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Distinct APCs Explain the Cytokine Bias of α-Galactosylceramide Variants In Vivo

Li Bai,*†,1 Michael G. Constantinides,*†,1 Seddon Y. Thomas,*† Rachel Reboulet,*† Fanyong Meng,*† Frank Koentgen,‡ Luc Teyton,§ Paul B. Savage,¶ and Albert Bendelac,*†

α-Galactosylceramide represents a new class of vaccine adjuvants and immunomodulators that stimulate NKT cells to secrete Th1 and Th2 cytokines. Synthetic variants with short or unsaturated acyl chains exhibit a striking Th2 bias in vivo but no evidence of defect in TCR signaling or stimulation of NKT cells in vitro. Using cd1d−/− mice, we demonstrated that distinct APC types explained the cytokine bias in vivo. Whereas NKT stimulation by α-Galactosylceramide required CD1d expression by dendritic cells (DCs), presentation of the Th2 variants was promiscuous and unaffected by DC-specific ablation of CD1d. This DC-independent stimulation failed to activate the feedback loop between DC IL-12 and NK cell IFN-γ, explaining the Th2 bias. Conversely, forced presentation of the Th2 variants by DC induced high IL-12. Thus, lipid structural variations that do not alter TCR recognition can activate distinct Th1 or Th2 cellular networks by changing APC targeting in vivo. *The Journal of Immunology, 2012, 188: 3053–3061.

Natural killer T cells are CD1d-restricted T cells that express a conserved semi-invariant Vα14(huVα24)-Jo18/Vβ8.7,2(huVβ11) TCR (1, 2). They constitute a separate lineage of innate-like T cells characterized by the expression of the signature transcription factor promyelocytic leukemia zinc factor, which is induced during thymic development and directs the acquisition of an innate effector program (3, 4). This program includes the ability to produce both Th1 and Th2 cytokines upon primary stimulation and changes in homing and recirculation with permanent downregulation of the lymph node homing receptor CD62L, upregulation of the adhesion/migration receptor CD44, changes in chemokine receptor profile, and constitutive activation of the integrin LFA-1 (5).

Most NKT cells recognize microbial lipids characterized by a glycuronosyl group in α-linkage to a ceramide as well as the synthetic analog α-galactosylceramide (αGC) acC26:0 psC18:0 (termed αGC) (6). These agonist ligands induce the reciprocal activation of NKT cells and CD1d-lipid presenting cells through CD40L/CD40 interactions (7, 8), resulting in the secretion of Th1 and Th2 cytokines and the creation of a rich proinflammatory milieu that can markedly enhance adaptive T and B cell responses. Consequently, NKT ligands have often demonstrated superior adjuvant properties in vivo compared with TLR ligands (9, 10).

Intriguingly, extensive structure–function studies have identified synthetic variants of αGC with a marked Th2 bias and others with a Th1 bias, raising the prospect of their use for cytokine-specific adjuvant therapy as well as immunomodulation of disease (11–13). On the basis of the assumption that they differentially engaged the NKT cell TCR, the Th2 variants have been referred to as “altered glycolipid ligands” in some reports (11, 14) by analogy to altered peptide ligands with similar cytokine-biasing properties. This hypothesis has received some support for one of the leading ligands, αGC acC24:0 psC9:0 (also called OCH), which was shown to induce a conformational change of the F′ pocket of CD1d with corresponding diminution of the binding properties of the TCR (14, 15). OCH failed to induce sufficient c-rel mRNA and CD40L on NKT cells in vivo and elicited diminished IFN-γ but conserved IL-4 from NKT cells (16). Whereas αGC-mediated NKT stimulation indirectly induces NK cells to release a second, long-lasting wave of IFN-γ secretion in vivo (17), OCH conspicuously failed to recruit NK cells. Because the late IFN-γ is dependent of CD40-mediated IL-12 production by dendritic cells (DCs), it was concluded that altered TCR signaling impaired both direct and indirect secretion of Th1 cytokines (18).

