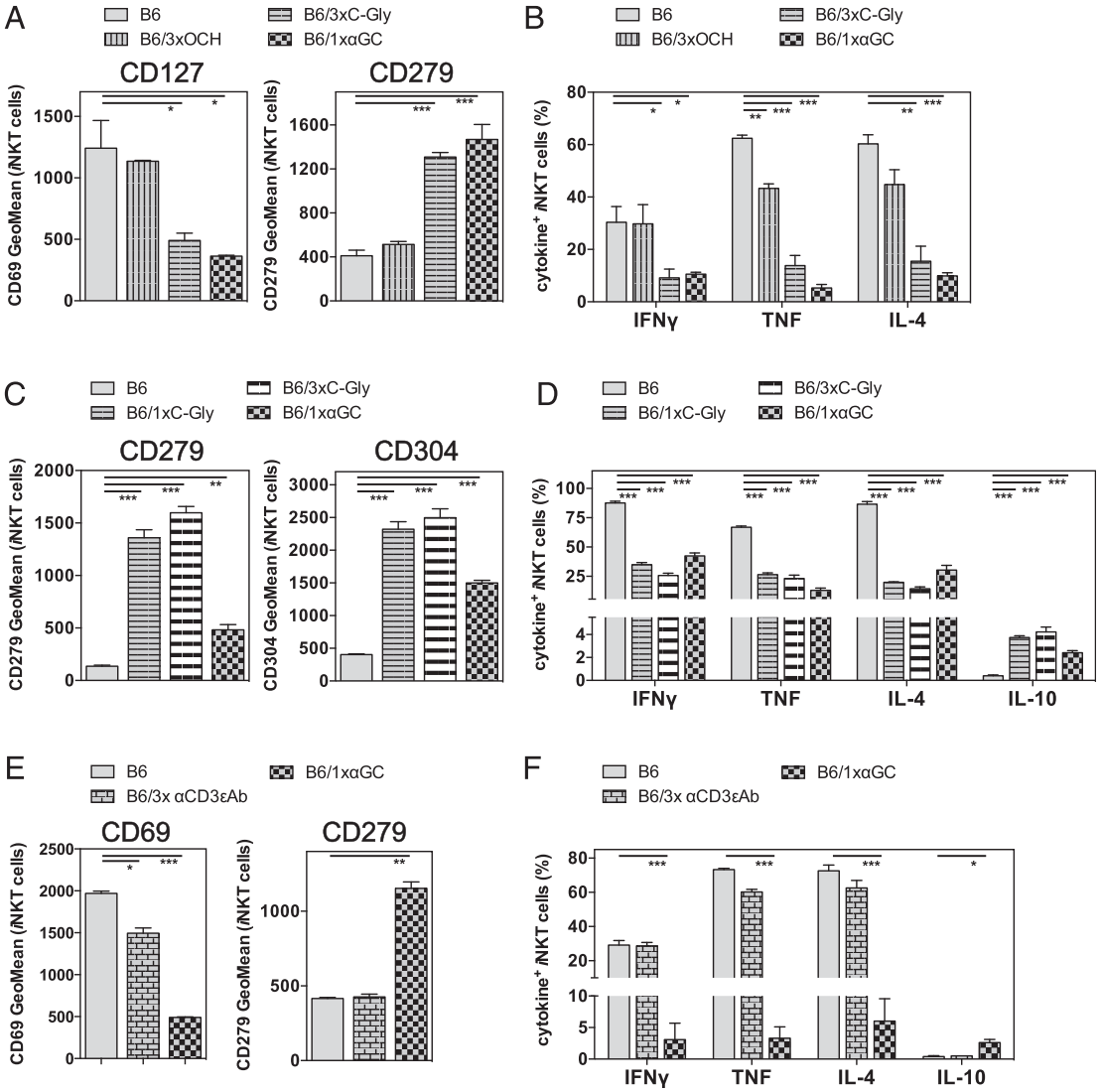


FIGURE 3. Repetitive injection or increased dose can augment *i*NKT cell hyporesponsiveness. (**A** and **B**) C57BL/6 (B6) mice were either left untreated or injected i.v. once with 4 μg αGalCer (1×αGC) or three times every other day with 4 μg OCH (3×OCH) or C-Gly (3×C-Gly) as indicated. One month later mice were injected i.v. with 1 μg αGalCer and 90 min later splenic *i*NKT 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 *i*NKT 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 *i*NKT cells were analyzed for the expression of CD69 (D) and of intracellular cytokines (E). Differences in the amount of IFN-γ$^+$ *i*NKT 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. $*p < 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

