NK T cells provide lipid antigen-specific cognate help for B cells

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The mechanisms of T cell help for production of antilipid antibodies are largely unknown. This study shows that invariant NK T cells (iNK T cells) and B cells cooperate in a model of antilipid antigen-specific antibody responses. We use a model haptenated lipid molecule, 4-hydroxy-3-nitrophenyl-αGalactosylCeramide (NP-αGalCer), to demonstrate that iNK T cells provide cognate help to lipid-antigen-presenting B cells. B cells proliferate and IgG anti-NP is produced from *in vivo*-immunized mice and *in vitro* cocultures of B and NK T cells after exposure to NP-αGalCer, but not closely related control glycolipids. This B cell response is absent in CD1d$^{-/-}$ and Jα18$^{-/-}$ mice but not CD4$^{-/-}$ mice. The antibody response to NP-αGalCer is dominated by the IgM, IgG3, and IgG2c isotypes, and marginal zone B cells stimulate better *in vitro* lipid antigen-driven proliferation than follicular B cells, suggesting an important role for this B cell subset. iNK T cell help for B cells is shown to involve cognate help from CD1d-instructed lipid-specific iNK T cells, with help provided via CD40L, B7–1/B7–2, and IFN-γ, but not IL-4. This model provides evidence of iNK T cell help for antilipid antibody production, an important aspect of infections, autoimmune diseases, and vaccine development. Our findings also now allow prediction of those microbial antigens that would be expected to elicit cognate iNKT cell help for antibody production, namely those that can stimulate iNKT cells and at the same time have a polar moiety that can be recognized by antibodies.

antibodies | CD1d | T cell helper | iNK T cell | α-galactosylceramide

Innate-like lymphocytes natural killer (NK) T cells, γδ T cells, marginal zone (MZ) B cells, and B1-B cells are important for early immune defense against viruses, bacteria, and tumors (1). They share common traits, such as germ-line transcript-encoded receptors with limited repertoires and low activation threshold. Activation of one of these cell types, invariant NK (iNK) T cells, has been most thoroughly evaluated after stimulation with αGalactosylCeramide (αGalCer), a synthetic mimic of a natural glycolipid that evokes rapid production of large amounts of cytokines, particularly IL-4 and IFN-γ (2), when presented by CD1d. CD1d-restricted iNK T cells are important in defense against bacterial, viral, and parasitic infections (3–5), and specifically react to purified bacterial lipid antigens such as *Borellia burgdorferi* diacylglycerol (BbGL-II) (6) and *Sphingomonas* species glycolipids (7). Once stimulated, iNK T cells activate many other cell types, including NK cells, dendritic cells (DCs), T cells, and B cells (8).

Murine iNK T cell activation *in vivo* with αGalCer induces IL-4-dependent expression of activation markers CD69, B7–2, and I-A^b on B cells (9), and human NK T cell activation *in vitro* with αGalCer induces IL-4/IL-13-dependent B cell proliferation and total IgM, IgG1 antibody production (10). Reduced antibody responses in CD1d$^{-/-}$ or Jα18$^{-/-}$ mice compared with WT mice during infection or autoimmune disease (3–5, 11) is also consistent with iNK T cells and B cell cooperation. Naturally occurring IgG antilipid antibodies have been detected during malaria infection (12), systemic lupus erythematosus (13), and diabetes (14), implying that lipid-specific B cells received T cell help for class switching in these models. Antibody production by protein-specific B cells is most efficient when the B cell receives cognate T cell help from T cells specific for peptides contained within the same protein internalized through the BcR (15), so in analogy to MHC II-restricted responses, class switched antilipid responses may depend on lipid-specific T cells. Vaccine studies show that murine MHC II-restricted anti-protein IgG1 and IgA responses are improved by *in vivo* coadministration of αGalCer (16) and have shown a requirement for CD1d on B cells (17), but direct, cognate help from CD1d-restricted lipid-specific T cells for a lipid-specific B cell has not been described.

Here, we use haptenated model lipid antigens to understand iNK T cell antilipid help for lipid specific B cells, mimicking the approach used to characterize CD4$^+$ T cell help for protein-specific B cells (18). We demonstrate cognate CD1d-restricted iNK T cell help for B cell proliferation and antibody production against the model haptened-lipid antigen. In this system, B cells responding to the hapten, 4-hydroxy-3-nitrophenyl (NP), are recognizing a component of the lipid antigen and mimic lipid-specific B cells. By definition, T cells do not recognize haptens, they only provide help if they recognize a component of a larger molecule conjugated to the hapten. Here that larger molecule is the lipid antigen, αGalCer. These results provide insights into the source of help driving lipid-specific antibody production with relevance for defense against microbial infections and vaccine protection.

Results

Synthesis and Biologic Activity of Haptenated-Lipid Antigens. We have synthesized hybrid haptenated lipids that contain a B cell recognition component, a hapten, and an iNK T cell recognition component, a glycolipid. The lipid components of these molecules include the well described iNK T cell agonist, αGalCer, or as a control, the closely related, but weak agonist, β-GalactosylCeramide (βGalCer). The structure of each molecule contains a hapten, nitrophenyl, conjugated by way of a six-carbon linker attached at C2 of the galactose [supporting information (SI) Fig. S1]. The sphingosine base is attached to the sugar ring, either in the α anomeric linkage in the active molecule (NP-αGalCer) or the β anomeric linkage in the control structure (NP-βGalCer) (Fig. 1*A*).

Initial *in vitro* studies compared the iNK T cell stimulating activity of the synthetic, haptenated antigens with the unmodified lipids. NP-αGalCer- or αGalCer-loaded, plate-bound mouse CD1d fusion protein stimulated two iNK T cell hybridomas, DN32D3 (Fig. 1*B*) and 24.9E (Fig. S2), to produce significantly ($P \leq 0.05$) more IL-2

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The authors declare no conflict of interest.