Other well-studied Th2 variants such as αGC acC20:2 psC18 (termed αGC acC20:2) (12) and αGC acC8:0 psC18 (termed αGC acC8) (13) did not seem to fall into the same category, however, as detailed biophysical and crystallographic studies demonstrated that their interactions with CD1d and the NKT cell TCR were indistinguishable from those of αGC (14, 19, 20). Other properties of these strong agonists might therefore account for their marked Th2 bias. Recently, a unifying set of chemical and cell biological properties was identified for all Th2 ligands, including αGC acC20:2, αGC acC8, and OCH. Their short or unsaturated lipid chains conferred higher solubility in the aqueous environment, which allowed them to directly load CD1d at the cell surface, whereas, in contrast, αGC and other Th1 ligands needed endosomal loading by lipid transfer proteins (20–22). Furthermore, after recycling of CD1d in the lysosomal compartment, the Th2
variants were displaced within seconds by endogenous lipids in a pH-dependent manner, whereas αGC remained stably associated with CD1d (22). As CD1d recycles actively between the cell surface and the lysosome (23), cells pulsed with short and unsaturated lipids lost their ability to stimulate NKT cells in culture faster than those pulsed with αGC (22). In addition, CD1d-αGC complexes were shown to localize preferentially to lipid rafts at the cell surface, whereas the “Th2” variant αGC aceC20:2 was largely excluded (20). These common cell biological properties of the Th2 variants suggested several possible mechanisms to explain their functional differences in vivo. For example, the location of CD1d-lipid complexes on membrane lipid rafts might somehow alter NKT cell stimulation toward more IFN-γ production or indirectly favor NK cell recruitment. IFN-γ production might also be selectively impaired upon interruption of TCR signaling because of the fast dissociation of CD1d complexed with short and unsaturated lipid ligands. In this study, we directly tested a third possibility, that the Th2 bias of the αGC variants in vivo might be the consequence of different APCs.

Materials and Methods

Mice

Cd19-Cre (B6.129P2-Cd19<sup>tm1(cre)Kgn</sup>), Lyz2-Cre (B6.129P2-Lyz2<sup>tm1(cre)Kgn</sup>), Cd4-Cre (B6 Tg(cd4-cre)1Cwi), and C57BL/6J mice were from The Jackson Laboratory. Cd11c<sup>−/−</sup> mice were obtained from Dr. A. Chervonsky at our institution. Cd4p<sup>Vα14-Jα18</sup> transgenic mice (25) were maintained in the laboratory. All mice were raised in a specific pathogen-free environment at the University of Chicago, and experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee.

Generation of B6.cd1d<sup>fl/fl</sup> mice

The mouse cd1 locus includes two cd1d genes, cd1d1 and cd1d2, which are 95% identical. Because cd1d2 is a pseudogene in the C57BL/6 (B6) strain (26), we used homologous recombination to flank exons 2 and 6 of

![FIGURE 1. Characterization of cd1d<sup>fl/fl</sup> mice.](http://www.jimmunol.org/Downloadedfrom)
ed1d1 with loxP sites in an ES line of B6 origin. After crossing to B6 mice expressing the Flp recombinase to remove the flrt-flanked Pga-neomycin sequence used for in vitro selection of recombined ES cells, the B6.ed1d1fl/fl conditional mutant line was generated.

B6.ed1d1fl/fl mice were crossed to cd19-cre, cd11c-cre and ly2-cre to obtain cd19 ΔΔ, cd11c ΔΔ, and ly2 ΔΔ/ΔΔ mice. Littermate controls included Cre-negative littermates and in some cases Cre-positive ed1d1fl/fl or Cre-positive ed1d1fl/+ littermates.

PCR protocol for typing the ed1d1fl/+ and the cre mice

B6.ed1d1fl/+ mice were genotyped by PCR (forward primer, 5'-ATG TAT CCA GAG TTT GTA CTT GGA A-3', reverse primer, 5'-TGG GGT CCA TTC CAG ATG CAA A-3'). Mice with loxP sites generated a 351-bp fragment instead of a 156-bp fragment in wild-type mice. Cre-deleter mice were genotyped by PCR with primers (forward primer, 5'-TGA CCG GTC GAT GCA ACG AGT-3'; reverse primmer, 5'-TTC CAT GAG TAC AGC AAC CTG G-3') that generated a 400-bp fragment.

