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CD40L-null NKT cells provide B cell help for specific antibody responses

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Abstract

CD1d-binding glycolipids exert potent adjuvant effects on T-dependent Ab responses. The mechanisms include cognate interaction between CD1d-expressing B cells and TCR-expressing Type I CD1d-restricted Natural Killer T cells (NKT). However, the critical NKT-derived factors that stimulate B cells are poorly understood. We tested the hypothesis that CD1d-driven CD40L expression by NKT cells influences humoral immunity. Bone marrow chimeras with $CD40L^{+/+}$ or $CD40L^{-/-}$ NKT cells were immunized with Ag plus CD1d ligand before measuring Ab responses. $CD40L^{-/-}$ NKT cells stimulated higher endpoint Ab titers than controls expressing CD40L. In contrast, immunization of $CD40L^{-/-}$ mice revealed that $CD40L^{-/-}$ NKT cells could not provide B cell help when Th cells lacked CD40L. We report that $CD40L^{-/-}$ NKT cells can provide help for Ab production and do so cooperatively with $CD40L^{+/+}$ Th cells. We suggest that the manner in which NKT cells provide B cell help is distinct from that of Th cells.

Keywords

Antibody; B lymphocyte; CD40L; NKT cell

Introduction

Type I CD1d-restricted NKT cells regulate adaptive immunity. In response to professional APC's presenting CD1d/glycolipid complexes, the semi-invariant TCR on NKT cells is activated and leads to cytokine production. NKT cell activation influences cellular [1] and humoral [2–4] immunity. Consequently NKT cells have been implicated in beneficial immune responses to cancer [5] and infectious pathogens [6], and in harmful immune responses in asthma [7].

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Author Contributions

H.S. designed and performed experiments, analyzed data and wrote the paper. S.J. performed experiments. M.L. designed the project, analyzed data and wrote the paper.

Conflict of Interest Disclosure

The authors declare no competing financial interests.

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There has been progress in understanding the mechanism by which NKT cells are induced to provide B cell help. NKT cells activated by the CD1d ligand alpha-galactosylceramide (α-GC) enhanced specific Ab response to a co-administered T-dependent Ag [2–4]. Cognate interaction between CD1d-expressing B cells and NKT cells appears necessary for NKT-enhanced Ab responses [8–10], but non-cognate help may also occur [11].

CD40L, a TNF super-family member, is best known for its up-regulation on Th cells following activation by class II/peptide and co-stimulatory molecules [12]. CD40 engagement by CD40L exerts multiple effects on B cells including germinal center formation, immunoglobulin isotype switch, somatic hyper-mutation, B cell memory and long-lived plasma cell differentiation [13]. The importance of CD40L/CD40 interactions in B cell help is underscored by spontaneous CD40L mutations which cause hyper-IgM syndrome, resulting in a failure in isotype switch, somatic hyper-mutation and B cell memory [14].

Several reports implicate CD40L in NKT-enhanced adaptive immunity [2, 4, 11, 15]. In two independent studies, NKT cells induced limited T-dependent Ab responses in class II-null mice lacking Th cells [2, 4]. In one study agonistic anti-CD40 mAb was required for Ab production [4]. In another, α-GC had no adjuvant effect on Ab responses in mixed bone marrow chimeras whereby NKT cells were $CD40L^{+/+}$ and B cells were $CD40^{-/-}$ [11]. Despite the available evidence, the absence of an in vivo system whereby only NKT cells lack CD40L expression has thus far impeded determining specifically whether NKT-expressed CD40L regulates humoral immunity.

Herein through immunization of $CD40L^{-/-}$ mice we confirm that $CD40L^{-/-}$ NKT cells cannot provide B cell help when Th cells lack CD40L expression. We then demonstrate through the use of mixed bone marrow chimeras that $CD40L^{-/-}$ as well as $CD40L^{+/+}$ NKT cells provide help for Ab production. These results demonstrate that $CD40L^{-/-}$ NKT provide B cell help for Ab production, but must do so cooperatively with $CD40L^{+/+}$ Th cells.

