Natural killer T (NKT)–B-cell interactions promote prolonged antibody responses and long-term memory to pneumococcal capsular polysaccharides

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Innate-like natural killer T (NKT) cells critically enhance cell and humoral immunity against infections through recognition of conserved microbial lipid antigens presented by CD1d-expressing antigen-presenting cells, and provision of CD40L and cytokine signals. Whereas NKT cells efficiently licensed dendritic cells to prime potent effector and memory T cells, studies based on model antigens such as alphagalactosylceramide-nitrophenyl conjugates concluded that help to B cells was associated with NKT follicular helper differentiation, but limited to short-term responses without induction of memory. We revisited this surprising conclusion in the context of the extracellular encapsulated pathogen *Streptococcus pneumoniae*, where recognition of lipid and capsular polysaccharide antigens by NKT cells and B cells, respectively, provide critical host protection. Using liposomal nanoparticles displaying synthetic lipid and polysaccharide antigens to elicit pure and direct NKT–B-cell interactions in vivo, we observed intense and prolonged antibody responses with isotype switch, affinity maturation, and long-lasting B-cell memory, despite modest or absent NKT follicular helper differentiation. Furthermore, conditional ablation of *Cd1d* demonstrated a requirement for a two-step process involving first cognate interactions with dendritic cells, for NKT cell activation, and then with B cells, for induction of isotype switch and memory. Thus, NKT help to B cells represents both a major arm of antimicrobial defense and a promising target for B-cell vaccines.

The microbial organism *Streptococcus pneumoniae* is a ubiquitous extracellular pathogen and a leading agent of pneumonia and meningitis causing 8–12% of all deaths in children aged <6 y worldwide (1). The protective role of antibodies is highlighted by the severity of infection in splenectomized or agammaglobulinemic patients and by passive transfer experiments (reviewed in ref. 2). Natural, circulating IgM antibodies to the phosphorylcholine (PC) residues decorating teichoic acid can provide a first barrier to infection (3), but the main protection is provided by antibodies elicited upon infection or nasopharyngeal carriage (4, 5). These antibodies target the capsular polysaccharides (PSs), leading to opsonization and complement fixation. Variations of these capsular PSs, which impact the ecological niche and the fitness of the microbial organism, define common serotypes recognized by highly specific antibodies.

Purified capsular PSs are T-independent type II antigens, which typically elicit short-lived IgM responses without germinal center formation, affinity maturation, isotype switch, or memory (reviewed in ref. 6). However, T-dependent IgG isotypes are commonly detected in patients with a history of infection by *Pneumococcus* or with nasopharyngeal carriage of this organism (4, 5), suggesting that PS-specific B cells can receive some form of cognate help, although the source of this help has remained unclear (reviewed in ref. 7). Capsular PSs are noncovalently associated with proteins and lipids in the underlying bacterial cell wall, implying that B cells carrying capsular PS-specific antibodies might internalize these potential T-cell antigens along with the PS antigens to recruit a form of "intermolecular" help, after endosomal digestion and loading onto MHC class II and CD1d molecules, respectively.

Lipid antigens are particularly relevant in the case of *S. pneumoniae*, because the pneumococcal cell wall contains α-glucosyldiacylglycerol (αGlcDAG) (8–10), a glycolipid that is structurally and functionally similar to the prototypical natural killer T (NKT) antigen alphagalactosylceramide (αGalCer). NKT-deficient mice lacking CD1d or the T-cell receptor (TCR) gene segment Jα18 exhibited diminished clearance of *Pneumococcus* and increased mortality after intratracheal administration of the pathogen, highlighting the importance of NKT cells in the context of live infection (8, 10, 11).