Flow cytometry

Splenic lymphocytes and thymus cells were isolated by mincing and passing through a 70-μm nylon cell strainer (Falcon). Hepatic lymphocytes were further purified by running through Percoll gradient (Sigma-Aldrich). Fluorochrome-labeled mAbs against mouse CD1d (1B1), F4/80 (BM8), CD11c (N418), CD11b (M1/70), CD24 (M1/69), Aβ (53-6.7), DX5 (DX5), CD40L (MR1), IL-4 (RA3.6B2), F4/80 (BM8), CD11c (N418), CD11b (M1/70), CD24 (M1/69), CDS (53-7.3), TCR BVβ (TR310), TCR BVβ 1.8+8.2 (MR5-2), TCRβ (H57-597), CD4 (GK1.5), CD8α (53-6.7), DX5 (DX5), CD40L (ML-1), IL-4 (11B11), IFN-γ (XMG1.2), and IL-12p40 (C15.6) were further purified by running through Percoll gradient (Sigma-Aldrich). Fluorochrome-labeled mAbs against mouse CD1d (1B1), B220 (RA3.6B2), F4/80 (BM8), CD11c (N418), CD11b (M1/70), CD24 (M1/69), Aβ (53-6.7), DX5 (DX5), CD40L (ML-1), IL-4 (11B11), IFN-γ (XMG1.2), and IL-12p40 (C15.6) were purchased from eBioscience or BioLegend. CD1d-PBS57 tetramers were obtained from the National Institute of Allergy and Infectious Diseases Tetramer Core Facility.

For intracellular cytokine staining, cells were stained with cell surface mAbs against mouse CD1d (1B1), B220 (RA3.6B2), F4/80 (BM8), CD11c (N418), CD11b (M1/70), CD24 (M1/69), Aβ (53-6.7), DX5 (DX5), CD40L (ML-1), IL-4 (11B11), IFN-γ (XMG1.2), and IL-12p40 (C15.6) were purchased from eBioscience or BioLegend. CD1d-PBS57 tetramers were obtained from the National Institute of Allergy and Infectious Diseases Tetramer Core Facility.

Characterization of ed1d1fl/fl mice

CD1d1fl/fl mice (Fig. 1A) were produced by homologous recombination in an ES line of B6 origin. CD1d1fl/fl cd4-Cre mice (cd4

FIGURE 2. Comparison of αGC with αGC acC8 in vivo. B6 mice were injected i.p. with 2 μg αGC or 5 μg αGC acC8 unless otherwise indicated. (A) Serum IL-4 at 2 h and serum IFN-γ at 24 h. (B) Serum IL-12p70 at 6 h. (C) Splenic TCRβ+/CD1d-PBS57+ NKT cells collected at 1, 2, and 6 h as indicated and directly stained for intracellular IL-4 and IFN-γ. Results compiled from three separate experiments. (D) Spleen cells collected at 6 h, stained with anti-DX5 and anti–IFN-γ, and displayed after gating on TCRβ+B220 cells. Left, representative FACS plots; right, summary figures showing the frequency of IFN-γ-secreting cells among NK cells and their mean fluorescence intensity (MFI) for IFN-γ. Results compiled from three separate experiments. (E) Mice received five injections (at 0, 1, 2, 3, and 5 h) of 5 μg or one injection of 25 μg αGC acC8. Sera were collected at 6 h for IL-12p70, 24 h for IFN-γ, and 2 h for IL-4 and were compared with sera of mice receiving one injection of 2 μg αGC at 0 h. Results compiled from two separate experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

**Results**

Characterization of cd1d1fl/fl mice

CD1d1fl/fl mice (Fig. 1A) were produced by homologous recombination in an ES line of B6 origin. CD1d1fl/fl Cd4-Cre mice (cd4

Statistical analysis

Different groups were compared using two-tailed t test: *p < 0.05, **p < 0.01, and ***p < 0.001.
CD1d(DDEL) mice showed a 90% reduction of CD1d expression on cortical CD4+CD8+ thymocytes and a corresponding 75–86% reduction of NKT cell numbers in the thymus, spleen, and liver (Supplemental Fig. 1A). This result constitutes a direct demonstration that NKT cell development is exquisitely and solely dependent on CD1d expression by DP thymocytes, as previously suggested by bone marrow chimera and transgenic studies (28–30). Furthermore, as expected in the presence of low concentrations of CD1d ligands, the few residual NKT cells expressed a bias toward expression of Vβ7 (Supplemental Fig. 1B), which confers the highest affinity for endogenous CD1d ligands when paired with Vα14-Jα18 (31).