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response 1 mo later after rechallenge with αGalCer. As shown in Fig. 4A and 4B, the phenotype and function of *i*NKT cells pretreated with αGalCer or SMC124 were comparable. Furthermore, pretreatment with EF77 also induced *i*NKT cell hyporesponsiveness, albeit to a lower degree (Fig. 4A, 4B). One of the key differences between the Th1 cytokine– versus Th2 cytokine–biasing *i*NKT cell Ags is the ability of the Th1-biasing Ags to *trans*-activate NK cells, downstream of *i*NKT 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 *i*NKT 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 *i*NKT cell hyporesponsiveness upon rechallenge (Fig. 4C, 4D). Similarly, in mice deficient for the IFN-γ receptor ($Ifngr^{-/-}$) the αGalCer-induced *i*NKT 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 *i*NKT cell hyporesponsiveness.

Cytokine-driven stimulation does not induce iNKT cell hyporesponsiveness

*i*NKT 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 *i*NKT cells (NKT1 cells) in C57BL/6 mice (9, 26, 27). Therefore, we addressed whether such cytokine-driven activation would also lead to *i*NKT cell hyporesponsiveness. To this end, we injected 40 μg LPS either once or

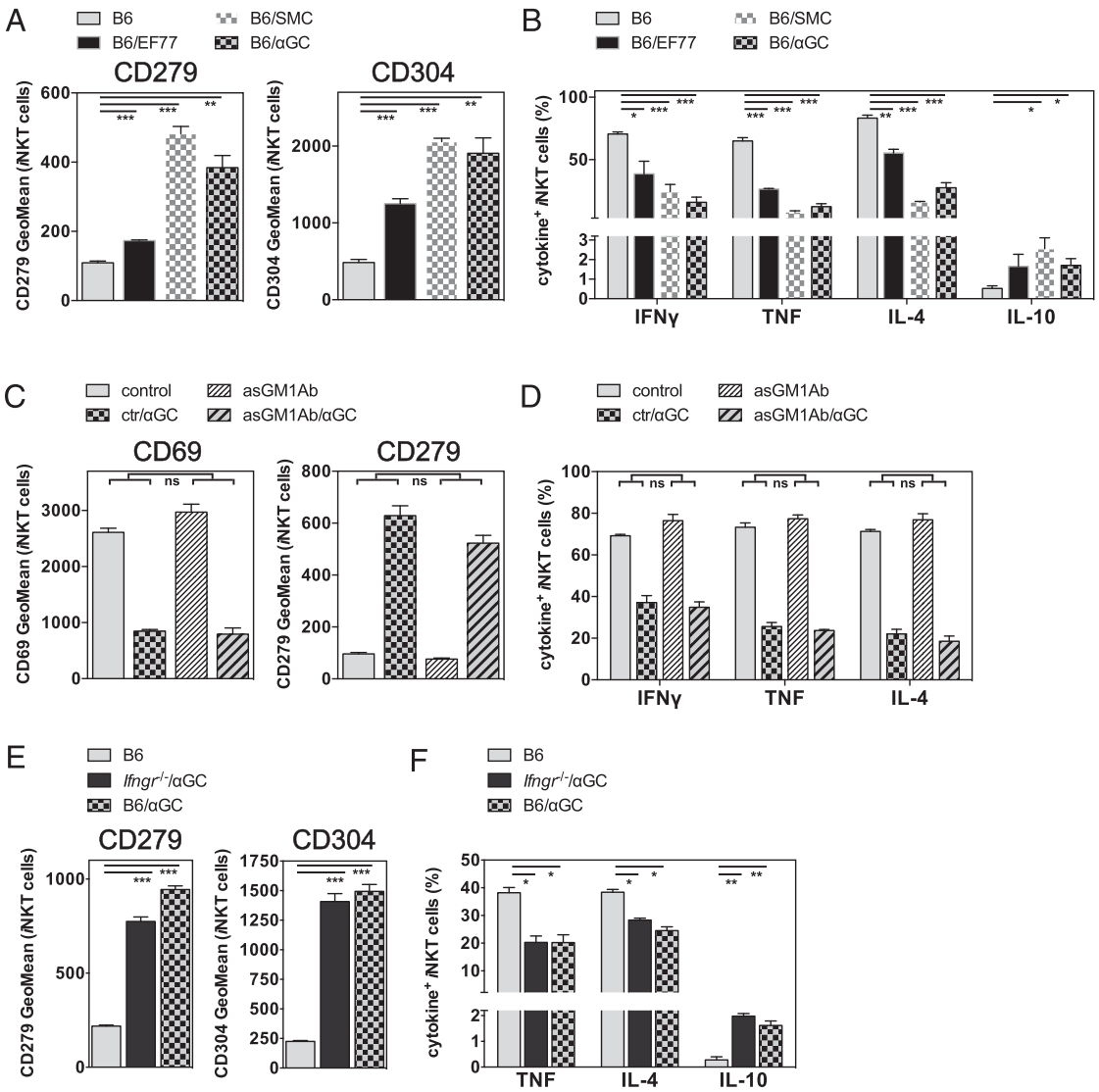


FIGURE 4. *i*NKT cell hyporesponsiveness is induced by Th1-biasing compounds. (**A** and **B**) C57BL/6 (B6) mice were either left untreated or injected i.v. with 4 μg EF77, SMC124 (SMC), or αGalCer (αGC) as indicated. One month later mice were injected i.v. with 1 μg αGalCer, and 90 min later splenic *i*NKT cells were analyzed for the expression of surface makers (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 *i*NKT cells were analyzed 90 min later for the expression of surface makers (C) and of intracellular cytokines (D). Statistically significant differences (ANOVA) of $B6_{(\text{control versus } \alpha GC)}$ versus the NK-depleted asialo $GM1_{(\text{control versus } \alpha GC)}$ 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 *i*NKT cells were analyzed 90 min later for the expression of surface makers (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. $*p < 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

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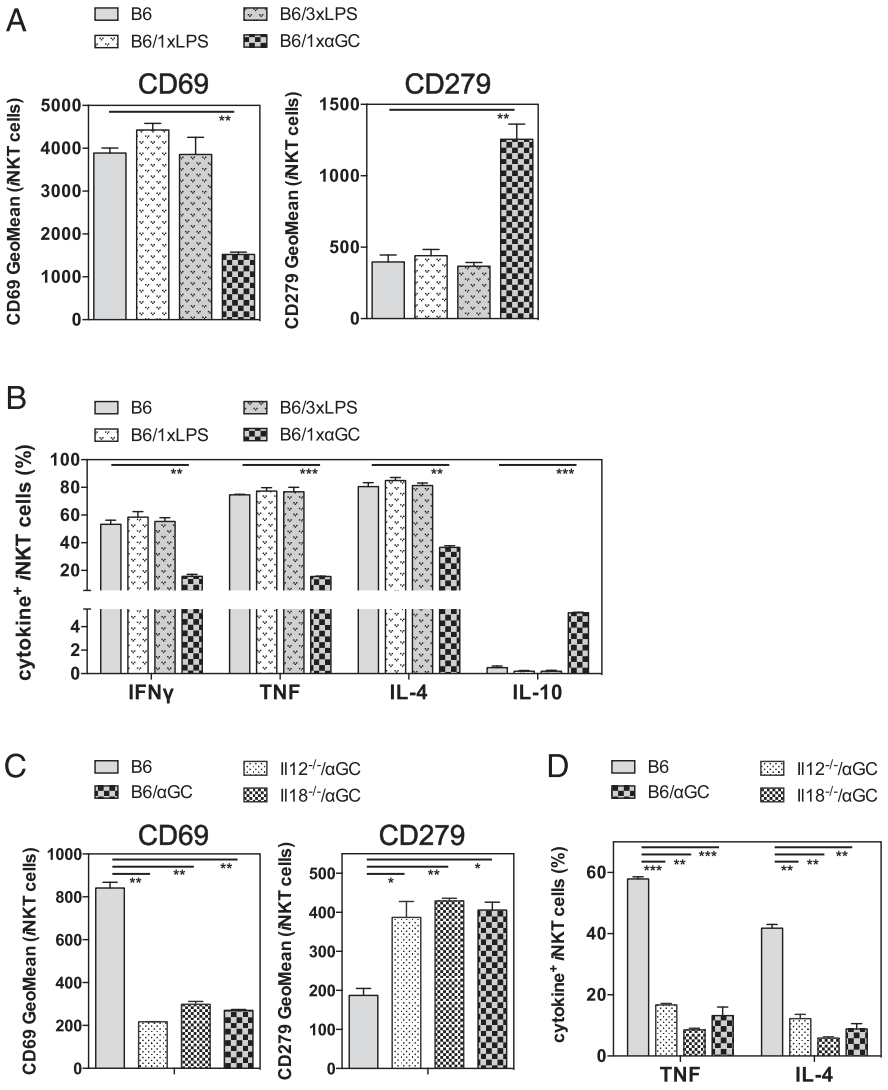