NKT cells can provide direct cognate help to antimicrobial B cells, as shown during infection of mice with *Sphingomonas*, a bacterium that carries agonist lipid antigens in its cell wall. Indeed, in mice reconstituted with a mixture of Igh allotype marked $CD1d^{-/-}$ and $CD1d^{+/+}$ bone marrow cells, production of IgG2a antibodies against the PDC-E2 microbial protein was preferentially (although not exclusively) elicited from the CD1d-expressing B cells (12). PDC-E2 is a membrane-associated bacterial protein that is not covalently linked to the membrane lipid antigen, suggesting intermolecular help.

Several studies using model antigens have also established that NKT cells could directly or indirectly help B cells mount antibody responses (13–23). Indirect help was channeled through the enhanced priming of conventional CD4 T cells by NKT cell-licensed dendritic cells (DCs), with subsequent MHC class II-restricted

Significance

Antibodies directed against microbial polysaccharides are a critical component of protective immune responses and vaccines. We used nanoparticles coexpressing pneumococcal capsular polysaccharides and a cell wall lipid antigen analog to model NKT–B-cell interactions. Our study demonstrated CD1d-restricted cognate interactions, isotype switch, affinity maturation, and long-term memory, despite the apparent failure of NKT cells to differentiate into follicular helper cells. The findings demonstrate the importance of nonconventional sources of help for B cells responding to polysaccharide antigens.

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help to B cells responding to the same protein antigen (18, 23). Direct help, involving cognate NKT–B-cell conjugate formation, has been studied in mice immunized with alphagalactosylceramide-nitrophenyl (αGalCer-NP), a conjugate of the NKT ligand αGalCer covalently linked to the B-cell antigen NP, or in MHC II-deficient or -nonresponder mice immunized with protein antigens associated with αGalCer (20–23). These studies suggested that NKT cells could induce the transcription factor Bcl6 and acquire a typical follicular helper program, entering B-cell follicles in a chemokine CXCR5-dependent manner to form long-lived conjugates with antigen-specific B cells, promoting both germinal center formation and extrafollicular foci and enhancing B-cell proliferation and antibody production. Surprisingly, despite the abundant CD40L and cytokine signals that NKT cells can supply, the B-cell response was of short duration, without generation of long-lasting plasma cells or induction of B-cell memory ("fast but does not last"), suggesting that NKT cell help was intrinsically limited compared with conventional CD4 T-cell help (21–24).

Here, instead of using NP conjugates, we have modeled NKT–B-cell interactions in the physiologically relevant context of the microbial pathogen, *S. pneumoniae*, and its major lipid and PS antigens. To avoid interferences by the multiple streptococcal components known to elicit or subvert immune responses and to preserve the particulate nature and the architectural relationship between the lipid and PS antigens, we engineered liposomal nanoparticles containing synthetic forms of the NKT and B-cell antigens, with the lipid inserted in the membrane bilayer and the PS displayed at the outer surface through a diacylglycerol anchor. In addition, we used mice carrying a conditional allele of Cd1d (25) to identify the antigen-presenting cells (APCs) required for NKT cell activation and for B-cell responses. The results demonstrate that, after initial interactions with CD1d-expressing DCs, NKT cells provided cognate help to B cells to promote antibody responses. In contrast with previous reports, and despite the absence of covalent linkage between the NKT and B-cell antigens, the antibody response was very long lasting, exhibited affinity maturation, and showed long-term memory. Furthermore, this strong cognate to B cells occurred despite minimal signs of NKT follicular helper (NKTfh) differentiation, suggesting a mostly extrafollicular response. Thus, the results considerably expand the significance and the scope of NKT cell help to B cells, particularly in the context of T-independent polyvalent microbial antigens. They also raise a simple and effective alternative to the conjugate PS vaccines currently used worldwide to protect children against pneumococcal infections.