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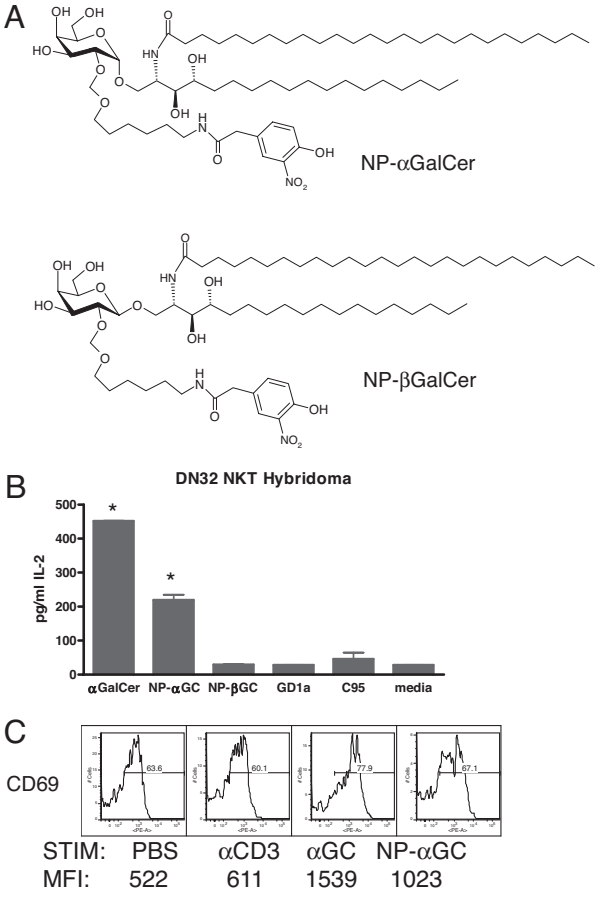

Fig. 1. Characterization of NP-aGalCer and NP-BGalCer. (*A*) NP-αGalCer and NP-βGalCer. (*B*) Haptenated NP-αGalCer, but not NP-βGalCer, stimulates iNK T cells *in vitro* and *in vivo*. CD1d-restricted hybridoma (DN32D3) is stimulated with 1.25 nM αGalCer, NP-αGalCer, GD1a, C95 dolichol, or media alone. IL-2 is measured by ELISA and is expressed as mean/SE of duplicate wells. Representative of two experiments is shown (*, significantly different from media). (*C*) iNK T TcR Tg splenic T cells were labeled *ex vivo* with αGalCer-loaded mouse CD1d tetramer, αCD3, and anti-CD69 2 h after PBS/0.05% BSA, 0.5 μg αGalCer, and 0.5 μg NP-αGalCer or 30 min after 0.5 μg αCD3 immunization. Data are expressed as MFI of CD69 expression on CD3, tetramer double positive NKT cells.

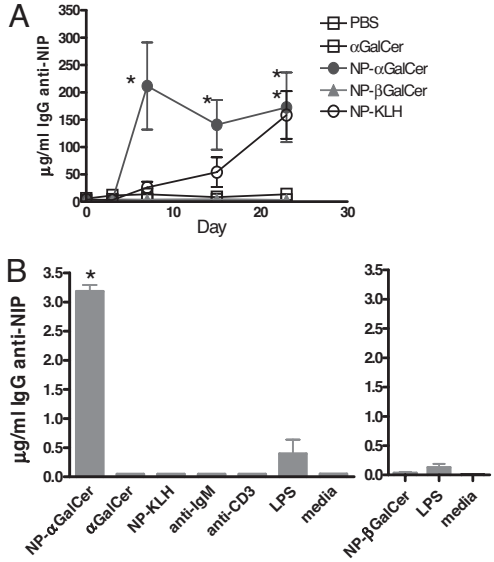

Fig. 2. NP-αGalCer immunization stimulates IgG anti-NIP. (*A*) Intraperitoneal administration of NP-αGalCer (0.5 μg per mouse) but not NP-KLH (2.2 μg per mouse), NP-βGalCer (0.5 μg per mouse), or αGalCer (0.5 μg per mouse) stimulates early anti-NIP IgG *in vivo*. (*B*) IgG anti-NIP from mixed splenic B1–8^{hi} B and iNK T TcR Tg T cell culture supernatants 7 days after media, 15 μg/ml αIgM, 1 μg/ml LPS, 0.2 μg/ml αCD3, 8.6 μg/ml NP-KLH, 1 ng/ml αGalCer, 1 ng/ml NP-αGalCer, or 1 ng/ml NP-βGalCer stimulation (*, significantly different from PBS/media group).

than media or NP-linked βGalCer. No antigen-presenting cells (APCs) are involved in this assay, eliminating any presentation advantage from receptor cross-linking or uptake. NP-αGalCer also activates iNK T cells *in vivo*. CD69 expression on NK T cells was higher after i.p. αCD3 [mean fluorescence intensity (MFI) 611], αGalCer (MFI 1539), and NP-αGalCer (MFI 1023) than after PBS (MFI 522) (Fig. 1*C*). Both *in vivo* and *in vitro* experiments confirm that NP hapten modification of αGalCer or β-GalCer does not alter their recognition by iNK T cells.