FIGURE 5. Cytokine-driven stimulation does not induce *i*NKT cell hyporesponsiveness. (**A** and **B**) C57BL/6 (B6) mice were either left untreated or injected i.v. once with 4 μg αGalCer (1×αGC) or with 40 μg LPS, either once (1×LPS) or three times every other day (3×LPS) as indicated. One month later mice were injected i.v. with 1 μg αGalCer, and 90 min later splenic *i*NKT 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 *i*NKT 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. $*p < 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

three times i.v. and analyzed the *i*NKT cell response 1 mo later. As shown in Fig. 5A and 5B, LPS, even when given three times, did not induce *i*NKT 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 *i*NKT 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 *i*NKT cell hyporesponsiveness (Fig. 5C, 5D). We also tested the secondary response of *i*NKT cells following exposure to infectious agents. MCMV can stimulate *i*NKT 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 *i*NKT cells (11, 31, 32). However, neither viral infection with MCMV nor bacterial infection with *S. yanoikuyae* induced any signs of hyporesponsiveness in the *i*NKT cells, based on the phenotype and cytokine response (Supplemental Fig. 3). Taken together, these data suggest that cytokine-driven activation of *i*NKT 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 nonhematopoietic, cells (12, 33, 34). To investigate the cellular requirements for the αGalCer-induced *i*NKT 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 *i*NKT 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 *i*NKT cell response was analyzed after rechallenge with αGalCer in vivo. The response of *i*NKT 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 *i*NKT cell hyporesponsiveness. The reciprocal KO→wt chimeras could not be analyzed because these mice would not have *i*NKT 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 *i*NKT 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

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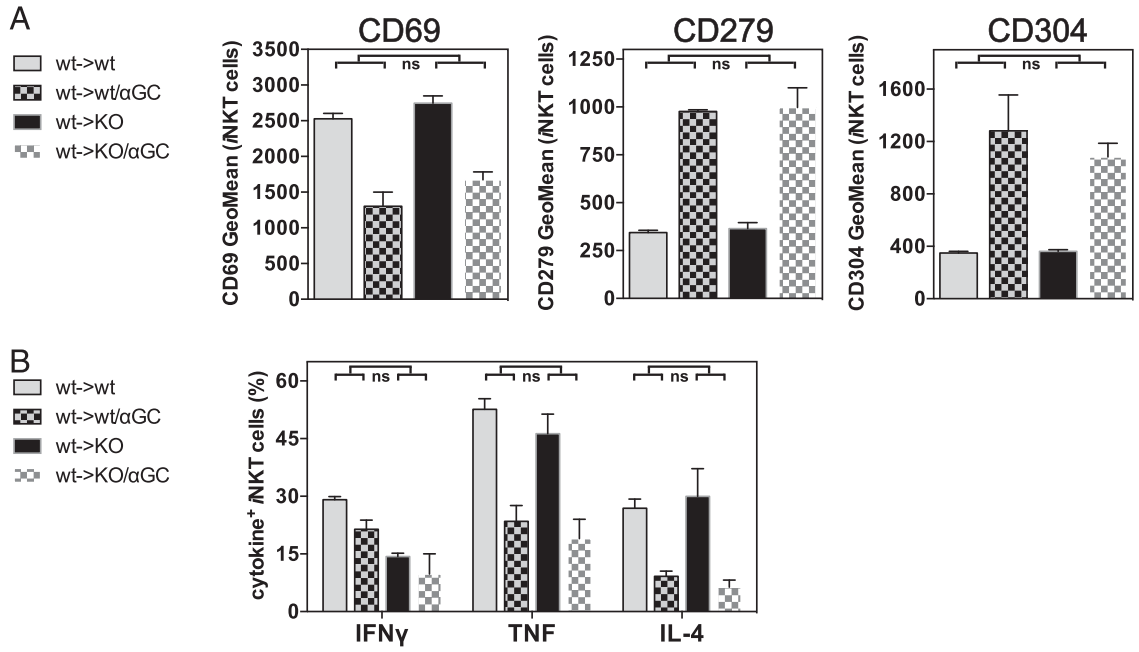


