Reduction of CD1d expression in vivo minimally affects NKT-enhanced antibody production but boosts B-cell memory

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Abstract

The CD1d-binding glycolipid α-galactosylceramide exerts potent adjuvant effects on T-dependent humoral immunity. The mechanism is driven by cognate interaction between CD1d-expressing B cells and TCR-expressing type I CD1d-restricted NKT cells. Thus, far positive effects of α-galactosylceramide have been observed on initial and sustained antibody titers as well as B-cell memory. Following vaccination, each of these features is desirable, but good B-cell memory is of paramount importance for long-lived immunity. We therefore tested the hypothesis that CD1d expression in vivo differentially affects initial antibody titers versus B-cell memory responses. CD1d+/+ and CD1d−/− mice were generated and immunized with antigen plus CD1d ligand before analysis of cytokine expression, CD40L expression, initial and longer term antibody responses and B-cell memory. As compared with CD1d+/+ controls, CD1d−/− mice had equivalent numbers of total NKT cells, lower cytokine production, fewer CD40L-expressing NKT cells, lower initial antibody responses, similar long-term antibody responses and higher B-cell memory. Our data indicate that weak CD1d antigen presentation may facilitate good B-cell memory without compromising antibody responses. This work may impact vaccine design since over-stimulation of NKT cells at the time of vaccination may not lead to optimal B-cell memory.

Keywords: antibody, B lymphocyte, CD1d, memory, NKT cell

Introduction

Type I CD1d-restricted NKT cells are central regulators of adaptive immunity. In response to professional antigen-presenting cells (APCs) presenting CD1d/glycolipid antigen complexes, the semi-invariant TCR on NKT cells is activated and leads to production of numerous cytokines (1). NKT cell activation regulates the cellular (2, 3) and humoral (4–6) arms of the immune response. Consequently, NKT cells have been implicated in beneficial immune responses to cancer (7, 8) and infectious pathogens (9–12) and in harmful immune responses in allergy and asthma (13, 14).

We and others have reported that when NKT cells are activated with the CD1d-binding ligand α-galactosylceramide (α-GC), there is an enhanced specific antibody response to a co-administered T-dependent antigen (4–6). These results led to several investigations regarding the mechanisms by which NKT cells affect humoral immunity. Three different laboratories, including ours, reported that cognate interaction between CD1d-expressing B cells and NKT cells was necessary for NKT-enhanced antibody responses (15–17), while another showed that NKT cells could provide non-cognate B-cell help (18). The effects of NKT cells on B cells have also received attention and it is now known that NKT activation contributes to short-lived primary antibody responses, memory B-cell-driven recall responses and to the development of long-lived plasma cells (4, 6, 19). Collectively, these studies indicated that NKT cells can influence the differentiation of antigen-experienced B cells in the germinal center reaction, leading to emergence of memory B cells and development of long-lived plasma cells.

Following vaccination, good antibody titers sustained by bone marrow resident long-lived plasma cells (20) are desirable to allow immediate protection during a pathogenic insult. However, good memory B-cell responses are also desirable. In response to a repeat antigen exposure, antigen-specific memory B cells expand rapidly and differentiate into large numbers of plasma cells that secrete high-affinity antibody and further boost the protective titer (21). At present, it is not clear if the strength of CD1d expression and NKT activation affects these processes differentially.
In this study, we generated mice with high and low CD1d expression against a background of equivalent development, phenotype and intrinsic function of NKT cells. This allowed weak and strong NKT activation in vivo following α-GC administration. We demonstrate through immunizations and adoptive transfer procedures that weak NKT activation is associated with similar NKT-enhanced antibody responses but improved B-cell memory as compared with strong NKT activation. These results suggest that maximal activation of NKT cells may not be desirable during vaccination regimens intended to induce good B-cell memory.