NP-αGalCer Stimulates T Cell Help-Dependent Antibody Responses. NP-αGalCer was next examined for immunogenicity by assessing antibody responses *in vivo*. Immunization with NP-αGalCer in PBS, but not negative controls [NP- keyhole limpet hemocyanin (KLH), NP-βGalCer, or PBS/BSA], stimulated both IgM (17 μg/ml IgM vs. 3 μg/ml with PBS on day 7; data not shown) and IgG anti-4-hydroxy-3-iodo-5-nitrophenylacetate (NIP) (212 μg/ml IgG vs. 14 μg/ml IgG with PBS on day 7) (Fig. 2*A*) in a dose-dependent fashion (Fig. S3*A*). The NP-αGalCer response is not bystander activation because immunization with αGalCer alone does not induce IgM or IgG anti-NIP (4 μg/ml IgG) above PBS/BSA (14 μg/ml IgG on day 7) (Fig. 2*A* and IgM; data not shown), nor does αGalCer or NP-αGalCer change total IgG titers (data not shown). NP-αGalCer immunogenicity was confirmed *in vitro* by using a mixed culture of NP-specific B1–8^{hi} (19) splenic B cells and iNK T cell receptor transgenic (20) splenic T cells (iNKT TcR Tg T cells). By day 7, NP-αGalCer stimulated 3.2 μg/ml anti-NIP IgG, whereas αGalCer, NP-KLH, NP-βGalCer, and media stimulated <0.05 μg/ml IgG anti-NIP (Fig. 2*B*). FACS analysis of *in vitro* cultures confirms the specificity of these cells' proliferation: LPS stimulates B cell (31%) but not T cell proliferation (2%), and αCD3 stimulates T cell (52%) but not B cell proliferation (8%) (Fig. S3*B*). NP-αGalCer (10 ng/ml) stimulates such robust proliferation from both B cells (29%) and T cells (29%), that the response is still evident at a 100-fold lower concentration of NP-αGalCer, 0.1 ng/ml (34% B cells, 17% T cells) (Fig. S3*C*). In comparison, NP-βGalCer stimulates moderate B cell (24%) and little T cell proliferation (5%) at a high dose of 10 ng/ml, and no proliferation (1% B cells and <1% T cells) at a lower dose of 0.1 ng/ml, suggesting high-dose NP-βGalCer may cross-link the BcR, but is not sufficiently recognized to be stimulatory to T cells. Thus, immunization with NP-αGalCer stimulates rapid, dose-dependent IgM and IgG anti-NIP, and T and B cell proliferation, consistent with NP-αGalCer-specific iNK T cell help for hapten-specific B cells.

Anti-NP Response Is CD1d and Vα14$^+$ NK T Cell, but Not CD4, Dependent. To confirm the importance of CD1d and iNK T cells for the anti-NIP IgG response to NP-αGalCer *in vivo*, mice lacking CD1d (B6 CD1d$^{-/-}$), mice lacking Vα14$^+$ iNK T cells (B6 Jα18$^{-/-}$), or WT B6 controls were immunized with NP-αGalCer. Only WT B6 mice made significant IgG (17 μg/ml) and IgM (89 μg/ml) anti-NP-αGalCer by day 6 (Fig. 3). No mice made any specific anti-NIP IgG or IgM by day 6 when immunized with controls αGalCer (<1 μg/ml) or PBS (<1 μg/ml) (data not shown). This finding confirms the requirement for CD1d/NP-αGalCer recognition by iNK T cells to facilitate specific anti-NIP antibody production. Human CD4$^+$ NK T cell clones are better *in vitro* inducers of total B cell IgG than CD4$^-$CD8$^-$ NK T cell clones (10), so mice lacking CD4 (CD4$^{-/-}$) were examined to evaluate whether

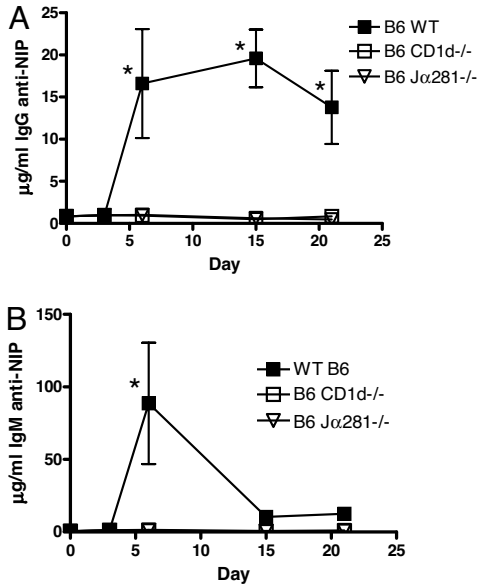

Fig. 3. CD1d and Jα18 iNK T cells, but not CD4, are required for *in vivo* IgG and IgM anti-NIP response to NP-αGalCer. IgG (*A*) and IgM (*B*) anti-NIP from B6 WT, B6 CD1d$^{-/-}$, or B6 Jα18$^{-/-}$ mice immunized with 0.5 μg per mouse NP-αGalCer, 0.5 μg per mouse αGalCer, or 200 μl of PBS/0.05% BSA, detected by ELISA between days 3 and 21 (n = 4–5 mice per group) (*, significantly different from WT).

CD4 expression on murine NK T cells is similarly required. Seven days after NP-αGalCer immunization, substantial IgG anti-NIP was detectable in both the B6 WT (146 μg/ml) and CD4$^{-/-}$ mice (147 μg/ml) despite the fact that CD4$^{-/-}$ mice had a reduced IgG anti-NIP response to NP-KLH/alum (below detection) compared with B6 WT mice (22 μg/ml IgG) (Fig. S4*A*). Thus, CD4 expression is required for peptide-specific, but not lipid-specific, T cell help in this system.