Materials and Methods

Mice

C57Bl/6 ($CD45.2^{+/+}$ and $CD45.1^{+/+}$) and $CD40L^{-/-}$ mice were from the National Cancer Institute (Bethesda, MD) and the Jackson Laboratory (Bar Harbor, ME) respectively. $J\alpha18^{-/-}$ mice were bred in the Animal Resource Center at the University of Oklahoma Health Sciences Center (OUHSC). All procedures were approved by the OUHSC Institutional Animal Care and Use Committee.

Flow Cytometry

Splenocytes and thymocytes were isolated as described previously [3]. Cells were incubated with media (containing: FCS, FcR-block; fluorochrome-conjugated mAbs; and CD1d tetramer), before washing, fixation and analysis with a Becton-Dickinson FACScalibur (Palo Alto, CA) [3]. Reagents were purchased from: BD Biosciences, San Jose, CA (anti-CD1d, -CD40L, -TCRβ); eBioscience, San Diego, CA (anti-CD4, -CD8, -CD11c and -CD19) and BioXcell, Lebanon, NH (anti-FcγR). CD1d/α-GC tetramers were provided by the NIAID Tetramer Facility (Emory University, Atlanta, GA).

Immunizations and serum collection

Mice were immunized *s.c.* (dose divided over both flanks) with 10 μg NP-KLH (Biosearch Technologies, Novato, CA) in 200 μl sterile endotoxin-free PBS or NP-KLH mixed with 4

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μg of α-galactosylceramide (α-GC, Axorra, Plymouth Meeting, PA) in PBS. Mice were bled at d 28 post-immunization and sera obtained. On d 28 mice were bled and then boosted with 10 μg of NP-KLH and bled again on d 35.

ELISA

Endpoint anti-NP Ig titers in serum were measured as described previously [3].

Bone Marrow Chimeras

Six weeks old C57Bl/6 CD45.1$^{+/+}$ mice were irradiated in split doses (700 then 500 Rad, 18 h apart). After a further 4 h, 10^6 donor bone marrow cells were transferred by the i.v. route to irradiated recipients. Donor cells consisted of 50:50 mixtures of: (i) Jα18$^{-/-}$ and C57Bl/6 cells; (ii) Jα18$^{-/-}$ and CD40L$^{-/-}$ cells. Recipients were engrafted for 12 wk before immunization.

Results

CD40L$^{-/-}$ NKT cells do not provide B cell help in the absence of CD40L$^{+/+}$ Th cells

As reported previously, the CD1d ligand α-GC exerts a potent adjuvant effect on specific Ab responses to T-dependent Ags (Figure 1A) ([3, 4, 9, 16]). When C57Bl/6 mice were immunized with NP-KLH alone or NP-KLH plus α-GC, NP-specific Ab titers were higher in the group receiving α-GC. The effect was significant in IgG1 titers as compared to IgM, IgG2b, IgG2c and IgG3 titers. Since CD40L is required for B cell help, experiments were performed to determine if NKT cells could stimulate Ab production in CD40L$^{-/-}$ mice.

Flow cytometry analysis revealed that thymic and splenic cells from CD40L$^{-/-}$ mice had a comparable frequency of TCRβ^{+}, CD1d-tetramer-binding NKT cell to C57Bl/6 controls (Figure 1B). Comparable numbers of thymocytes and splenocytes were also recovered from C57Bl/6 and CD40L$^{-/-}$ mice. CD40L expression was detected on NKT cells from C57Bl/6 mice but not CD40L$^{-/-}$ mice.

Following immunization with NP-KLH plus α-GC, CD40L$^{-/-}$ mice produced NP-specific IgM, but largely failed to produce IgG (Figure 1C). End-point NP-specific IgG1, IgG2b, IgG2c and IgG3 titers were significantly lower in CD40L$^{-/-}$ mice than in C57Bl/6 mice. These data show that CD40L$^{-/-}$ NKT cells did not provide B cell help when Th cells lacked CD40L expression.

CD40L$^{-/-}$ NKT cells provide B cell help in the presence of CD40L$^{+/+}$ Th cells

Mixed bone marrow chimeric mice were designed so that NKT cells were unable to express CD40L (Figure 2A). Flow cytometry revealed that >95% of splenocytes in the chimeric mice were donor-derived (Figure 2B). The Jα18$^{-/-}$/C57Bl/6 and Jα18$^{-/-}$/CD40L$^{-/-}$ chimeras had a similar frequency of B cell, T cells, and DCs (Figure 2C). The chimeras also had equivalent re-constitution of donor-derived NKT cells and expression of CD1d (Figure 2D). Re-constitution of NKT cells to the frequency observed in C57Bl/6 mice did not occur, but intact function and ability to enhance Ab responses has been demonstrated by our group [17].