Methods

Reagents

Nitrophenol-conjugated keyhole limpet hemocyanin (NP-KLH) and NP-BSA were purchased from Biosearch Technologies Inc. (Novato, CA, USA). α-GC was purchased from Axonra Inc. (Enzo Life Sciences, Plymouth Meeting, PA, USA). HRP-conjugated anti-igG1, anti-igG2b and anti-igG2c and biotin-conjugated-anti-igG6 were purchased from Southern Biotechnology (Birmingham, AL, USA). Fluorochrome-conjugated mAbs were purchased from BD Biosciences (San Jose, CA, USA) (CD1d, CD40L and TCRβ) and eBioscience (San Diego, CA, USA) (CD4, CD8, CD11c and CD19). The FcγR-blocking mAb (2.4G2) and unlabeled anti-Thy1.2 and anti-CD4 mAbs (for depletion) were purchased from BioXpress (West Lebanon, NH, USA). CD1d/α-GC tetramers were provided by the NIAID Tetramer Facility (Emory University, Atlanta, GA, USA). Phorbol myristate acetate (PMA) and ionomycin were purchased from Sigma Chemical (St Louis, MO, USA). PE-, B220-, MHCIi-, CD8- and NK1.1-specific microbeads were purchased from Miltenyi Biotech (Auburn, CA, USA).

Mice

Female C57Bl/6 CD45.2+/+ and C57Bl/6 CD45.1+/+ mice were purchased from the National Cancer Institute (Bethesda, MD, USA). IgHa mice were purchased from the National Cancer Institute (Bethesda, MD, USA). CD1d+/+ male CD1d−/− mice were bred in the Animal Resource Center at Emory University, Atlanta, GA, USA. Phorbol myristate acetate (PMA) and ionomycin were purchased from Sigma Chemical Co (St Louis, MO, USA). PE-, B220-, MHCIi-, CD8- and NK1.1-specific microbeads were purchased from Miltenyi Biotech (Auburn, CA, USA).

Isolation of splenocytes

Splenocytes were harvested into RPMI buffer and a single-cell suspension obtained by mechanical disruption. Erythrocytes were removed by incubation with ammonium chloride lysis buffer (0.16 M NH₄Cl, 0.17 M Tris-HCl, pH 7.4) for 2 min at 37°C. After washing in culture media, cell viability was confirmed as >98% by trypan blue exclusion. Cells were enumerated using a Nexcelom cell counter (Lawrence, MA, USA).

Flow cytometry

Cells were incubated at 4°C (mAbs) or room temperature (tetrarmers) at a density of 10⁷ cells ml⁻¹ in RPMI plus 10% FCS with 2.4G2 mAb at a final concentration of 20 μg ml⁻¹. Fluorochrome-conjugated mAbs were added at a 1:100 to a 1:500 dilution as appropriate or with APC-conjugated CD1d tetramer at a 1:250 dilution. After 1 h, unbound mAb and tetramer was removed by washing and centrifugation. Cells were fixed with 1% w/v para-formaldehyde in PBS and analyzed using a Becton-Dickinson FACSCalibur (Palo Alto, CA, USA).

Cytokine assays

Two hundred microliters of splenocytes at a density of 10⁷ cells ml⁻¹ were added to round-bottom sterile microtiter plates and followed by addition of media or media containing α-GC at a final concentration of 50, 250 or 500 ng ml⁻¹. Plates were incubated under 5% CO₂ for 48 h before collection of supernatants, which were stored at −80°C until required. Samples were assayed using a Bio-Rad Bio-Plex multi-cytokine assay kit according to manufacturer's instructions (Bio-Rad, Hercules, CA, USA). Alternatively, NKT cells were enriched using Miltenyi microbeads in conjunction with an AutoMacs cell sorter. CD8+, B220+ and MHC II+ cells were removed by negative selection and NK1.1 cells enriched by positive selection. NKT cells constituted ~50% of the final preparation and NK cells constituted the remainder. Enriched NKT cells were stimulated with plate-bound anti-CD3 mAb and soluble anti-CD28 mAb for 48 h before collection of supernatants. Samples were analyzed using an IFNγ or IL-4 sandwich ELISA (BD Biosciences, Mountain View, CA, USA) because residual microbeads interfered with the Bio-Plex assay.
Immunizations and experimental schedule

Female mice of 6–10 week of age were used and five mice per group were immunized unless indicated otherwise. A single subcutaneous (s.c.) immunization was administered over both flanks on day 0 immediately following collection of pre-bleed sera. Unless indicated otherwise, immunizations consisted of 10 µg NP-KLH in 200 µl sterile endotoxin-free PBS or NP-KLH mixed with 4 µg of α-GC. Mice were then bled at day 28 post-immunization and sera obtained. On day 28, mice were bled and then boosted s.c. with 10 µg of NP-KLH in PBS and bled on day 35 unless indicated otherwise. Our previous publications have established this timeline as suitable for the experiments described herein (6, 16).