Results

Antibody Response to Liposomal Nanoparticles Carrying Lipid and Capsular PS Antigens. To study the interplay between NKT cells and B cells in a relevant microbial context, while eliminating potential confounding factors associated with the whole microbial organism, we engineered liposomal nanoparticles that mimicked the natural display of NKT and B-cell antigens by *S. pneumoniae*. The tetrasaccharide unit repeat of the capsular PS (serotype 14) (26) was linked to a diacylglycerol group (compound PBS150) (Fig. 1) for insertion into liposomal membranes along with the NKT lipid ligand PBS57, a modified α-galactosylceramide. Fig. 2*A* shows the primary and secondary responses elicited after two sequential injections of antigenic liposomes at days 0 and 39. IgM antibodies against the PS were observed in the first week, followed by IgG3 in the second week, consistent with the T-independent nature of these isotypes. "Help-dependent" isotypes IgG1 and IgG2c began to appear after 2 wk. All of the isotypes achieved very high titers, peaking 1 wk after the second injection at up to 10^3- to 10^4-fold above preimmunization levels. These circulating antibodies persisted for an extended period, as substantial titers were still observed 7 mo after the last injection, suggesting the presence of long-lived antibody-secreting cells. The antibodies elicited by the liposomes did not react against lipid components and, consistent with previous studies in mice and humans, were specific for the pneumococcal PS serotype 14 (Fig. 2*B*). Furthermore, omission of the NKT ligand PBS57 from liposomes partially decreased IgM titers and prevented the IgG1 antibody switch, essentially causing reversion to a pattern of T-independent B-cell response (Fig. 2*C*). Altogether, these results indicated that the presence of NKT ligands in PS-presenting liposomal particles enabled the generation of elevated and prolonged titers of IgM and IgG antibodies to pneumococcal PS.

Cognate CD1d-Restricted NKT–B-Cell Interactions. To test whether direct CD1d-restricted interactions with B cells were required for NKT cells to promote antibody responses, we immunized *Cd1d*$^{fl/fl}$ (flanked by lox site) *Cd19-cre* mice (lacking CD1d expression on B cells) and their littermate controls. Fig. 3*A* shows that genetic ablation of CD1d in B cells not only abrogated the switched antibody response, as shown with IgG1 isotypes, but also decreased

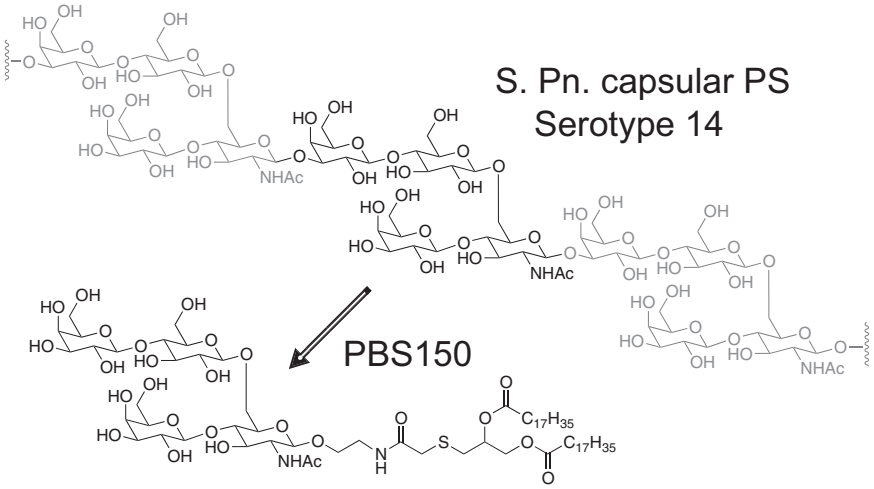

Fig. 1. DAG-anchored pneumococcal capsular polysaccharide. The tetrasaccharide repeat of *S. pneumoniae* capsular polysaccharide serotype 14 (*Upper*) was linked to a diacylglycerol as shown (*Lower*, compound PBS150) for insertion into liposomal membranes.