FIGURE 6. *i*NKT cell hyporesponsiveness can be induced by bone marrow–derived cells. (**A** and **B**) Lethally irradiated C57BL/6 (wt) or *Cd1d*$^{-/-}$ (KO) mice were reconstituted with C57BL/6 bone marrow (wt→wt or wt→KO), rested for 12 wk, and then either left untreated or injected i.v. with 4 μg αGalCer (αGC). One month later mice were injected i.v. with 1 μg αGalCer, and 90 min later splenic *i*NKT cells were analyzed for the expression of surface markers (A) and intracellular cytokines (B). Statistically significant differences (ANOVA) of wt→wt$_{(\text{control versus } \alpha GC)}$ versus the wt→KO$_{(\text{control versus } \alpha GC)}$ groups are indicated. Representative data from one of two independent experiments are shown.

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 *i*NKT 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 *i*NKT cells with αGalCer in vivo and analyzed the *i*NKT cell response directly ex vivo on the single cell level. By this approach, the response of *i*NKT 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 *i*NKT cells in the host even when the transferred B cells were derived from *Cd1d*$^{-/-}$ 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 *i*NKT 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 *i*NKT 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 *i*NKT cell hyporesponsiveness in vivo, we injected αGalCer into B cell–deficient $\mu MT^{-/-}$ mice (17, 36) and analyzed the *i*NKT cells 1 mo later. *i*NKT cells from both $\mu MT^{-/-}$ 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 *i*NKT cell hyporesponsiveness.

iNKT cell hyporesponsiveness does not require DCs

A recent study reported that CD8α$^+$ DCs are the dominant APC type for activating *i*NKT cells with injected Ags (18–21, 37). Therefore, we addressed whether DCs also are necessary for the αGalCer-induced *i*NKT 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$^+$ and CD8$^+$ CD11c$^+$ DCs in the spleen within 24 h (Refs.11, 20 and Supplemental Fig. 4). αGalCer was injected into control and DC-depleted CD11c-DOG mice and 4 wk later the *i*NKT cell response was analyzed after rechallenge with αGalCer in vivo. However, *i*NKT 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^{high} DCs are not necessary to induce αGalCer-induced *i*NKT cell hyporesponsiveness.

Discussion

Initial activation of *i*NKT 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 *i*NKT cell hyporesponsiveness. First, our data demonstrate that Th0- and Th1-biasing GSL Ags can induce *i*NKT cell hyporesponsiveness, but not a Th2-biasing Ag or cytokine-driven *i*NKT cell activation owing to TLR engagement as a result of LPS exposure or infections. Second, although presentation of αGalCer by hematopoietic cells can induce *i*NKT cell hyporesponsiveness, we did not find a nonredundant function either for B cells or DCs for these changes.