Measuring relative engraftment of donor Jα18$^{-/-}$ versus C57BL/6 or CD40L$^{-/-}$ cells directly was not possible because all donor strains were CD45.2$^{+/+}$. C57Bl/6 (CD45.2$^{+/+}$) mice were therefore irradiated and transferred with a 50/50 mix of donor Jα18$^{-/-}$ (CD45.2$^{+/+}$) and CD45.1$^{+/+}$ donor cells. Engraftment was such that a 68/32 average ratio of CD45.2$^{+/+}$/CD45.1$^{+/+}$ cells was observed (Figure 2E). This shows that while NKT cells in

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Jα18$^{-/-}$/CD40L$^{-/-}$ chimeras could not express CD40L, the capacity for expression by Jα18$^{-/-}$-derived non-NKT cells was minimally affected.

Following immunization, endpoint anti-NP IgG1, IgG2b and IgG2c titers were measured (Figure 2F-H). IgG2a was not assayed since C57Bl/6 mice express IgG2c rather than IgG2a [18]. In the Jα18$^{-/-}$/C57Bl/6 mice, α-GC enhanced the primary IgG1 response similar to that observed in C57Bl/6 mice [3, 4], but had little impact on the secondary Ab response (Figure 2F). In contrast, α-GC significantly enhanced the primary and secondary IgG1 response following the booster in Jα18$^{-/-}$/CD40L$^{-/-}$ mice (Figure 2F). Perturbation of CD40L expression by non-NKT cells was not problematic because Ab responses in NP-KLH-immunized Jα18$^{-/-}$/C57BL/6 and Jα18$^{-/-}$/CD40L$^{-/-}$ chimeras were comparable. IgG1 was the dominant Ab titer, and α-GC did not significantly alter IgG2b or IgG2c titers (Figure 2G-H), consistent with data in C57Bl/6 mice. This shows that CD40L on NKT cells is dispensable for NKT-enhanced Ab production and may even limit Ab production.

Discussion

We have demonstrated that NKT cells, despite dependence on interaction with B cells via CD1d/TCR interaction [8–10] do not provide B cell help in a manner dependent on CD40L expression by NKT cells. We reported previously that CD40 ligation with agonistic mAbs could result in limited Ab production in Ag/α-GC-immunized class II$^{-/-}$ mice lacking Th cells [4]. We originally interpreted the result to suggest that CD40L on NKT cells could contribute to enhanced Ab responses. However, in Jα18$^{-/-}$/CD40L$^{-/-}$ chimeras, CD40L$^{-/-}$ NKT cells provided adequate help for Ab production following immunization with NP-KLH α-GC and the response was modestly enhanced as compared to controls. These two observations allow refinement of previous interpretations such that CD40L$^{-/-}$ NKT cells provide help for Ab production, but a CD40L signal from another source (Th cells) is required.

The enhancing effect of CD40L$^{-/-}$ NKT cells on the secondary Ab response may appear paradoxical because CD40L is required for induction of B cell memory and isotype switch to produce IgG. However, the amount of CD40L expression and therefore the number of CD40 molecules engaged on the B cell determines its fate. A heightened engagement of CD40 with agonistic mAbs inhibited B cell memory [19]. Constitutive expression of CD40L in transgenic mice led to diminished germinal center formation following immunization [20]. In this context, our data may be explained by a model in where CD40L expression by NKT cells is dispensable for NKT-enhanced Ab production and high expression of CD40L exerts a limiting effect on the response.