Retro-orbital eye bleed and serum preparation

Mice were anesthetized using a vaporized 4% isofluorane/96% oxygen mixture and 100 µl blood was collected by retro-orbital bleed with heparinized microcapillary tubes (Fisher Scientific, Hampton, NH, USA). Samples were transferred immediately to polypropylene micro-centrifuge tubes. Blood samples were incubated for 30 min at room temperature then allowed to clot overnight at 4°C before centrifugation at 13 000 × g for 15 min at 4°C. Sera were withdrawn with a pipette and stored in aliquots at −20°C.

Enzyme-linked immunosorbent assay

ELISA plates (Nunc, Maxisorp; Nalge Nunc Inc., Rochester, NY, USA) were coated with NP-19-BSA (Biosearch Technologies Inc., Novato, CA, USA) at 10 µg ml⁻¹ in binding buffer (0.1 M Na₂HPO₄, pH 9.0) overnight at 4°C. Plates were incubated for 1 h at room temperature with 1.0% w/v BSA in PBS/0.05% v/v Tween 20. Sera were diluted 100- or 10 000-fold in PBS/0.05% v/v Tween and subjected to 2-fold serial dilutions before adding to NP-KLH-coated pre-blocked plates. Plates were incubated overnight at 4°C with diluted sera before washing four times in PBS/0.05% v/v Tween 20. Plates were incubated for 1 h at room temperature with HRP-conjugated anti-mouse IgG1, IgG2b or IgG2c as a final concentration of 0.2 µg ml⁻¹. In some experiments, allelo-specific antibodies were detected using biotin-anti-IgH²⁺ (BD Biosciences) in conjunction with HRP-streptavidin (Jackson ImmunoResearch, Jacksonville, FL, USA). Plates were washed and developed for 5 min at room temperature using 90 µl of 2,2’-azino-bis (3 ethylbenzthiazoline-6-sulphonic acid) substrate per well (KPL, Gaithersburg, MD, USA). Reactions were stopped by addition of 110 µl of a 10% w/v SDS solution. Plates were analyzed using a Dynex MRX Revelation plate reader. End-point titers were determined as optical density (O.D.) < 0.01 at 405 nm (equivalent to O.D. of 1/200 dilution of pre-bleed sera). Individual antibody titers were plotted as geometric means using GraphPad Prism software. Differences in antibody titers between two experimental groups were assessed for statistical significance using a non-parametric Mann–Whitney U-test.

Adoptive transfers

Two distinct adoptive transfer procedures were used in this study for transferring B-cell memory (i) whole splenocytes isolated from donor mice that had been immunized 60 days previously with 10 µg NP-KLH mixed with 4 µg of α-GC s.c. Twenty five million cells in 100 µl sterile PBS were injected para-orbitally into CD45.1 congenic recipient mice. The recipient mice received a boost of 20 µg ml⁻¹ NP-KLH in PBS administered 24 h after the adoptive transfer. Sera were collected at 7 and 21 days post-boost. (ii) B-cell-enriched splenocytes (>90% B cells) were isolated from immunized donor mice. T cell and NKT cells were depleted by incubating the splenocytes on ice with 10 µg anti-Thy1.2 and 10 µg anti-CD4 mAb per spleen. The splenocytes were washed and then T and NKT cells were removed by complement-mediated lysis. Cells were incubated with 66 µg ml⁻¹ of LoTox rabbit complement (Cedarlane Laboratories, Burlington, NC, USA) for 45 min at 37°C. This method was reported by us previously and gives comparable B-cell purification to magnetic sorting (16). The cells were then washed in sterile PBS and counted. Twenty five million cells in 100 µl sterile PBS were injected para-orbitally into IgH²⁺ congenic recipient mice. The recipient mice received a boost of 20 µg ml⁻¹ NP-KLH 24 h after the adoptive transfer. Sera were collected at 7 and 21 days following administration of the booster vaccine.