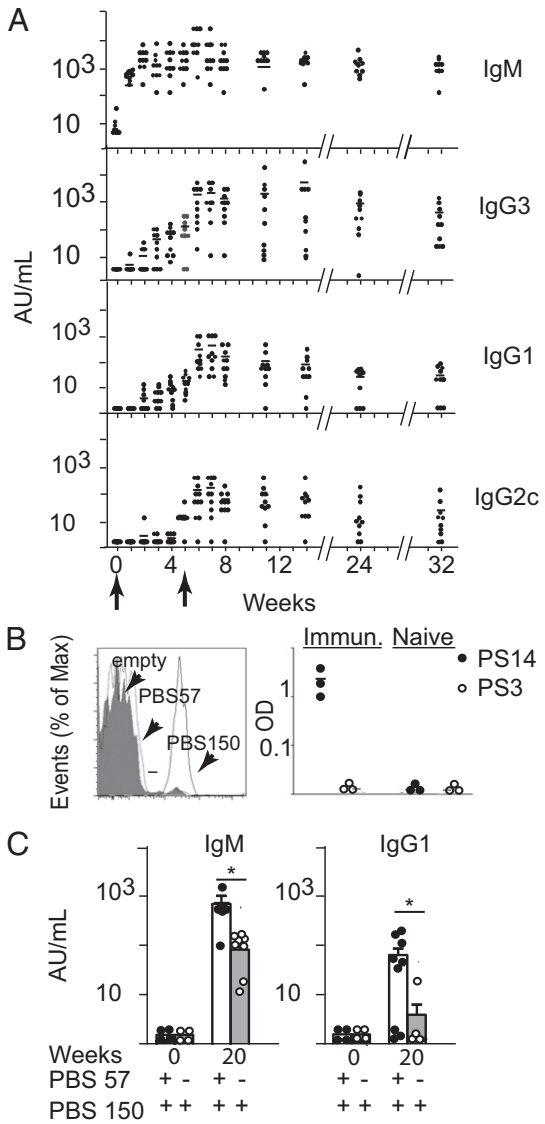

Fig. 2. Anti-PS antibody response elicited by pneumococcal liposomes. (*A*) Kinetics of serum anti-PS14 antibody isotypes after immunization of B6 mice at indicated time points (arrows) with pneumococcal liposomes carrying 4 μg PS14 and 1 μg NKT lipid. Titers are shown as arbitrary units per milliliter by reference to a standard hyperimmune serum. Results represent two independent experiments with 10 mice each. (*B*, *Left*) FACS analysis of serum IgG1 antibodies binding to glass beads coated with PS14-carrying liposomes compared with empty liposomes and liposomes carrying the NKT ligand PBS57. (*Right*) ELISA analysis of serum antibodies against purified pneumococcal polysaccharide in individual naïve or immunized mice. (*C*) Serum IgM and IgG1 antibodies against PS14 measured 20 wk after immunization with liposomes carrying both PBS57 and PBS150, or PBS150 alone.

the titers of IgM by >10-fold, essentially reverting the pattern to that of a T-independent type II response. Notably, the failure to enhance IgM levels and to switch isotypes occurred despite the activation and cytokine secretion by NKT cells, which presumably recognized their lipid ligand on DCs and macrophages. Indeed, *Cd1d*$^{fl/fl}$ *Cd19-cre* mice exhibited a normal IL-4 and IFN-γ cytokine burst as measured in the serum at 2 h and 24 h (Fig. 3*B*). Staining of B cells confirmed that substantial depletion of CD1d was achieved in *Cd1d*$^{fl/fl}$ *Cd19-cre* mice, although modest residual amounts could be detected by comparison with CD1d KO mice (Fig. 3*C*). These results demonstrated that cognate CD1d-restricted interactions between NKT cells and B cells, rather than bystander exposure to cytokines, were essential for potentiation of the B-cell response and induction of isotype switch.