The importance of CD1d for a response to NP-αGalCer by B and T cells was confirmed by using CD1d-blocking antibodies *in vitro*. Flow cytometry of mixed, carboxyfluorescein succinimidyl ester (CFSE)-labeled, *in vitro* cultured B1–8^{hi} B and iNK TcR Tg T cells reveals that anti-CD1d blocking antibody reduces T cell proliferation in response to NP-αGalCer from 24% to 5% and B cell proliferation from 23% to 14% (Fig. S4*B*). Only 3–5% of the B cells in these cultures are specific for NP (19), so not all B cells will respond or be affected by T helper cells. The reduction in B cell proliferation after CD1d blocking provides further evidence that iNK T cells are contributing to B cell proliferation in this system.

iNK T Cell Help for B cells Involves Cognate B–T Interactions. T cell help stimulates B cell proliferation, antibody production, or class switch most efficiently if provided by a T cell recognizing its cognate antigen presented by the B cell. To determine whether iNK T cells are capable of providing efficient, specific, cognate T cell help we compared immunization of mice with an NP-linked lipid (NP-βGalCer) or protein (NP-KLH) antigen plus αGalCer. Mixed together, the NP B cell antigen and the αGalCer iNK T antigen should end up in the same cells, leading to cognate help. Contrarily, immunizing mice with T and B cell antigens in disparate locations increases the chance that the antigens will be taken up by different APCs, allowing only noncognate help. We found that day-9 IgG anti-NIP titers were comparable in mice separately immunized with NP-KLH and αGalCer (25 μg/ml) or NP-KLH mixed with αGalCer (21 μg/ml) (Fig. 4*A*). However, mice produced lower IgG anti-NIP when immunized separately with NP-βGalCer and αGalCer (below detection) compared with mixed NP-βGalCer and αGalCer (25 μg/ml) (Fig. 4*B*).

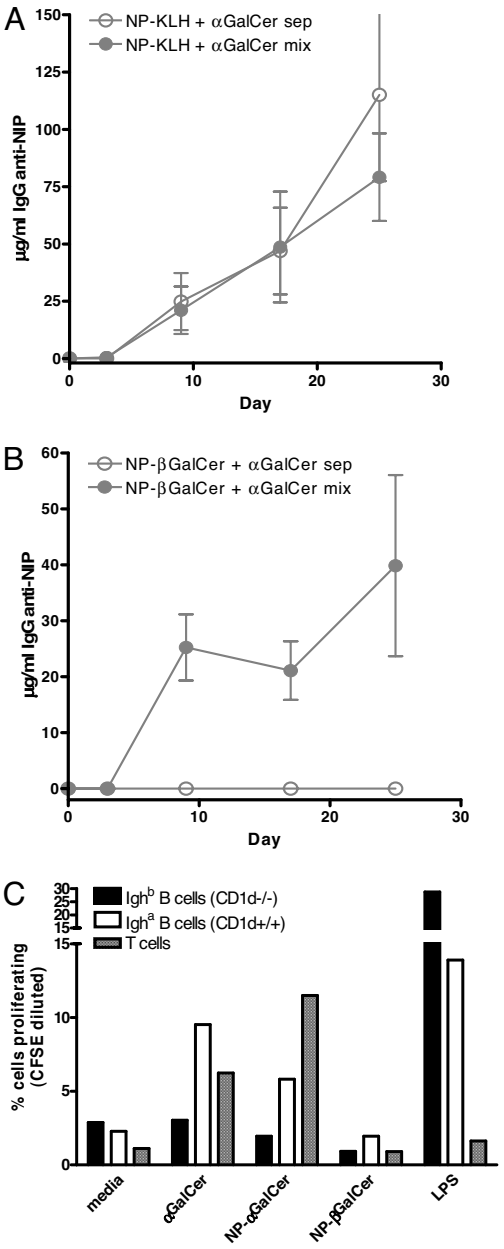

Fig. 4. iNK T cells provide *in vivo* and *in vitro* cognate help for antihapten antibody production. (*A*) B6 WT mice were immunized with two separate injections of 2.2 μg of NP-KLH (i.p.) and 0.5 μg of αGalCer (s.c.) or one injection of 2.2 μg of NP-KLH mixed with 0.5 μg of αGalCer (i.p. only). (*B*) Comparable mice were immunized with two separate injections of 0.5 μg of NP-βGalCer (i.p.) and αGalCer (s.c) or one injection of 0.5 μg of NP-βGalCer mixed with αGalCer (i.p. only) (n = 5 per group). (*C*) Three-day culture of CFSE-labeled 1 × 10^5 Igh^a B6 CD1d$^{+/+}$ splenic B cells, 1 × 10^5 Igh^b B6 CD1d$^{-/-}$ splenic B cells, and 1 × 10^5 iNK T TcR Tg splenic T cells plus media, 50 ng/ml NP-αGalCer, 50 ng/ml αGalCer, 50 ng/ml βGalCer, or 1 μg/ml LPS. IgM^a+ CD1d$^{-/-}$ B cells, IgM^b+ CD1d$^{+/+}$ B cells, and Thy1.2+ T cells were identified by FACS. Proliferation represents percentage of cells that have divided from a pool of triplicate wells. Representative of two experiments is shown.