Induction of *i*NKT 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

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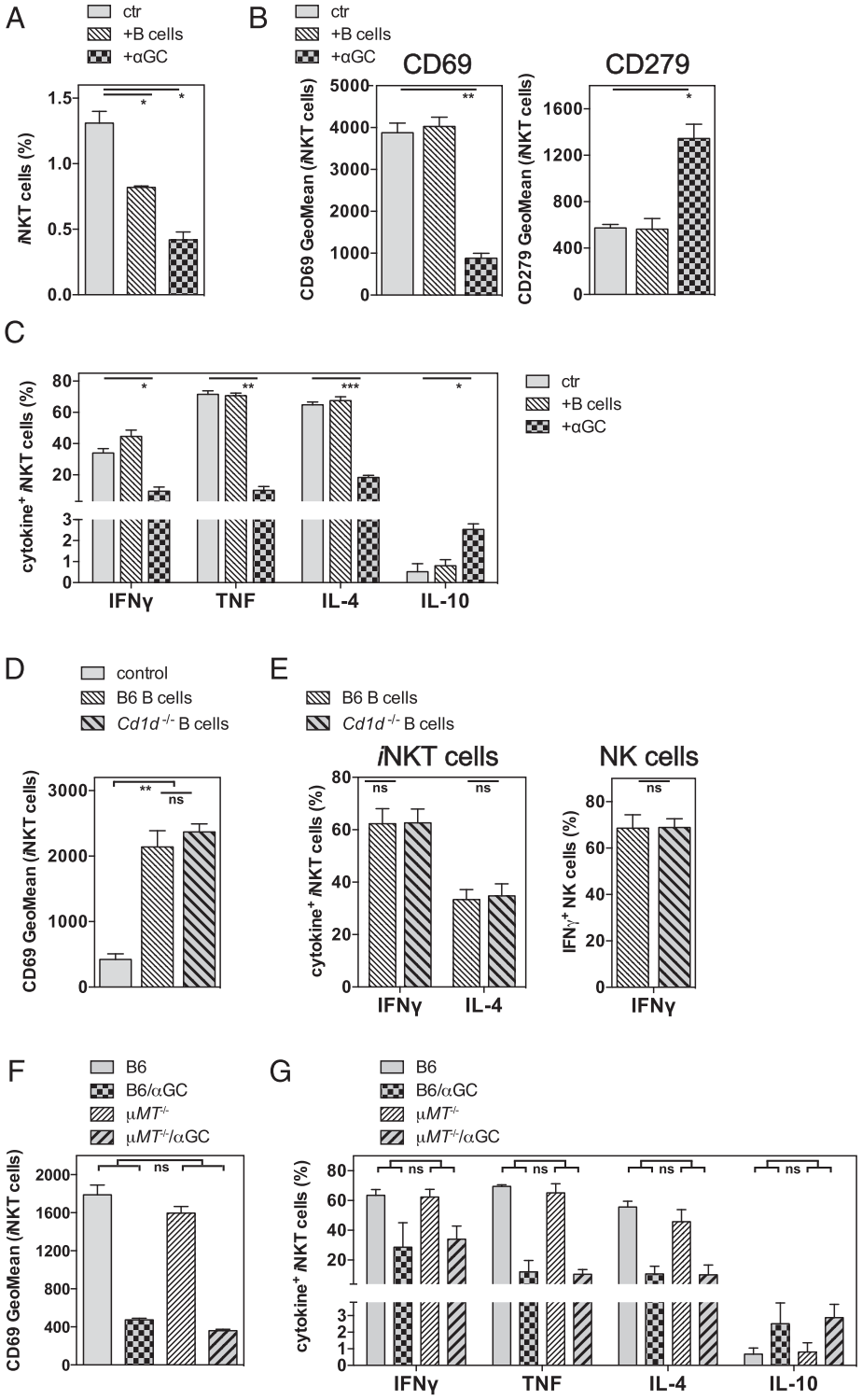