Results

To allow experimental control of the number of CD1d/glycolipid complexes available in vivo, we bred female CD1d⁻/- C57Bl/6 mice to male CD1d⁻/- C57Bl/6 mice to generate CD1d⁻/- C57Bl/6 mice. We then genotyped the parents and offspring (Fig. 1A) and compared phenotypes of CD1d⁰⁺ and CD1d⁻/- mice (Fig. 1B–F). CD1d⁻/- mice expressed exactly 50% of the average number of CD1d molecules on the surface of splenocytes as compared with CD1d⁰⁺ controls (Fig. 1B). However, the frequency of NKT cells in the periphery was comparable between the two substrains (Fig. 1C). The absolute number of NKT cells was also comparable since the total number of splenocytes recovered from CD1d⁰⁺ and CD1d⁻/- mice was similar. We recovered 6.8 ± 0.66 × 10⁷ versus 7.4 ± 2.56 × 10⁷ total splenocytes, respectively (n = 7). Furthermore, NKT frequencies and numbers were comparable in the thymi of CD1d⁰⁺ and CD1d⁻/- substrains (Supplementary Figure 1 is available at International Immunology Online).

NKT cells from CD1d⁰⁺ and CD1d⁻/- mice exhibited comparable ratios of CD4⁺ and CD4⁻/CD8⁻ cells (Fig. 1D), comparable TCR Vβ gene usage (Fig. 1E) and comparable expression of CD69, CD62L and NKG2D (Fig. 1F). Importantly, unlike naive helper T cells, ‘naive’ NKT cells are constitutively CD62L⁺, CD69⁰⁺ and a substantial subset express the activation receptor NKG2D (23, 24). CD1d⁰⁺ and CD1d⁻/- mice were then treated with α-GC before collecting sera and measuring cytokine production (Fig. 1G). CD1d⁻/- mice had higher serum concentrations of IFNγ and IL-4 than CD1d⁰⁺ mice. These data show that CD1d⁻/- and CD1d⁻/- mice are a useful tool for controlling the amount of CD1d glycolipid presentation in vivo against a background of consistent NKT development and export to the periphery.

To confirm that NKT cells activated by CD1d⁻/- and CD1d⁻/- APCs had differential activation, we assessed IFNγ and IL-4 production following α-GC treatment in vitro (Fig. 2). Several studies have shown that IFNγ and IL-4 are produced directly
Fig. 1. Comparison of NKT cells from CD1d^{+/+} and CD1d^{-/-} mice. Female CD1d^{+/+} C57Bl/6 mice were bred to male CD1d^{-/-} C57Bl/6 mice to generate CD1d^{-/-} C57Bl/6 mice. (A) Shows sample PCR results to amplify Neo cassette and CD1d gene sequences in parents and offspring. (B–F) Splenocytes from age-matched female CD1d^{+/+} and CD1d^{-/-} C57Bl/6 mice were analyzed by flow cytometry as detailed in Methods. (B) Shows CD1d expression in splenocytes. (C) Shows staining and gating strategy to detect and enumerate type I α-GC-specific splenic NKT cells. (D) Shows expression of CD4 and CD8 by NKT cells (TCRβ/CD1d-tetramer gate applied). (E) Shows TCR Vβ expression by NKT cells. (F) Shows percent of NKT cells expressing markers of activation (CD69, NKG2D and CD62L). Mean fluorescence intensity of these markers was comparable (not depicted). (G) Shows IL-4 and IFNγ secretion following i.p. administration of α-GC (4 μg per mouse in 100 μl of PBS/0.05% polysorbate-20). IL-4 and IFNγ were measured at 6 and 22 h, respectively. Data in (A–F) are representative of four independent experiments. Graph in (C) shows pooled data from 10 mice per substrain. Data in (G) are representative of two independent experiments.
by NKT cells following α-GC stimulation (1). We stimulated splenocytes in vitro with α-GC and measured secreted cytokines after culture for 48 h (Fig. 2A and B). We observed that as compared with CD1d+/+ mice, CD1d+/- mice were seriously compromised in their ability to produce IFNγ and IL-4 following α-GC stimulation. As expected, α-GC was unable to stimulate IFNγ production by splenocytes from CD1d-/- controls.