αGalCer alone does not induce nonspecific IgG anti-NIP, and immunization with NP-KLH or NP-βGalCer in the absence of adjuvant or an iNK T cell ligand does not induce IgG anti-NIP either (Fig. S5). Further, NP-βGalCer mixed with αGalCer and NP-KLH mixed with αGalCer give equivalent IgG anti-NIP responses (25 and 21 μg/ml, respectively) (Fig. 4 *A* and *B*) to those

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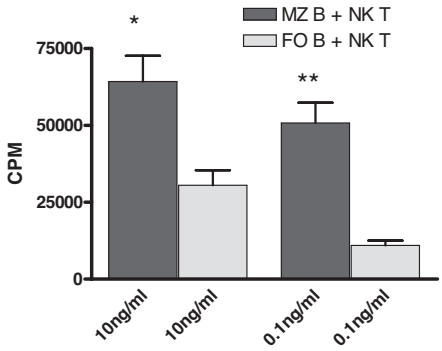

Fig. 5. MZ B cells stimulate stronger proliferation than FO B cells when mixed with iNK T TcR Tg T cells plus NP-αGalCer. A total of 1×10^5 splenic MZ B cells or 1×10^5 splenic FO B cells were mixed with 1×10^5 iNK T TcR Tg splenic T cells and incubated for 3 days with 10 ng/ml NP-αGalCer or 0.1 ng/ml NP-αGalCer. Media were <200 cpm for all cell types. Individual populations proliferate <4,000 CPM in response to NP-αGalCer, and αCD3 and LPS controls confirmed active T and B cell populations but are not shown for simplicity. Proliferation was measured by ^{3}[H]thymidine incorporation. Pool of three experiments is shown. *, significantly different from FO B cells + 10 ng/ml Ag; **, significantly different from FO B cells + 0.1 ng/ml Ag.

elicited by NP-αGalCer (15 μg/ml) (Fig. S5). Reversing the locations of immunizations (NP-βGalCer s.c. + αGalCer i.p.) also does not induce IgG anti-NIP antibody (data not shown).

To confirm cognate interactions between CD1d$^+$ B cells and iNK T cells, complementary *in vitro* studies mixed Igh^b CD1d$^{-/-}$ B cells, Igh^a CD1d$^{+/+}$ B cells, and iNK TcR Tg T cells, which provides a unique opportunity to observe CD1d-deficient and CD1d-sufficient B cells in the presence of iNK T cells and antigen in the context of an identical environment. CD1d$^{-/-}$ (Igh^b) B cells do not proliferate above background in the presence of NP-αGalCer plus T cells, but CD1d$^{+/+}$ (Igh^a) B cells plus T cells proliferate well when stimulated with either αGalCer (10% B cells, 6% T cells proliferating) or NP-αGalCer (6% B cells, 12% T cells proliferating) (Fig. 4*C*). There is no specific proliferation advantage for B cells stimulated with NP-αGalCer over αGalCer because WT mice have similarly low precursor frequencies for each antigen. Clearly, only CD1d$^+$ B cells stimulate iNK TcR Tg T cells and receive cognate help in this mixed culture. In parallel control studies mixing individual B cell populations and iNK TcR Tg T cells, only CD1d$^{+/+}$ B cells stimulated NK T cell proliferation when incubated with αGalCer or NP-αGalCer, whereas CD1d$^{-/-}$ B cells do not (data not shown). B cells alone do not proliferate in response to αGalCer or NP-αGalCer (data not shown). In summary, these *in vitro* and *in vivo* systems both clearly demonstrate a cognate component for B and iNK T cell interactions in response to a lipid antigen.

Characterization of the B Cell Immune Response to NP-αGalCer. We determined whether iNK T cells preferentially help MZ B cells by comparing FACS-sorted B1–8^{hi} splenic MZ to sorted follicular (FO) B cells mixed 1:1 *in vitro* with iNK TcR Tg T cells plus antigen. MZ B cells stimulate greater proliferation than FO B cells when cultured with iNK T cells and 10 ng/ml (64,259 vs. 30,522 cpm) or 0.1 ng/ml NP-αGalCer (50,785 vs. 10,965 cpm) (Fig. 5). Further studies will detail the relationship between these two populations.

To gain additional insight into which B cell subpopulations are activated, we measured the antibody isotypes produced in response to NP-αGalCer immunization. Typically, class switch to IgG2a/c and IgG3 is facilitated by IFN-γ, whereas IgG1 and IgG2b are facilitated by IL-4 and TGF-β, respectively (21). B6 WT mice immunized with NP-αGalCer produce more IgM (1:110,000 titer), IgG2c (1:101,250 titer), and IgG3 (1:31,250 titer) anti-NIP than IgG1 (1:9,538 titer) or IgG2b (1:8,000 titer) anti-NIP (Fig. 6*A*), typical of a predominant influence from IFN-γ. In comparison,

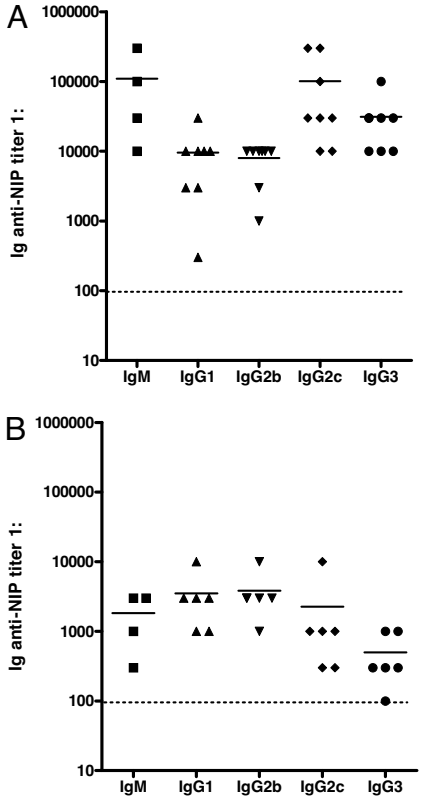

Fig. 6. NP-αGalCer preferentially induces IgM, IgG2c, and IgG3 anti-NIP. Serum collected 7–10 days after immunization of C57BL/6 WT mice with 0.5 μg NP-αGalCer in PBS/0.05% BSA (*A*) or 50 μg NP-KLH in alum (*B*) was tested by ELISA for NIP-specific antibodies. Serum titer represents the first dilution with OD >3× background. ELISA limit of detection is 1:100. Shown is pool of two experiments. Each point = one mouse (n = 5–9 mice per condition).

immunization with NP-KLH + alum induces a profile more typical of an IL-4-driven response with dominant IgG1 (1:3,500 titer) and IgG2b (1:3,833 titer) (Fig. 6*B*). At the same time, it is evident that immunization with NP-βGalCer plus αGalCer (mixed, i.p.) induces titers and a profile very similar to NP-αGalCer alone (IgM > IgG2c > IgG3 > IgG2b, IgG1), whereas NP-KLH plus αGalCer (mixed, i.p.) induces high titers that have a mixed profile similar to both the protein in alum and the NP-αGalCer immunization (IgG2c > IgG1 > IgM, IgG2b > IgG3) (Fig. S6).