FIGURE 7. B cells are not required to induce *i*NKT cell hyporesponsiveness. (**A**–**C**) C57BL/6 (B6) mice were either left untreated (ctr) or injected i.v. with 4 μg αGalCer (+αGC) or with 5×10^6 αGalCer-loaded (250 ng/ml, 2 h) B cells (+B cells) as indicated. One month later mice were injected i.v. with 1 μg αGalCer, and 90 min later splenic *i*NKT cells were analyzed for frequency (A), the expression of surface makers (B), and of intracellular cytokines (C). Statistically significant differences of treated groups versus the control group are indicated. (**D** and **E**) C57BL/6 mice were either left untreated (control) or injected i.v. with 5×10^6 αGalCer-loaded (250 ng/ml, 2 h) B cells derived from C57BL/6 (B6) or $Cd1d^{-/-}$ mice as indicated. Fifteen hour later purified splenocytes were incubated for 2 h in vitro in the presence of protein transport inhibitors before *i*NKT cells (D and E) and NK cells (E) were analyzed for the expression of surface makers (D) and intracellular cytokines (E). (**F** and **G**) C57BL/6 (B6) or $\mu MT^{-/-}$ 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 *i*NKT cells were analyzed for the expression of CD69 (F) and of intracellular cytokines (G). Statistically significant differences (ANOVA) of $B6_{(\text{control versus } \alpha GC)}$ versus the $\mu MT^{-/-}{}_{(\text{control versus } \alpha GC)}$ groups are indicated. Representative data from one of at least two independent experiments are shown. $^*p < 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

(9, 17) and the plakoside A analogs EF77 and SMC124 (7–9, 12). In contrast, the Th2-biasing compound OCH (16, 22) did not induce *i*NKT cell hyporesponsiveness (Figs. 2A, 2B, 3A, 3B). It has been suggested that the ability of an Ag to induce *i*NKT cell hyporesponsiveness correlates with its antigenic strength (23, 38). However, our data do not support this model. First, C-Gly was able induce long-term *i*NKT cell hyporesponsiveness, whereas OCH, which has more avid binding to the *i*TCR when complexed to CD1d than C-Gly, was not able to induce hyporesponsiveness (Figs. 2A, 2B, 3A, 3B). Second, a strong activation of *i*NKT cells with an agonistic anti-CD3ε Ab, either once or repetitively, did not lead to *i*NKT cell hyporesponsiveness (Fig. 2C, 2D, 3D, 3F). Rather, our data indicated that the induction of long-term *i*NKT cell hyporesponsiveness is a particular feature of Th0- and Th1-biasing *i*NKT 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 *i*NKT cell Ags (39–41), but not a Th2-biasing Ag (39), may induce long-term *i*NKT 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 *i*NKT cell response only seven days after the initial challenge, the timing of the analysis

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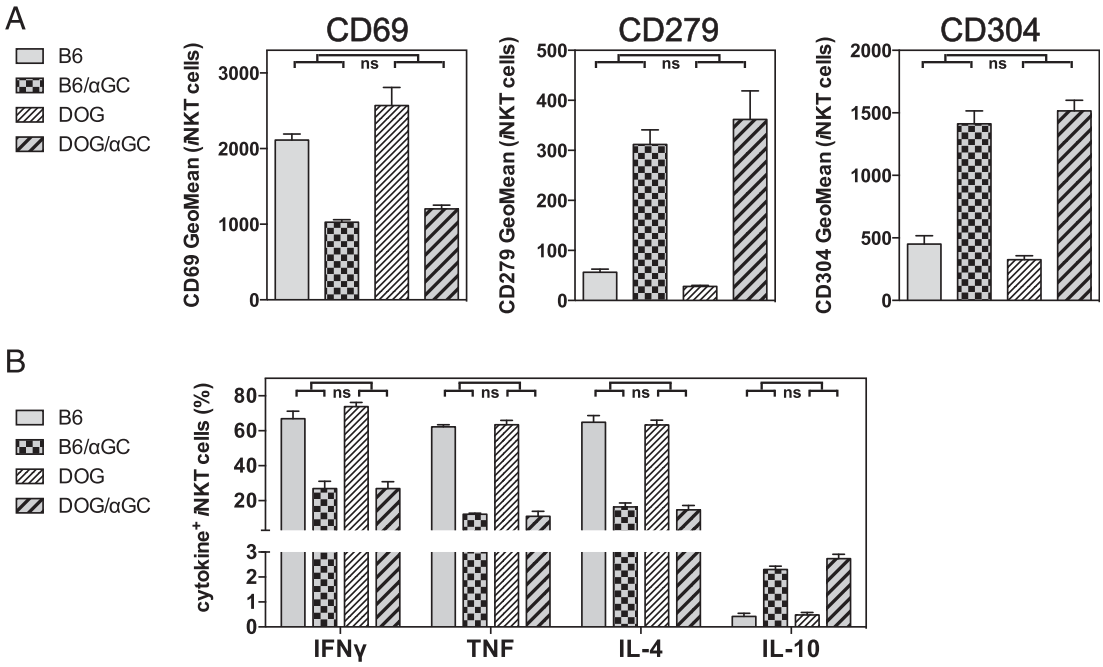