Since CD40L expression by NKT cells was dispensable for the α-GC-enhanced Ab response, we tested other candidate molecules known to be expressed by NKT cells. Using a similar mixed bone marrow chimera approach to that described herein, we were unable to assign a contribution to the inducible co-stimulator (ICOS) molecule *(data not shown)*, or to NKT-derived IL-4 or IFNγ [17]. ICOS-blocking mAbs were administered to the Jα18$^{-/-}$/C57Bl/6 and the Jα18$^{-/-}$/CD40L$^{-/-}$ chimeras since ICOS$^{-/-}$ mice are NKT-deficient. Under those conditions, there was no effect of ICOS blockade on the Ab response, but incomplete ICOS blockade could not be excluded. In a recently published study, we showed that NKT-derived IL-4 and IFNγ were dispensable for IgG1 responses against anthrax toxin, but could exert modulatory effects on IgG2c [17].

The surprising lack of effect of NKT expressed CD40L, ICOS or secreted IL-4 or IFNγ suggests either functional redundancy, whereby Th cells provide adequate amounts of several signals provided by NKT cells, or that NKT cells provide B cell help in a manner

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distinct from Th cells. This could include use of different receptor pairings between the B cell and NKT cell, different soluble factors including other cytokines, or a mechanism whereby NKT cells boost Th cell priming to increase B cell help. Thus far available information suggests room for each of these hypotheses since cognate and non-cognate interactions seem to be important for NKT-enhanced B cell help [2, 4, 8, 9, 11].

In previous studies, adoptive transfer of $CD1d^{+/+}$ versus $CD1d^{-/-}$ B cells into B cell-deficient μMT mice was used to demonstrate that CD1d expression by B cells was required for NKT-mediated help [9]. Production of Ab specific for NP-haptenylated α-GC was compromised in $B7.1^{-/-}$ and $CD28^{-/-}$ mice [10], but the lack of NKT development in those mice precluded using a bone marrow chimeric system. Bone marrow chimera approaches also showed that if class II and CD1d were expressed on different B cells then NKT cells could still provide B cell help [11], with the interpretation favoring non-cognate interactions. We therefore favor a hypothesis whereby CD1d expression by B cells is important for eliciting help from NKT cells but propose that help is not delivered directly, but perhaps through effects of NKT cells on Th function. Delineating these mechanisms is a current goal of our laboratory and will impact efforts to include NKT-activating ligands in vaccines.

Non-standard abbreviations

NKT	natural killer T cell
α-GC	alpha-galactosylceramide
KLH	keyhole limpet hemocyanin
NP	nitrophenol

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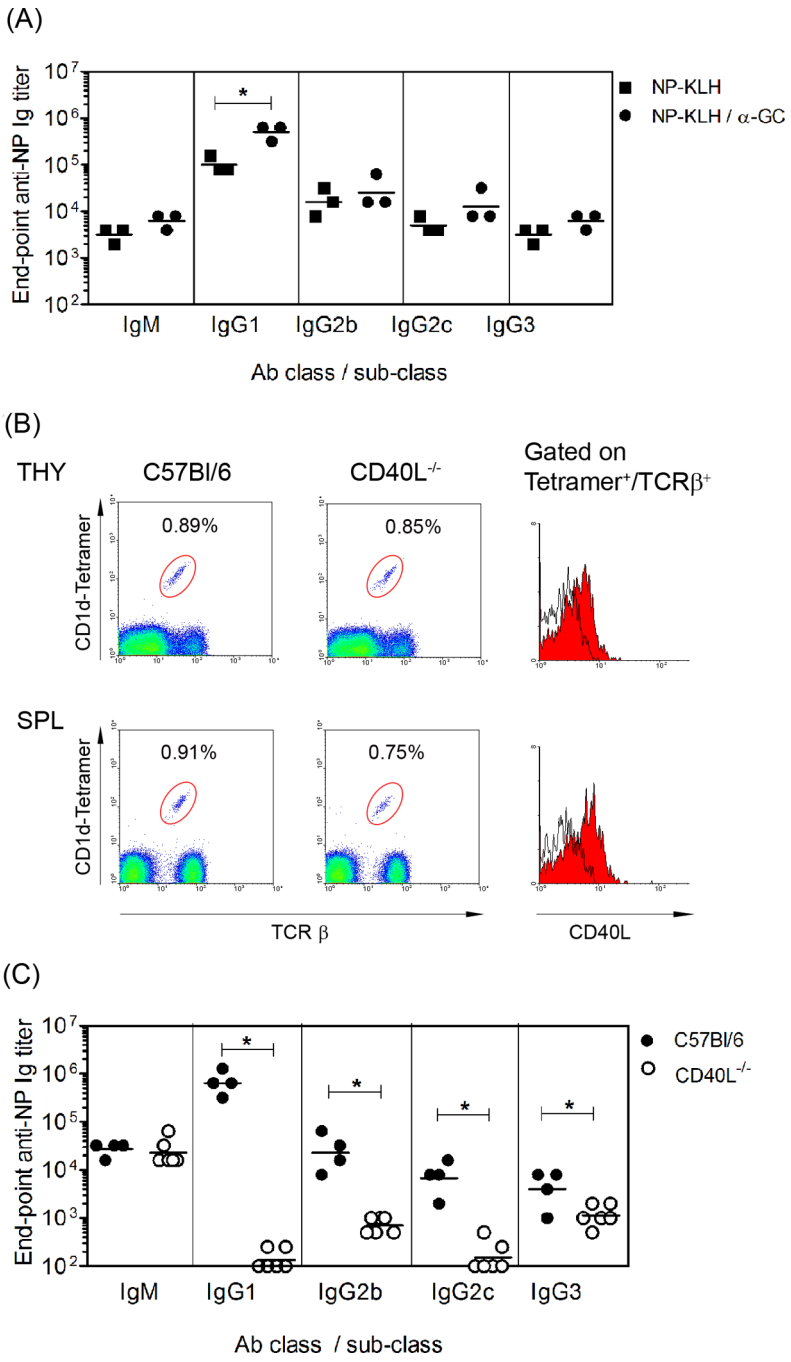