Costimulation Requirements for B Cell Help from iNK T Cells. Cognate T cell help for MHC/peptide presenting B cells requires costimulatory molecules on the B and T cells and T cell cytokines. In this model, B7–1/2$^{-/-}$ mice immunized with NP-αGalCer produced less IgG anti-NIP (4 μg/ml) than similarly immunized WT mice (21 μg/ml) by day 7 (Fig. 7*A*). WT mice produce more IgG anti-NIP when immunized with NP-KLH/alum than B7–1/2$^{-/-}$ mice, and neither mouse makes a response to PBS (Fig. 7*A*). Also, CD40L$^{-/-}$, IL-4$^{-/-}$, and IFNγ$^{-/-}$ mice were immunized *in vivo* with NP-αGalCer or NP-KLH/alum. By day 7, CD40L$^{-/-}$ mice produced less IgG anti-NIP than WTs after NP-αGalCer (3 vs. 171 μg/ml) or NP-KLH/alum (3 vs. 688 μg/ml) immunization (Fig. 7 *B* and *C*). IFNγ$^{-/-}$ mice produced less IgG anti-NIP than B6 WT mice in response to NP-αGalCer or NP-KLH/alum (Fig. 7 *B* and *C*). IL-4$^{-/-}$ mice produced equal or slightly greater amounts of IgG anti-NIP than B6 WT mice (Fig. 7*B*) and showed no changes in isotype ratios (Fig. S7*A*) in response to NP-αGalCer. In addition, adding CD40L-blocking antibody *in vitro* reduced NP-αGalCer-treated B cell proliferation by 37% over isotype control but had no effect on the NK T cell response (Fig. S7*B*). Thus, CD40L, B7–1/2,

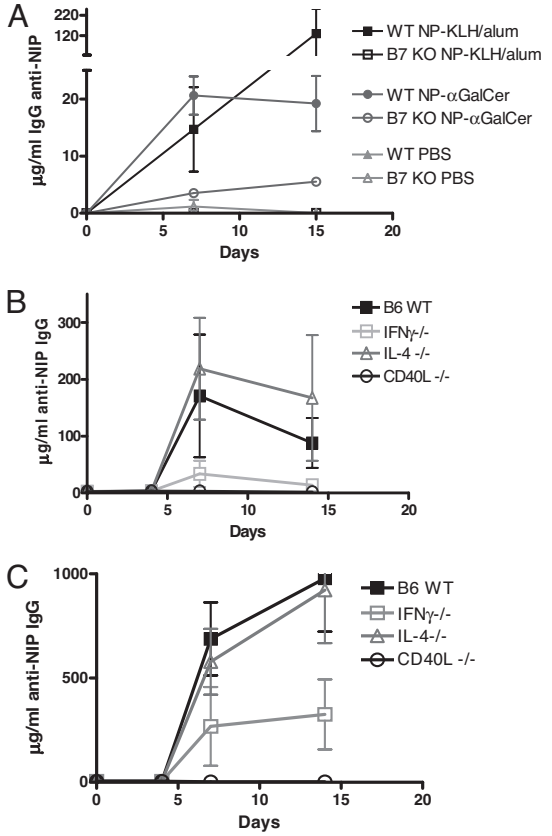

Fig. 7. B7–1/2, CD40L, and IFN-γ are important for the *in vivo* antibody response to NP-αGalCer. (*A*) B6 WT and B7–1/2$^{-/-}$ mice immunized with 0.5 μg NP-αGalCer, 50 μg NP-KLH/alum, or PBS/0.05% BSA. (*B* and *C*) B6 WT, IFNγ$^{-/-}$, IL-4$^{-/-}$, and CD40L$^{-/-}$ mice immunized with 0.5 μg NP-αGalCer/PBS/0.05% BSA (*B*) or 50 μg NP-KLH/alum (*C*). Shown is mean/SE of two to three mice per group and one representative of two experiments.

and IFN-γ, but not IL-4, are important for iNK T cell-mediated B cell help for NP-αGalCer responses.

Discussion

T cell help for B cells is a key paradigm for facilitating protein-specific antibody production, yet the mechanism of lipid-specific antibody production has remained uncharted. We used a model hapten lipid antigen very similar to the well established haptenated-protein antigen used by many investigators to decipher the nature of CD4$^+$ T cell help for protein-specific B cells (18). By measuring B cell responses against a CD1d-presented iNK T cell-restricted lipid ligand (αGalCer) linked to the hapten NP, we determined the mechanisms of class-switched antigen-specific responses that require both BCR cross-linking by the hapten-bearing lipid antigen and T cell help provided by TCR-activated, CD1d-restricted iNK T cells. B and iNK TcR Tg T cell *in vitro* cultures proliferate only to immunogens such as NP-αGalCer, which contain an iNK T cell activation component, and NP-αGalCer-induced IgG and IgM depends on CD1d-restricted iNK T cells, not diverse NK T cells. This finding suggests that the B cell must receive both a B cell receptor cross-linking signal and a second signal from a helper iNK T cell to make proliferation and class-switched IgG antigen-specific responses. T-independent type II B cell antigens require polyvalency for their proliferative effect, but the stimulatory NP-αGalCer in this system is monovalent.