CD11c(DDEL) mice lacked CD1d on splenic DCs, which are CD11c(high), and also on macrophage subsets that express low levels of CD11c such as the F4/80+ red pulp macrophages (Fig. 1B). Ly2(DDEL) mice lacked CD1d on macrophage subsets including the F4/80+ red pulp macrophages but expressed normal levels on CD11c(high) DCs. Cd19(DDEL) mice lacked CD1d on B cells. In contrast with the cd4(DDEL) mice, the cd11c(DDEL), ly2(DDEL), and cd19(DDEL) mice exhibited normal development and distribution of NKT cells (Fig. 1C). Furthermore, the intrinsic cytokine secretion properties of NKT cells assessed by stimulation with PBS57-pulsed BMDCs in vitro were preserved, as was the rapid release of IL-4 upon injection of anti-CD3 in vivo, a characteristic properties of NKT cells (Fig. 1D, 1E) (32). Thus, the cd11c(fl/fl) mouse allowed deletion of CD1d in selective APC subsets without altering the frequencies or functional properties of NKT cells.

Comparison of αGC and αGC acC8 in vivo

The structure and nomenclature of αGC variants used in this study is shown in Supplemental Fig. 2. The Th2 bias of αGC variants with short acyl chain was previously defined in vitro in whole-spleen cell stimulation assays (13). A detailed comparison of their functional properties was performed in a time-course study in vivo. Unless otherwise indicated, mice were injected i.p. with doses of αGC and αGC acC8 that were previously shown to elicit similar activation of NKT cells in vivo, 2 μg for αGC and 5 μg for αGC acC8. Fig. 2A showing serum IL-4 and IFN-γ at their respective peaks at 2 and 24 h illustrates the opposite cytokine bias of αGC and αGC acC8. The Th2 bias of αGC acC8 correlated with the lack of detectable IL-12p70 and contrasted with the abundant amount released after αGC injection (Fig. 2B). These findings are superimposable to those previously reported for the other Th2 variants αGC acC20:2 and αGC psC9 (33).

Although the two lipids induced comparable intracellular IL-4 and IFN-γ in splenic NKT cells at 2 h, the cytokine secretion elicited by acC8 was shifted to earlier time points for acC8 and declined, whereas the cytokines induced by αGC were still increasing between 2 and 6 h (Fig. 2C). Splenic NK cell production of IFN-γ was markedly inferior for αGC acC8 compared with αGC both in frequency and in mean fluorescence intensity at 6 h (Fig. 2D).

Repeating the injections of αGC acC8 five times over a 5-h period or increasing the dose from 5 to 25 μg resulted in a further increase in IL-4 but completely failed to reverse the defect in IL-12 and IFN-γ, indicating that the Th2 bias was due to the dose or the duration of exposure to the lipid in vivo (Fig. 2E).

Further kinetic studies indicated that NKT cell stimulation by αGC was simply delayed by 2–3 h compared with αGC acC8, without significant alterations in the quality of this stimulation. For example, TCR downregulation and the induction of CD25, CD40L, and programmed death-1 (PD-1) all occurred earlier after injection of αGC acC8 than after αGC (Fig. 3). Full expression of PD-1 after αGC as reported by others (34) only occurred at 12 h, beyond the time frame of this experiment.

Differential APC requirements for αGC and αGC acC8 in vivo

Cd11c(DDEL) mice showed no detectable serum IL-4 or IFN-γ after injection of αGC, suggesting failure to activate NKT cells (Fig. 4A). This conclusion was confirmed by direct intracellular staining showing absent or massively decreased IL-4 and IFN-γ in splenic and liver NKT cells (Fig. 4B, 4C) and absent IFN-γ in splenic and liver NK cells (Fig. 4D, 4E). Thus, despite the broad tissue distribution of CD1d expression in mice, NKT cell stimulation was absolutely dependent on CD1d expression by CD11c-expressing cells.
Lyz2 Δ/Δ mice exhibited a modest but in some cases significant reduction of cytokines released in the serum or present in the cytosol of NKT and NK cells (Fig. 4). Taken together with the total ablation of NKT cell activation in cd11c Δ/Δ, Lyz2 Δ/Δ, and cd19 Δ/Δ mice and littermate controls, these results suggested that a cell type coexpressing cd11c and Lyz2 (e.g., a macrophage or DC subset) contributed partially to the presentation of αGC and might be preferentially involved in the serum release of IL-4.

Finally, no detectable changes were observed in cd19 Δ/Δ mice (Fig. 4), indicating that presentation by B cells was not significantly involved in NKT or NK cell activation.