To determine, if NKT cells from CD1d+/+ and CD1d+/- mice had the same intrinsic capacity for IFNγ and IL-4 production, we enriched NKT cells by magnetic sorting and stimulated them with anti-CD3 and anti-CD28 mAbs (Fig. 2C and D). Under these conditions, NKT cells from CD1d+/+ and CD1d+/- mice had a similar capacity for IFNγ production. IL-4 production was lower in the CD3/CD28-stimulated NKT cells from CD1d+/- mice than the CD1d+/+ controls. However, the response was not compromised as drastically as that observed for stimulation with CD1d+/- APCs. Enriched NKT cells, although CD1d expressing, do not respond to α-GC in the absence of APCs (24). As a further control, flow cytometry analysis of the splenocytes was performed before cell culture. Cells from CD1d+/+ and CD1d+/- mice had comparable numbers of CD1d-expressing APCs. Specifically, we observed 44.9 ± 2.0% versus 49.7 ± 2.4% CD1d/CD19-expressing cells (n = 3) and 1.33 ± 0.16% versus 1.31 ± 0.15% CD1d/CD11c+ cells (n = 3) for CD1d+/+ and CD1d+/- mice, respectively. These data show that NKT cells from CD1d+/+ and CD1d+/- have similar intrinsic function and that the number of available CD1d/antigen complexes largely determines the amount of NKT IFNγ and IL-4 production.

To further confirm differential NKT activation by CD1d+/+ and CD1d+/- APCs, we assessed expression of CD40L following α-GC treatment (Fig. 3). We observed that a larger frequency of NKT cells in CD1d+/+ mice expressed CD40L following in vitro α-GC treatment than NKT cells from CD1d+/- mice (Fig. 3). The α-GC treatment did not induce a significant increase in CD40L expression by T cells suggesting that CD1d antigen presentation does not directly influence CD40L-mediated B cell help by T cells. In an additional control experiment, we treated splenocytes from CD1d+/+ mice

Fig. 2. CD1d antigen presentation regulates IFNγ and IL-4 secretion by NKT cells. (A, B) Splenocytes from CD1d+/-, CD1d+/- and CD1d-/- C57Bl/6 mice were cultured with media (vehicle) or with α-GC-containing media for 48 h. (C, D) NKT cells were enriched using magnetic beads and cultured for 48 h with anti-CD3 and anti-CD28 mAbs. Culture supernatants were harvested and cytokine concentrations measured. Data show the mean ± SD IFNγ and IL-4 concentrations in the cell supernatants. Data are representative of four independent experiments.
and CD1d\(^{+/+}\) mice with PMA/ionomycin to bypass CD1d glycolipid presentation and TCR engagement. We then assessed CD40L expression and observed that it was equivalently expressed on untreated and PMA/ionomycin-treated NKT cells and T cells from CD1d\(^{+/+}\) and CD1d\(^{+/−}\) mice (Supplementary Figure 2 is available at International Immunology Online). This shows that NKT cells and T cells from CD1d\(^{+/+}\) and CD1d\(^{+/−}\) mice had the same intrinsic capacity for CD40L expression and provision of B-cell help. These data demonstrate that CD1d glycolipid presentation determines the number of NKT cells expressing CD40L.