Requirement for NKT–DC Interactions. The above results did not address whether CD1d expression by B cells was sufficient for the isotype-switched response. Indeed, prior studies have established that initial interaction of T cells or NKT cells with antigen-presenting DCs was required for acquisition of a follicular helper program and migration into B-cell follicles (reviewed in ref. 27). Using *Cd1d*$^{fl/fl}$ *Cd11c-cre* mice, which lack CD1d expression on DCs and on some CD11c^{intermediate} (CD11c^{int}) macrophage subsets such as F4/80 splenic macrophages, we found that the IgM response was decreased and the isotype-switched IgG1 antibodies were abrogated (Fig. 3*A*). In these mice, the amount of cytokines released in the serum by NKT cells was partially decreased, as expected (Fig. 3*B*). These results support a two-step process whereby NKT cells must be primed by DCs or macrophages before interacting with B cells.

NKT Cell Activation. NKT cells are found in the T-cell zone as well as in the red pulp and possibly also the marginal zone of the spleen, but they are conspicuously absent from B-cell follicles (28–30). Previous studies indicated that NKT cells could acquire

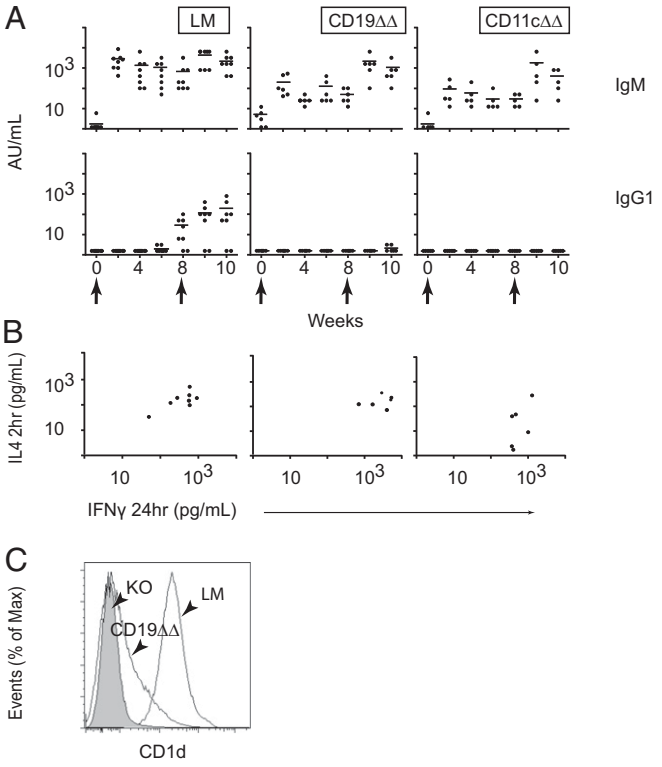

Fig. 3. Cognate NKT cell interactions with both DC and B cells are required for isotype-switched anti-PS antibody response. (*A*) Serum anti-PS antibodies after immunization of *Cd1d*$^{fl/fl}$*Cd19-cre* (CD19ΔΔ), *Cd1d*$^{fl/fl}$*Cd11c-cre* (CD11cΔΔ), and littermate controls (LM) with pneumococcal liposomes carrying 4 μg PS14 and 1 μg NKT lipid at indicated times (arrows). (*B*) Early serum IL-4 (2 h) and IFN-γ (24 h) after pneumococcal liposome injection in *Cd1d* conditional mutants and their littermate controls. Results are representative of two independent experiments with four to seven mice per group. (*C*) FACS analysis of surface expression of CD1d by CD19-gated splenocytes of wild type (LM), CD1d KO (KO), and CD19ΔΔ mice.