Taken together, these findings established that NKT cell stimulation by αGC in vivo was strictly dependent on αGC presentation by DCs and also, to a lesser extent, a subset of CD11c+ lysM+ macrophages or DCs.

In striking contrast with αGC, the Th2 variant αGC acC8 induced equivalent stimulation in controls and in cd11c Δ/Δ mice (Fig. 5A, 5B). Although αGC acC8 does not induce the late (NK-dependent) IFN-γ in the 24-h serum, the intracellular IFN-γ produced by NKT cells was similar in cd11c Δ/Δ and littermate controls. Furthermore, Lyz2 Δ/Δ and cd19 Δ/Δ mice also showed the same stimulation as littermate controls. Thus, in keeping with the promiscuous ability of αGC acC8 to load CD1d at the cell surface independently of either lysosomal recycling or lipid transfer proteins, there was no dedicated APC-type required in vivo. Furthermore, the absence of CD1d expression by DC did not alter the pattern of IL-4 and IFN-γ production by NKT cells.

Other Th1 and Th2 αGC variants exhibited patterns similar to αGC and αGC acC8
Other Th1 variants such as αGC C-glycoside (35) and PBS57 (27) exhibited a dependence of CD1d expression by DC such as αGC, whereas αGC acC20:2 (12), another well characterized Th2 variant, followed the same pattern as αGC acC8 (Fig. 5C, 5D). These

**FIGURE 4.** APC requirement for αGC in vivo. All mice were injected with 2 μg αGC i.p. (A) Serum IL-4 at 2 h and serum IFN-γ at 24 h in cd11c Δ/Δ, Lyz2 Δ/Δ, and cd19 Δ/Δ mice and littermate controls. Data are normalized to the mean of littermate controls. (B) Gated TCRβ+CD1d-PBS57+ NKT cells in spleens of wild-type and Δ/Δ mice were directly stained for intracellular IL-4 and IFN-γ at 4 h postinjection. Top, Representative FACS plots; bottom, summary figures. (C) Same as (B) for liver NKT cells collected at 4 h. (D) DX5 and intracellular IFN-γ staining on gated TCRβ+ B220− spleen cells at 6 h postinjection. Left, Representative FACS plots; right, summary figure. (E) Results as in (D) for liver NK cells at 6 h. Results are compiled from two independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
results indicate that the Th1 bias of NKT ligands is tightly associated with their selective presentation by DC.

**Forced interaction of NKT cells with αGC acC8-pulsed DCs induced IL-12**

Previous studies indicated that the CD8α⁺langerin⁺ subset of DCs was the main cell type producing IL-12 after αGC injection in vivo (24, 36). As IL-12 secretion by DCs required CD40L expression by NKT cells (18, 37, 38) and the Th2 variants potently induced CD40L on NKT cells, one potential explanation for the failure of the Th2 variants to induce IL-12 might be that they were not presented by DCs in vivo or that the frequency of DC encounters was decreased because of promiscuous presentation by other, more numerous cell types. Direct intracellular staining for IL-12 confirmed that CD8α⁺ DCs were the main IL-12p40 expressing cell type at 6 h postinjection of αGC (Fig. 6A). This experiment clearly established that αGC acC8 also induced IL-12p40, albeit on far fewer DCs than αGC.

Furthermore, if the Th2 bias was simply because of a DC-intrinsic defect in presentation of αGC acC8, then injecting mice with a mixture of αGC and αGC acC8 should result in strong IL-12 production and NK cell activation. Fig. 6B and 6C demonstrated the opposite result (i.e., a strict dominance of the Th2 bias). Thus, the Th2 bias likely resulted from the diversion of NKT cells away from DC toward interactions with other αGC acC8-presenting cell types.