B cells are 100–1,000 times more efficient at presenting antigen than professional APCs if the antigen is limiting and is specifically internalized via the B cell receptor (22). Efficient B cell responses

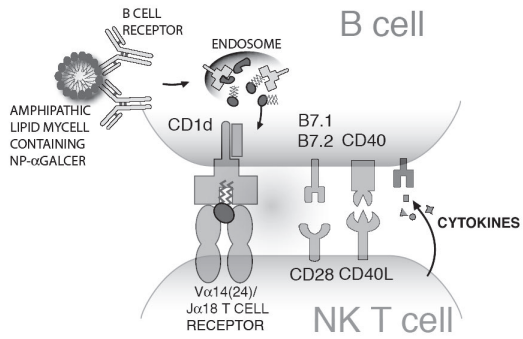

Fig. 8. iNK T cells provide cognate help for lipid-specific B cells. Recognition of CD1d by the TcR on the iNK T cell, plus engagement of costimulatory molecules CD40 and B7–1/2, are important for cognate iNK T cell help for B cell antibody and proliferation responses to lipid antigens. IFN-γ also contributes to isotype class switch during the B cell antibody response.

are facilitated by expression of costimulators on T cells recognizing the antigen presented by the B cell, known as cognate T cell help. Noncognate, or bystander help, requires higher doses of antigen, colocalization of an activated T cell, an APC, and the relevant B cell and is less likely to lead to prolonged memory B cell responses (23). Our results suggest that presentation of lipid antigen by the same B cell that has had its BCR cross-linked recruits more effective cognate iNK T cell help.

Cognate T cell help for protein-specific B cells usually requires B and T cell costimulatory molecules such as CD40L/CD40 (24) and B7–1 + 2/CD28 (25) in combination with T cell-secreted cytokines such as IFN-γ and IL-4 (26). Our results suggest a model where CD1d-restricted iNK T cells provide cognate help for lipid-presenting B cells by way of CD40L and B7–1/2 costimulatory molecules in addition to IFN-γ production (Fig. 8). We also found that sorted MZ B cells stimulate more proliferation in response to NP-αGalCer than FO B cells. NP-αGalCer also stimulates predominantly IgM, IgG3, and IgG2c, which is consistent with an IgM, IgG3-dominant MZ B cell response. These results suggest that MZ B and iNK T cell cooperation may occur in the antibody response to certain lipid antigens, but more study is needed to confirm this interaction.

CD1d$^{-/-}$ mice make reduced levels of organism lysate-specific antibody during *Plasmodium berghei* and *Borrelia hermsii* infection (3, 4), suggesting an important role for both iNK T cells and B cells in these infections. CD1d-binding lipid antigens purified from *B. burgdorferi* (6) may, like our model NP-αGalCer lipid antigen, stimulate iNK T cells and at the same time contain B cell epitopes that could induce protective antibodies during infection. This mechanism may ultimately help predict which glycolipid antigens would be B cell immunogens. Few of these antigens have yet to be identified, suggesting there may even be some natural selection against them, as a form of immune evasion. Given the limited polymorphism of CD1, iNK T cells will be a higher-frequency provider of T cell help for antigens than any peptide-specific, haplotype-restricted T cell population, making CD1 an appealing system for vaccine development. These studies underscore the emerging role for CD1 and iNK T cells in the B cell responses that are important in infection, autoimmunity, and tumor immunity.

Materials and Methods

Lipids. For synthesis of nitrophenyl donor **I**, reagents and conditions were (*i*) $SOCl_2$, 50°C, 1 h; 6-aminohexanol, CH_2Cl_2, 3 h, and (*ii*) DMSO, Ac_2O/AcOH, 2 d. For synthesis of αGalCer with nitrophenyl hapten, compound **II** was synthesized as described (27); activation of thiomethyl group of compound **I** with benzensulfinylpiperidine (BSP)/trifluoromethanesulfonic anhydride (Tf_2O) was as described (28); $Ce(OTf)_3$, $MeNO_2/H_2O$; NaOMe/MeOH. For synthesis of βGalCer with nitrophenyl hapten, compound **III** was synthesized as described (29), and compound **IV**

was synthesized as described (27). Glycosylation reaction was performed with BSP/Tf_2O, as described (28); NaOMe/MeOH; H_2S/Py, room temperature (r.t.); hexacosanoyl chloride, CH_2Cl_2, NEt_3, r.t, 5h; *tret*-$BuMe_2SiCl$, imidazole, CH_2Cl_2, 12 h. Activation of thiomethyl group of compound **I** with BSP/Tf_2O was as described (28); $Ce(OTf)_3$, $MeNO_2/H_2O$; NaOMe/MeOH. αGalCer was synthesized as reported (30). GD1a and C95 dolichol were generously provided by D. Branch Moody (Brigham and Women's Hospital).

Mice. C57BL/6 WT, C57BL/6 $CD4^{-/-}$, $CD40L^{-/-}$, IL-$4^{-/-}$, and IFN$\gamma^{-/-}$ mice were obtained from Jackson Laboratories. C57BL/6 Vα14/Jα18 NKT cell-deficient mice (J$\alpha18^{-/-}$, formerly J$\alpha281^{-/-}$) created by M. Taniguchi (Riken Research Center for Allergy and Immunology, Yokohama, Japan) were provided by J. Stein-Streilein (Massachusetts Eye and Ear Infirmary, Boston). C57BL/6 $CD1d^{-/-}$ mice and C57BL/6 Vα14 Tg mice (iNK T TcR Tg) created by A. Bendelac (University of Chicago, Chicago) were provided by Mark Exley (Beth Israel Hospital, Boston). C57BL/6.SJL congenic B1–8^{hi} B cell receptor knock-in mice, previously created by insertion of a high-affinity NP-specific BcR transgene into the BCR coding region to maintain class switch components, were provided by M. Nussenzweig (Rockefeller University, New York). C57BL/6 B7–$1/2^{-/-}$mice were provided and housed by A. Sharpe (Brigham and Women's Hospital/Harvard Medical School). Mice bred in-house had genotypes confirmed by PCR or phenotypes confirmed by FACS before use.