FIGURE 8. *i*NKT cell hyporesponsiveness does not require DCs. (**A** and **B**) CD11c-DOG mice were depleted <95% of $CD4^+$ and $CD8^+$ $CD11c^+$ DCs in the spleen as described. C57BL/6 (B6) or DC-depleted CD11c-DOG (DOG) mice were either left untreated or injected i.v. with 4 μg αGalCer (αGC). One month later mice were injected i.v. with 1 μg αGalCer, and 90 min later splenic *i*NKT cells were analyzed for the expression of surface markers (A) and of intracellular cytokines (B). Statistically significant differences (ANOVA) of $B6_{(\text{control versus } \alpha GC)}$ versus the DC-depleted $DOG_{(\text{control versus } \alpha GC)}$ 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 *i*NKT cell Ag C20:2 can induce a short-lived hyporesponsiveness in *i*NKT 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 *i*NKT cell hyporesponsiveness. Therefore, at this time the reason for the lack of *i*NKT cell hyporesponsiveness induced by the Th2-biasing Ag OCH is not known. It has been suggested that Th1-biasing Ags are characterized by prolonged *i*NKT 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 *i*NKT cells (12, 40, 43). Taken together, our data support a model whereby only Th0/1-biasing Ags have the capability to induce long-term *i*NKT 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 *i*NKT cells, as shown in the present study for C-Gly.

Besides Ag-driven activation via the TCR, *i*NKT 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 *i*NKT 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 *i*NKT cells hyporesponsiveness (data not shown). Taken together, these data support the conclusion that proinflammatory cytokines are not involved in the induction of αGalCer-induced *i*NKT cell hyporesponsiveness. In contrast, other reports suggested that some, but not all, bacterial infections could induce *i*NKT 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 *i*NKT 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 *i*NKT 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 *i*NKT 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 *i*NKT 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 $CD8\alpha^+$ DCs are the critical APC for

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the initial presentation of all Ags in vivo, irrespective of their Th1- or Th2-biasing properties (37). Nonetheless, the requirements for the immediate *i*NKT cell activation are not necessarily identical with the requirements for long-term effects leading to *i*NKT 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 *i*NKT cells (7). In contrast, when we analyzed the *i*NKT 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 *i*NKT cell phenotype and cytokine response (Fig. 7B, 7C). However, we noticed a tendency for a reduced *i*NKT 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 $\mu MT^{-/-}$ mice directly demonstrated that B cells are not required for the induction of *i*NKT cell hyporesponsiveness in vivo (Fig. 7F, 7G). We cannot exclude the possibility that the few B-1 cells remaining in $\mu MT^{-/-}$ mice (50, 51) could be responsible for the observed induction of *i*NKT cell hyporesponsiveness. However, we consider this unlikely, in light of the systemic nature of anergy induction (7, 9), the fact that *i*NKT 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 *i*NKT 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-DOG mice (11) indicated that $CD11c^{high}$ DCs are not required to induce αGalCer-induced *i*NKT cell hyporesponsiveness in vivo (Fig. 8). Collectively, our data demonstrate that although presentation of αGalCer by hematopoietic cells is sufficient to cause *i*NKT 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 *i*NKT 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 *i*NKT cell hyporesponsiveness in vivo. It is likely, however, that hyporesponsiveness by *i*NKT 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). How these changes might be correlated with a long-term decrease in the ability of *i*NKT cells to respond to Ag stimulation remains unknown.

In summary, we demonstrate in this study that Th0- and Th1-biasing Ags, but not a Th2-biasing Ags, can induce long-term *i*NKT 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 *i*NKT 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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