The nature of the preferred APCs for αGC acC8 and αGC was further investigated in vivo and in vitro. Two hours after the i.p. injection of lipid in vivo, different APCs were purified and immediately cocultured with the αGC-responsive IL-2–producing hybridoma DN32.D3 as a readout of Ag presentation. Whereas both DCs and F4/80 macrophages were effective presenters of αGC, B cells were not (Fig. 7A). In marked contrast, αGC acC8 was efficiently presented by B cells as well as by other cell types (Fig. 7B). In other experiments, CD11c⁺ DCs, B220⁺ B cells, and F4/80⁺ macrophages were purified from un.injected mice and incubated in vitro with fresh Vα14 transgenic NKT cells in the presence of lipid for 48 h. Notably, when forced to interact exclusively with DCs, αGC acC8 elicited the same amount of IFN-γ and IL-12 as αGC in this culture system, unambiguously demonstrating its intrinsic ability to elicit a Th1 profile (Fig. 7C). αGC acC8 also tended to elicit a little more IL-4 than αGC, but this 1.5-fold increase was not statistically significant. In this NKT-APC coculture assay, highly purified B cells were able to stimulate NKT cells when pulsed with αGalCer acC8, but little stimulation was observed with αGalCer, consistent with a relative defect in CD1d presentation of αGalCer by B cells. Furthermore, αGalCer acC8 induced markedly more IL-4 relative to IFN-γ when presented by B cells than by DCs, consistent with the importance of IL-12 production by DCs for positive feedback on IFN-γ production.

**Discussion**

The generation of mice carrying a conditional allele of cdll1 allowed a dissection of the role of different CD1d-expressing cells both in the development and in the peripheral activation of NKT cells. The results directly established the essential role of CD1d expression by thymocytes for NKT cell development, as previously suggested by bone marrow radiation chimera and CD1d transgenic experiments (28–30). They also demonstrated the dispensable role of CD1d expression by DCs, macrophages, or B cells for the development of NKT cells and the acquisition of their cytokine profile. The most novel insight derived from these studies was the in vivo demonstration of different APC requirements for αGalCer and its short and unsaturated acyl variants, which provided a simple and unifying explanation for the Th2 bias of the variants.

Whereas the presentation of αGC and other Th1 variants such as PBS57 and αGC C-glycoside was entirely restricted to DC/ macrophage cell types, presentation by DC/macrophages was negligible compared with other cell types after injection of the Th2 variants αGC acC8 and αGC acC20:2 in vivo. Because only DC and macrophages can produce the IL-12 that is critical for amplification of Th1 responses and the recruitment of NK cells, these findings are sufficient to explain the Th2 bias associated with the main variants studied so far.
The importance of αGC presentation by DC in vivo has been previously suggested, based on methods using a diphtheria toxin receptor transgenically driven by a cd11c promoter (24) or by a langerin promoter (36) to allow DC-specific killing after administration of diphtheria toxin. Notably, whereas serum IFN-γ was nearly absent, IL-4 was much less reduced in these experiments, contrasting with the total ablation of both cytokines in the cd11cΔΔ mice. The difference may reflect the role of macrophage subsets that were reportedly spared in the diphtheria toxin receptor models (24) but, as a result of their intermediate expression of CD11c, had lost CD1d expression in the cd11cΔΔ mice. The ablation of serum IFN-γ by mice lacking langerin+ DCs, which largely overlap with the CD8α subset and are located in the marginal zone of the spleen (36), further underscores the specialized role of these IL-12 producers in the recruitment of NK cells. In contrast, NKT cell production of both IL-4 and IFN-γ was preserved after deletion of langerin+ DCs, in keeping with the ability of other DC subsets to load αGC and stimulate NKT cells.

In the current study, the total ablation of NKT cell activation in cd11cΔΔ mice, as monitored by intracellular cytokines, unambiguously established that DCs, and possibly a small subset of macrophages, constituted the exclusive cell-type mediating a GC-induced stimulation in vivo. This selective presentation is at least in part a consequence of two peculiar features of αGC compared...
with the Th2 variants. αGC is transported by serum lipoproteins and its uptake by DCs and macrophages is mediated by the lipoprotein receptor (22, 39, 40). αGC requires lysosomal lipid transfer proteins, which are most active in DCs and macrophages, for CD1d loading (41, 42).

Although previous studies of B cell-deficient μMT mice suggested enhanced (24) or unmodified (43) stimulation by αGC, the cd19 ΔΔ mice demonstrated that B cell presentation was not significantly involved for systemic αGC-mediated stimulation. This finding was consistent with the inefficient capture and presentation of αGC by B cells when the lipid was injected alone and with its efficient presentation upon BCR-mediated uptake and endosomal delivery of Ag + lipid-coated beads (44). The uptake and presentation of the Th2 variants in vivo followed drastically different rules. The stimulation was entirely unaffected in cd11c ΔΔ mice compared with wild-type mice, demonstrating that NKT cells did not require professional APC for activation. This finding was fully consistent with previous studies, indicating that short or unsaturated αGC variants were rapidly loaded onto CD1d expressed at the cell surface and that lysosomal recycling was not only unnecessary but in fact was detrimental to sustained presentation (22). Although the removal of CD1d from DCs and macrophages did not impair the stimulation of NKT cells by the Th2 variants, our in vitro and in vivo experiments demonstrated that these cell types could present the Th2 variants efficiently. In fact, CD8α DCs loaded with αGC acC8 or αGC acC20:2 released the same amount of IL-12 as those loaded with αGC in the presence of fresh NKT cells. Conversely, B cells stimulated NKT cells more effectively when loaded with the Th2 variants than with αGC, and consistent with their inability to secrete IL-12, they induced relatively more IL-4 than IFN-γ compared with DCs.