Having previously established that CD1d glycolipid presentation of α-GC could boost antibody responses to T-dependent antigens (5, 6), we tested the effect of altering the number of CD1d molecules expressed by APCs. We compared anti-NP antibody responses in CD1d\(^{+/+}\) and CD1d\(^{+/−}\) mice immunized with NP-KLH/α-GC and boosted with NP-KLH (Fig. 4A). We observed that α-GC was better able to boost primary responses in the CD1d\(^{+/+}\) than in the CD1d\(^{+/−}\) mice. Antibody responses obtained 1 week after the day 28 booster were similarly boosted more efficiently by α-GC in CD1d\(^{+/+}\) than in CD1d\(^{+/−}\) mice (Fig. 4B). Further examination of titers on day 140 after the initial immunization showed no significant differences between CD1d\(^{+/+}\) and CD1d\(^{+/−}\) mice. We reported previously that antibody production is not affected by administration of α-GC to CD1d\(^{+/−}\) mice (5, 6). We did not observe differences in antibody subclass with IgG1 remaining dominant under all experimental conditions (data not shown). IgG2b and IgG2c titers accounted for 8 and 3% of the total titer, respectively, and were not significantly different between CD1d\(^{+/+}\) and CD1d\(^{+/−}\) mice. IgG2a was not assayed since C57Bl/6 mice express IgG2c rather than IgG2a (25). These results indicate that high CD1d expression levels result in higher initial antibody titers than lower CD1d expression levels. However, long-term antibody production is similar, showing that induction of long-lived plasma cells is not significantly impacted by low versus high expression of CD1d.

We then determined if long-term B-cell memory was affected by the amount of CD1d glycolipid presentation. The most reliable method for examining B-cell memory was to

**Fig. 3.** CD1d antigen presentation regulates CD40L expression by NKT cells. Splenocytes from CD1d\(^{+/+}\) and CD1d\(^{+/−}\) C57Bl/6 mice were cultured with media (vehicle) or with α-GC-containing media for 18 h. Cells were then harvested and stained with anti-TCR\(β\) mAb, α-GC-loaded CD1d tetramer and anti-CD40L mAb and analyzed by flow cytometry. Plot on left demarcates NKT cells (red gate) and T cells (black gate). Data on right show TCR\(β\) versus CD40L expression with and without α-GC treatment. Data are representative of three independent experiments.
determine if there was a difference in the transferable memory-driven antibody response between CD1d$^{+/+}$ and CD1d$^{+/−}$ mice. CD1d$^{+/+}$ and CD1d$^{+/−}$ C57Bl/6 mice were bled and then immunized s.c. with 10 µg NP-KLH or NP-KLH plus 4 µg α-GC. After 28 days, mice were bled (A, primary) and given a booster vaccine (10 µg NP-KLH s.c.) before collecting sera on day 35 (B, secondary) and day 140 (C, tertiary). Each data point represents the end-point anti-NP IgG1 titer for a single mouse. Data are representative of three independent experiments.

**Fig. 4.** Similar antibody titers in CD1d$^{+/+}$ and CD1d$^{+/−}$ mice. CD1d$^{+/+}$ and CD1d$^{+/−}$ C57Bl/6 mice were bled and then immunized s.c. with 10 µg NP-KLH or NP-KLH plus 4 µg α-GC. After 28 days, mice were bled (A, primary) and given a booster vaccine (10 µg NP-KLH s.c.) before collecting sera on day 35 (B, secondary) and day 140 (C, tertiary). Each data point represents the end-point anti-NP IgG1 titer for a single mouse. Data are representative of three independent experiments.

**Fig. 5.** Comparison of transferable B-cell memory in immunized CD1d$^{+/+}$ and CD1d$^{+/−}$ mice. CD1d$^{+/+}$ and CD1d$^{+/−}$ mice were immunized with NP-KLH plus α-GC and after 60 days, splenocytes were harvested. (A) Total splenocytes were adoptively transferred to CD45.1 C57Bl/6 recipients before administration of NP-KLH and collection of sera after 7 and 21 days. ELISAS were performed to determine end-point anti-NP IgG1 titers. (B) B cells were adoptively transferred to IgH$^a$ C57Bl/6 congenic mice before administration of NP-KLH and collection of sera on day 21. ELISAS were performed to determine NP-specific IgH$^a$ antibody responses. NP-specific IgH$^a$ was not detected (not depicted). Data show (A) mean ± SEM end-point titer or (B) absorbance at a one in 200 dilution of sera for three mice per group.