Figure 1. NKT cells do not provide B cell help in CD40L$^{-/-}$ mice

(A) C57Bl/6 mice were immunized with NP-KLH or NP-KLH plus α-GC. After 28 days, all mice received a booster vaccine (NP-KLH). Sera were collected on day 35 and endpoint IgM, IgG1, IgG2b, IgG2c and IgG3 titers determine by ELISA. (B) Thymocytes and splenocytes were obtained from CD40L$^{-/-}$ and C57Bl/6 mice and then analyzed by flow cytometry. Dot plots (left) show CD1d tetramer^{+}/TCRβ^{+} cells. Histograms (right) show expression of CD40L by gated CD1d tetramer^{+}/TCRβ^{+} cells. Data show representative analyses from two CD40L$^{-/-}$ mice and numerous (>50) C57Bl/6 mice. (C) C57Bl/6 and CD40L$^{-/-}$ mice were immunized with NP-KLH plus α-GC on d 0 and boosted with NP-KLH on day 28 before bleeding on day 35. Endpoint IgM, IgG1, IgG2b, IgG2c and IgG3

titers in the sera collected on day 35 were then determined by ELISA. Each data point in (A) and (C) represents an individual mouse and line indicates geometric mean titer. Statistically significant differences between groups were determined using Mann Whitney U test.