Altogether, the results suggest that the overriding cause of the Th2 bias of the short and unsaturated variants of αGC is their wide ranging and promiscuous presentation by non–IL-12–producing cell types, which stems from their ability to directly load CD1d at the cell surface. This wide ranging presentation decreases the frequency of IL-12–producing DC. It is also elicited less IL-12 production by DCs, this was mostly because they induced relatively more IL-4 than IFN-γ.

mechanisms, however, although these would appear less likely to play a prominent role in the Th2 bias of αGC variants. We did observe that the Th2 variants induced a stimulation that was somewhat less persistent than αGC, but the difference reflected in part a simple time shift of 2–3 h because of the faster loading of these lipids, and the upregulation of CD40L, CD25, PD-1, IL-4, and IFN-γ by NKT cells was comparable for both types of lipids. Furthermore, repeated injections of the short acyl variant did not restore serum IL-12 or IFN-γ. Although the Th2 variants elicited less IL-12 production by DCs, this was mostly because of a decrease in the frequency of IL-12–producing DC. It is also possible that the preferential subcellular location of CD1d-αGC on lipid rafts may somehow enhance the recruitment of NK cells, although this effect would have to be independent of the ability to induce CD40L, IL-4, and IFN-γ in NKT cells or IL-12 in DCs. Furthermore, the injection of a mixture of αGC and αGC acC8, which would lead to preferential loading of DC by αGC and of other APCs by αGC acC8, showed clear dominance of the Th2 variant consistent with the diversion of NKT cells away from IL-12–producing DC.

In conclusion, the common physicochemical properties of the Th2 variants, mainly their increased solubility in aqueous compartment, not only underly their cell biological properties, but also in turn dictated their targeting to vastly different sets of APCs in vivo. αGC, which selectively targeted DC and macrophages, is emerging as a choice adjuvant for the cross-priming of CTLs. However, our results predict that the ability of αGC to directly recruit NKT cell help to B cells is likely compromised by inefficient B cell presentation in vivo. By contrast, although the Th2 variants may be inferior at CTL priming they should provide superior recruitment of NKT cells for cognate B cell help and Ab production. In addition, similar differences are likely to govern NKT cell responses to natural endogenous and exogenous lipid ligands differing in lipid length and saturation (45, 46). The cd11cΔΔ mouse provides therefore a powerful system to dissect the complex cellular interactions involved in NKT cell-mediated adjuvant effects in cellular and humoral immune responses.

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Disclosures

The authors have no financial conflicts of interest.

References


Legends to Supplemental Figures

Fig. S1. Loss of NKT cells in Cd4 Δ/Δ mice.

(A) Residual CD1d expression (MFI mean±SEM) in gated CD4\(^+\)CD8\(^+\) DP thymocytes of Cd4 Δ/Δ mice compared to WT thymocytes as indicated (upper histogram); FACS analysis of CD24\(^\text{low}\) CD1d-PBS57 tetramer\(^+\) NKT cells in the thymus and summary of NKT cell numbers in thymus, spleen and liver (normalized to WT=100\%) of individual WT and Cd4 Δ/Δ mice. (B) FACS histogram of V\(\beta\)7\(^+\) and V\(\beta\)8.1/8.2\(^+\) NKT thymocytes gated as in A in WT and Cd4 Δ/Δ mice (upper) and summary data (bottom).

Fig. S2. Structure and nomenclature of αGC variants used or referred to in this study.

The reference compound αGC has a 26C acyl (ac) chain and 18C phytosphingosine (ps) chain.
αGC (acC26:0 psC18:0)

PBS57

αGC acC8:0 (acC8:0 psC18:0)

αGC acC20:2 (acC20:2 psC18:0)

C-glycoside

OCH (acC24:0 psC9:0)