**Fig. 5A.** Diagram showing the experiment for transferable B-cell memory in immunized CD1d$^{+/+}$ and CD1d$^{+/−}$ mice. CD1d$^{+/+}$ and CD1d$^{+/−}$ mice were immunized with NP-KLH plus α-GC and after 60 days, splenocytes were harvested. (A) Total splenocytes were adoptively transferred to CD45.1 C57Bl/6 recipients before administration of NP-KLH and collection of sera after 7 and 21 days. ELISAS were performed to determine end-point anti-NP IgG1 titers. (B) B cells were adoptively transferred to IgH$^a$ C57Bl/6 congenic mice before administration of NP-KLH and collection of sera on day 21. ELISAS were performed to determine NP-specific IgH$^a$ antibody responses. NP-specific IgH$^a$ was not detected (not depicted). Data show (A) mean ± SEM end-point titer or (B) absorbance at a one in 200 dilution of sera for three mice per group.
thus compounded effects of transferred B-cell memory and (ii) serum antibody measurements were not specific for donor-derived antibody. We therefore transferred donor B cells expressing the IgH\textsuperscript{a} allele into IgH\textsuperscript{b} congenic mice. We observed that a greater donor-derived memory-driven antibody response was observed in recipients of cells from CD1d\textsuperscript{+/−} donors compared with CD1d\textsuperscript{+/+} donors following administration of the NP-KLH booster (Fig. 5B). This confirmed that the increased antibody response was attributable to donor-derived memory B cells.

We performed ELISA analyses, whereby plates were coated with BSA with different numbers of NP hapten per protein molecule (NP-3-BSA and NP-26-BSA). This allowed us to distinguish between high-affinity and total NP-specific antibody. By comparing A405 values for binding of the same samples to NP-3-BSA and NP-26-BSA, we observed that 75.5 ± 22.5% of the O.D. was accounted for by high-affinity antibody in samples from CD1d\textsuperscript{+/−} mice. In contrast, samples from CD1d\textsuperscript{+/−} mice had a value of 94.6 ± 13.0% and those from CD1d\textsuperscript{+/+} mice had a value of 58.7 ± 24.8%. This indicates that the average affinity of the antibody in the memory B-cell population was marginally affected by CD1d expression, but the differences were not significant.

### Discussion

We have demonstrated that NKT activation with the CD1d-binding glycolipid α-GC can differentially affect initial antibody responses and B-cell memory. This suggests that vaccines could and perhaps should be tailored to ensure optimal NKT activation for inducing protective antibody as well as long-term B-cell memory.

When NKT cells were subjected to a weaker stimulation in vivo using CD1d\textsuperscript{+/−} APCs initial antibody responses were lower than that observed for CD1d\textsuperscript{+/+} controls. Antibody titers following administration of a booster vaccine were lower the CD1d\textsuperscript{+/−} mice, but the responses were similarly sustained. While CD1d-dependent NKT activation boosts antibody responses and the induction of long-lived plasma cells (4–6), the data herein indicate that weaker NKT stimulation does not adversely affect antibody production or its longevity. In contrast, weaker NKT stimulation led to enhanced B-cell memory as compared with the control group. This was evidenced by greater transferable B-cell memory.

Analysis of antibody in serum samples following adoptive transfer suggest that somatic hypermutation in germinal centers is not greatly affected by the strength of NKT activation. A preliminary experiment suggested that the number of IgD−/antigen-binding memory B cells emerging from the germinal center is higher in the CD1d\textsuperscript{+/−} mice than in the CD1d\textsuperscript{+/+} mice (Lang et al., unpublished observation). We therefore propose that NKT cells may affect the number of B cells entering or emerging from the germinal center but without having direct effects on somatic hypermutation. Further study of this issue is warranted.