NKTfh differentiation upon activation by their lipid ligand and subsequently moved to B-cell follicles where they engaged in interactions with B cells (21, 22). We confirmed that NKT cells activation readily occurred after injection of liposomes carrying PS and lipid antigens, including up-regulation of PD-1 and inducible cell costimulator (ICOS), but failed to observe a significant population of PD-1^{high}CXCR5^{high} (PD-1^{hi}CXCR5^{hi}) "germinal center T follicular helper (GC-Tfh)-like" NKT cells, at least compared with reference staining of Peyer's patch T cells, which are spontaneously enriched in GC-Tfh cells (Fig. 4 *A–C*). Furthermore, we demonstrated that this activation depended on CD1d expression by CD11c-expressing DCs or macrophages, because it was massively decreased in *Cd1d*$^{fl/fl}$ *Cd11c-cre* mice. In *Cd1d*$^{fl/fl}$ *Cd19-cre* mice lacking CD1d on B cells, however, NKT activation was readily observed with a kinetics and persistence similar to that of littermate controls. This persistence suggested that cognate interactions with B cells were neither required for the induction nor the maintenance of NKT cell activation, although it might also be associated with residual CD1d protein on the surface of *Cd1d*$^{fl/fl}$ *Cd19-cre* B cells.

Affinity Maturation. To test whether NKT cells also promoted affinity maturation of anti-PS antibodies, we compared the binding of IgG3 and IgG1 antibodies to liposomes carrying high or low levels of PS-DAG, respectively, 20% and 0.5% of liposomal lipids. Fig. 5 shows that IgG3 and IgG1 antibodies produced early in the response bound poorly to PS-0.5% compared with PS-20%. However, late antibodies after boost bound nearly as well to PS-0.5% as to PS-20% in several individual mice, demonstrating affinity maturation. Thus, NKT cell help can lead to affinity maturation of antibodies to capsular PS.

Memory Formation. The hallmark of T-dependent help to B-cell responses is the formation of long-term B-cell memory. Fig. 6 shows that, when challenged 8 mo after the initial primary and secondary immunizations, the average titer of IgG1 antibodies was increased by ~100-fold within 7 d, a much faster and greater response than naïve littermates exposed to the challenge. Thus, NKT cell help induces potent long-term B-cell memory.

Discussion

Our studies of the B-cell response to microbial PS antigens stand in contrast with previous studies using model antigens such as αGalCer-NP, which concluded that NKT cells readily acquired follicular helper differentiation based on CXCR5 expression but were intrinsically inefficient at promoting isotype switch, affinity maturation, long-term antibody production, and memory (21–24). These results were surprising because the same studies suggested that NKT cells readily acquired a SLAM-associated protein-dependent, Bcl6-driven follicular helper program, engaged in prolonged interactions with antigen-specific B cells, and promoted germinal centers and extrafollicular foci. In addition, NKT cells are well known to provide strong CD40 and cytokine-mediated signals, for example to DCs (31, 32). Our results differ in two important respects. We observed very little induction of PD-1^{hi}CXCR5^{hi} GC-Tfh-like NKT cells (<1–2%), suggesting a mostly extra-GC response, yet NKT cell-mediated cognate help to B cells was potent, comparable to a typical follicular helper response. The different outcome in our study may be related to one of several differences between the experimental systems used. These include the distinct chemical nature of the B-cell antigens, i.e., NP vs. PS antigens, and their different mode of association with NKT ligands as covalent conjugates, simple