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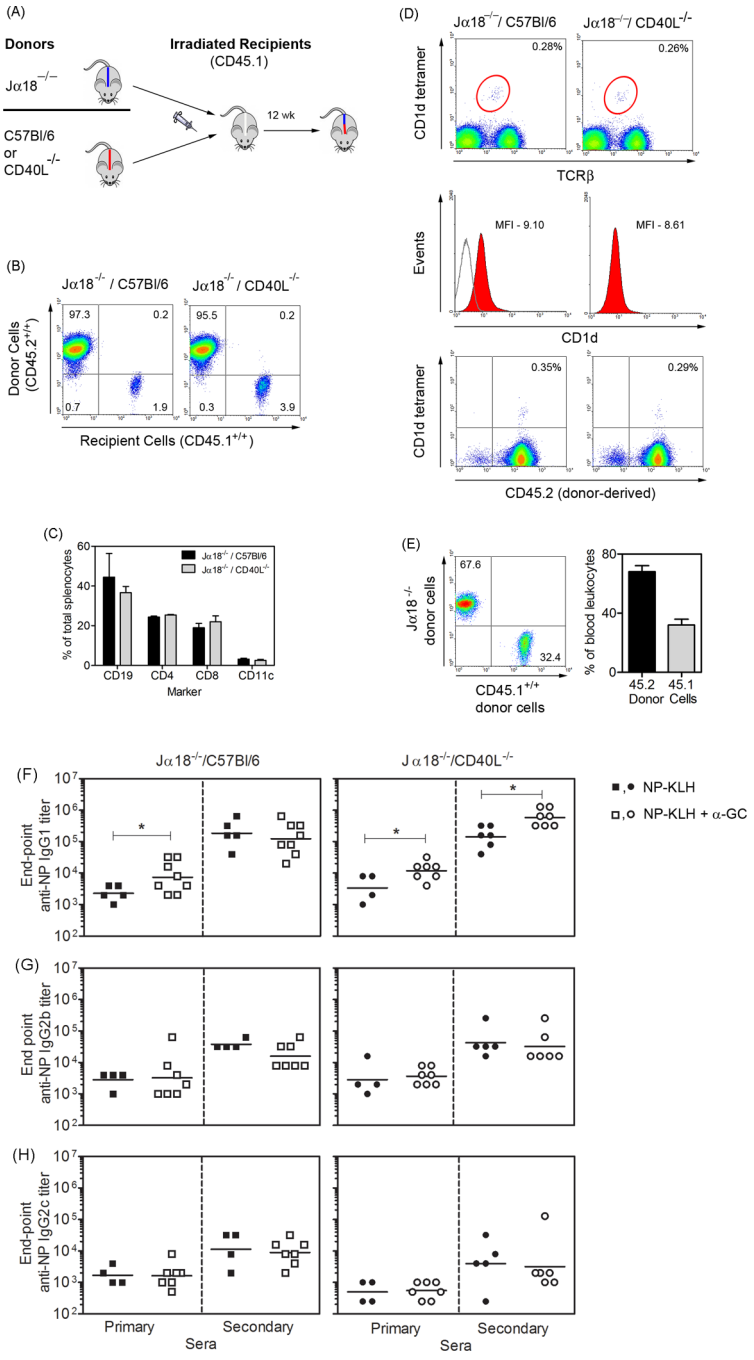

Figure 2. NKT-derived CD40L is dispensable for Ab production

(A) Outlines the strategy used for generating mixed bone marrow chimeras. Jα18$^{-/-}$ mice have a gene deletion in the TCR locus and do not rearrange the Vα14/Jα18 invariant TCR found on Type I NKT cells. (B) Spleens were obtained from immunized Jα18$^{-/-}$/C57Bl/6 and Jα18$^{-/-}$/CD40L$^{-/-}$ chimeric mice and analyzed by flow cytometry for CD45.2$^{+/+}$ donor cells and residual CD45.1$^{+/+}$ recipient cells. (C) Graph shows expression of B cell, T cell and dendritic cell markers for each of the chimeras. (D) Dot-plots in upper panels show CD1d tetramer^{+}/TCRβ^{+} NKT cells. Histograms in middle panels show CD1d expression. Anti-CD1d and isotype control mAb binding are represented by filled and empty histograms respectively. Dot-plots in lower panels show donor-derived (CD45.2$^{+/+}$) versus recipient-

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derived ($CD45.2^{-/-}$) tetramer-binding cells. Data in B-D are representative of three of each chimera. (E) $CD45.2^{+/+}$ (C57Bl/6) mice were lethally irradiated and engrafted with a 50/50 mixture of donor bone marrow cells from $J\alpha18^{-/-}$ mice ($CD45.2^{+/+}$) and $CD45.1^{+/+}$ mice. After 12 weeks peripheral blood leukocytes were analyzed by flow cytometry for CD45.2 and CD45.1 expression. Graph shows relative percentages of CD45.1 and CD45.2 cells in the periphery of 5 re-constituted recipients. (F-H) $J\alpha18^{-/-}$/C57Bl/6 and $J\alpha18^{-/-}$/$CD40L^{-/-}$ mixed chimeras were immunized as indicated. Sera were collected on d 28 (primary) and 35 (secondary) and ELISA assays were performed to analyze NP-specific (F) IgG1, (G) IgG2b and (H) IgG2c titers. Each data point represents an individual mouse and the lines indicate geometric mean titer. Two outliers were removed from the NP-KLH, primary sera group in (F). Data shown are representative of 2 independent experiments. Statistically significant differences between control and experimental mice were determined using Mann Whitney U test.