Antibodies. Murine-specific antibodies were anti-CD19 PerCP-Cy5.5 (1D3), anti-TCRB APC (H57–597), anti-IgM^b phycoerythrin (PE) (AF6–78), anti-Thy1.2 APC (53–2.1), anti-IgM^a biotin (DS-1) plus SA-PerCP, anti-CD3e FITC (145–2C11), and anti-CD69 PE (H1.2F3), anti-CD21/CD35 FITC (7G6), anti-CD23 PE (B3B4), anti-CD45R/B220 Cy-Chrome (RA3–6B2), anti-CD4 FITC (L3T4), anti-CD8α PE (53–6.7), NA/LE anti-CD40L (MR1), NA/LE anti-CD1d (1B1), and NA/LE anti-IFN-γ (cat 554408) plus isotype controls (all BD Biosciences PharMingen). Cells were preblocked with unlabeled anti-FcRIII, II (clone 2.4G2). ELISA detection was by HRP-labeled anti-mouse IgG, IgM, IgG1, IgG2c, IgG2b, and IgG3 (all Southern Biotech). Mouse CD1d fusion protein-APC (National Institutes of Health Tetramer Facility, Bethesda) was loaded with αGalCer or buffer before use.

***In Vitro* Lipid Binding Assay.** Assay was performed as described (31), except with Protein G-coated plates (Pierce). Briefly, plates coated overnight with 0.5 μg per well of CD1d-Fc fusion protein/PBS were blocked with 1% soy milk/PBS. Lipids or controls sonicated into 0.1% BSA/PBS were incubated on the plate overnight at 37°C. Lipid binding was detected as IL-2 production after overnight incubation at 37°C with 1×10^5 cells per well CD1d-restricted, αGC-specific iNK T cell hybridomas [DN32D3 and 24.9.E (31)].

***In Vivo* Challenge/Serum Collection.** Eight- to 14-week-old mice were immunized i.p. with 20–0.5 μg of sonicated lipid, NP (12)-KLH (Biosearch Technologies), or NP (20)-KLH (Biosearch Technologies) in 0.1% BSA/PBS. KLH protein dose was adjusted for MW and haptenation ratio. Differences in lipid and protein structure/form preclude a direct dose comparison. Serum collected by intraocular bleed was stored at −20°C.

Murine IL-2 ELISA. Ninety-six-well plates (Costar 3369) coated overnight with anti-IL-2 (BD PharMingen 554424) in 0.1 M $NaHCO_3$, pH 8.2 were blocked with 10% FCS/PBS for 2 h at 25°C. Supernatant added for 2 h at 25°C was detected with 1 μg/ml biotinylated anti-mIL-2 (BD PharMingen) in 10% FCS/PBS for 45 min at 25°C. Assay was incubated with 2.5 μg/ml Avidin-peroxidase (Sigma A-3151) for 30 min at 25°C, then developed with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate (Sigma A-1888) in 0.1 M citric acid, pH 4.35 and at OD_{405}. Concentration was extrapolated from a standard curve of purified mouse IL-2 (BD PharMingen 550069).

Murine Serum Antibody ELISA. NP-conjugated antigen challenge induces a heteroclitic response, where the resulting antibodies have higher affinity for NIP, so antibody is detected with an NIP-specific ELISA. Plates (Immulon 2 HB) were coated with 1 μg NIP (5)-OVAL in PBS and blocked with 10% soy milk/0.05% Tween/PBS. Serum was serially diluted in 0.1% soy milk/PBS and antibody detected with HRP-labeled goat anti-mouse IgG, IgM, IgG1, IgG2c, and IgG3 (Southern Biotech) developed with ABTS. Concentration was extrapolated from IgG anti-NP (clone Pevchγ1) or IgM anti-NP (clone J558) (provided by A. Ferguson, Boston University School of Medicine, Boston) standard curves.

***In Vitro* Cell Proliferation Assay.** B and T cells were purified by pan-B or pan-T MACS bead separation (Milteny-Biotec) according to the manufacturer's instructions. T and B cell populations were >85% pure. iNK T TcR Tg total splenic T cells were >40% iNK T cells (data not shown). MZ and FO B cells were sorted by the Dana-Farber Cancer Institute (Boston) Flow Cytometry Core: MZ B cells, $CD19^+$, $CD21^{hi}$, $CD23^{lo}$; FO B cells, $CD19^+$, $CD21^{lo}$, $CD23^{hi}$. Purified B and T cells mixed at 1:1 ratio (1×10^5 cells per well each) and labeled with 0.5 μM CFSE (Sigma 21888) for 9 min in PBS were then quenched with FCS and washed extensively before culture. Proliferation was assessed as CFSE dilution on day 3 or incorporation of 1μCi ^{3}[H]thymidine per well after 16 h. ^{3}[H] plates were harvested on a Tomtec harvester and counted on a Wallac 1205 Betaplate reader.

Statistics. Significant differences assessed by two-tailed Student's *t* tests or ANOVA tests with post hoc group comparisons using EXCEL or PRISM software. $P \leq 0.05$ is considered significantly different.

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