T\textsubscript{H}2 cytokines are well recognized to promote production of IgG1, the dominant isotype in our experiments. NKT-derived IL-4 correlated with IgG1 production (by comparison of responses in CD1d\textsuperscript{+/−} and CD1d\textsuperscript{+/+} mice). However, we expect that NKT-derived IL-4 is dispensable because bone marrow chimera experiments, whereby NKT cells did not express IL-4 revealed similar IgG1 titers to IL-4-expressing controls (Devera and Lang, unpublished observations). Furthermore, chimeras whereby NKT cells could not express IFN\textgamma did not reveal significant effects on production of IgG1, although modest effects on other subclasses may occur (Devera and Lang, unpublished observations). It therefore appears that NKT-derived cytokines do not significantly impact NKT-enhanced antibody responses.

CD40L, a member of the tumor necrosis factor (TNF) superfamily, is best known for its up-regulation on CD4\textsuperscript{+} helper T cells following activation by class II/peptide and co-stimulatory molecules on the APC (27). Engagement of CD40 by CD40L results in trimerization (28), inducing TNF-receptor-associated factor-dependent signaling (29–31) and exerting multiple effects on B cells including germinal center formation, immunoglobulin isotype switch, somatic hypermutation, memory B-cell induction and differentiation of long-lived plasma cells (32). The importance of the CD40L/CD40 pairing in B-cell help is underscored by spontaneous mutations in CD40L, which cause hyper IgM syndrome, resulting in a failure in isotype switch, somatic hypermutation and induction of B-cell memory (33–35). However, while CD40L is required for B-cell memory induction, an overabundance of CD40 signaling can effectively abrogate the response (36–38). Although CD40L can be expressed by CD1d/α-GC-activated NKT cells (39, 40), the fact that overstimulation of CD40L can inhibit B-cell memory, indicates that any role of NKT cell CD40L in humoral immunity cannot be predicted solely based on its known role in helper T cell function.

We previously reported that a combination of ovalbumin peptide (OVA), α-GC and an agonistic anti-CD40 mAb could induce an OVA-specific IgG response in class II-null mice lacking class II-restricted T cells (5). Another group confirmed our findings, using tetanus toxoid as the antigen, but did not require anti-CD40 mAb for an antibody response (4). The lack of an in vivo system whereby CD40L expression by the NKT cell can be experimentally controlled without preventing expression by other lineages has been a major impediment to determining if (and how) NKT-expressed CD40L regulates humoral immunity.

In the present study, we assessed expression of CD40L following stimulation with CD1d\textsuperscript{+/+} and CD1d\textsuperscript{+/−} APCs and by bypassing the APC using PMA and ionomycin as the stimulation. The results revealed that NKT cells in the CD1d\textsuperscript{+/−} and CD1d\textsuperscript{+/+} mice had similar intrinsic capacity for CD40L expression, but modestly lower expression of CD40L following stimulation with α-GC. NKT cells from stimulated CD1d\textsuperscript{+/−} mice therefore had a phenotype less synonymous with that of a ‘helper T cell’ than that of NKT cells from CD1d\textsuperscript{+/+} mice. The data are therefore suggestive that CD40L-expressing NKT cells could contribute to NKT-enhanced antibody responses. However, we have derived bone chimera mice, whereby NKT cells could not express CD40L. In that experiment, CD40L was dispensable for NKT-enhanced antibody production (Shah and Lang, unpublished data). This indicates that the mechanisms by which NKT cell provide B-cell help may be quite distinct from that of class II-restricted T\textsubscript{H} cells, which rely on CD40L for B-cell help. Studies are
ongoing in our laboratory to identify the NKT-derived factors that provide help for antibody production.

In summary, we propose that the mechanisms by which NKT cells enhance protective antibody responses following vaccination should be considered and that strategies may require calibrating NKT activation such that good B-cell memory is induced without compromising the production of circulating antibody that can offer immediate protection. The means to do this may not be immediately obvious since in a previous study, we reported that a lower dose of α-GC than used herein had a poor adjuvant effect on primary antibody responses but left recall responses intact and comparable to other adjuvants (6). This suggests that it is possible to direct NKT cells in vivo toward stimulation of B-cell memory. However, the challenge for researchers is to design NKT-based vaccination strategies that will induce optimal antibody production that is immediately protective while ensuring an adequate infection-stimulated memory response.

**Supplementary data**

Supplementary Figures 1 and 2 are available at *International Immunology* Online.

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**References**