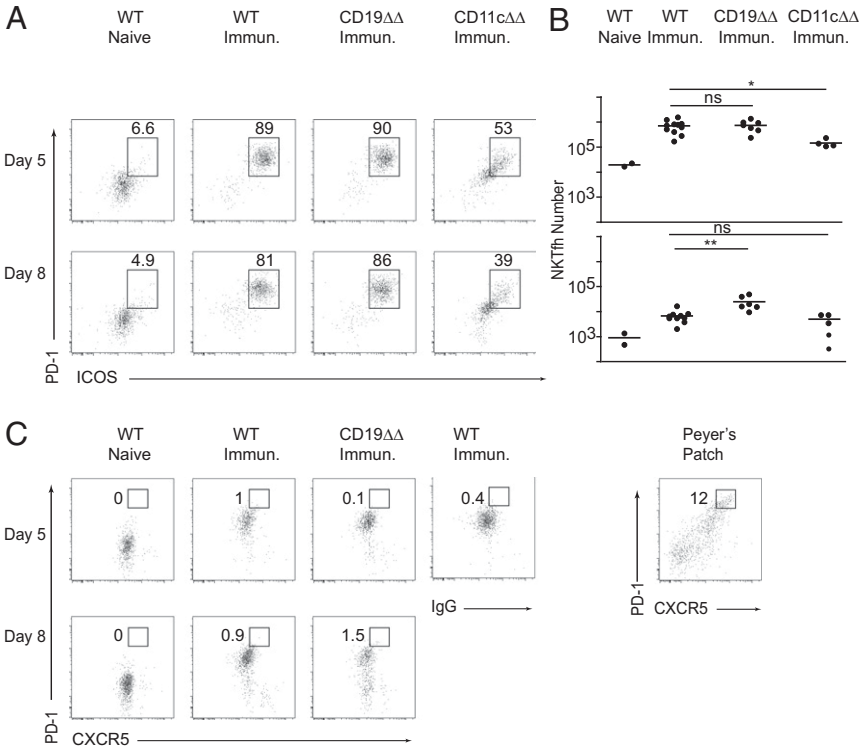

Fig. 4. The NKT follicular helper response requires CD1d expression by DCs but not B cells. (*A* and *B*) FACS staining of splenic NKT cells after pneumococcal liposome immunization of Cd1d conditional mutants and their littermate (LM) controls, as indicated. Results are shown as percentages for representative mice (*Left*) and absolute numbers for each individual mouse (*Right*) of ICOS^{hi}PD-1^{hi} cells among gated CD1d-αGC tetramer^{+} NKT cells at day 5 (*Upper*) and day 8 (*Lower*). Results are representative of three independent experiments with five to seven mice per group. (*C*) Same analysis as in *A* for CXCR5 and PD-1. Staining controls used to set the PD-1^{hi}CXCR5^{hi} GC-Tfh gate included WT immunized NKT cells stained with IgG isotype instead of anti-CXCR5, as indicated, and Peyer's patch CD4 T cells (rightmost dot plot), which are naturally enriched in GC-Tfh cells.

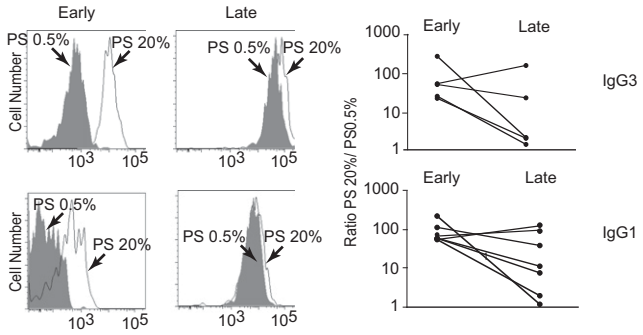

Fig. 5. Affinity maturation of the NKT cell-helped anti-PS antibody response. (*Left*) FACS analysis of serum IgG3 and IgG1 antibody (1/200 dilution) binding to liposomes carrying low (0.5%) or high (20%) density PS in the early and late (after boost) response of a representative mouse showing affinity maturation. (*Right*) Ratios of reciprocal antibody titers to PS-20% over PS-0.5% for individual mice in the early and late response, as indicated. Results are representative of two independent experiments with five to seven mice each.

mixtures, or liposomes. In particular, it is notable that αGalCer-NP conjugates expressed only one NP group per lipid molecule and might not induce a level of B-cell receptor (BCR) cross-linking comparable to that observed with PS liposomes. Indeed, αGalCer-NP did not induce anti-nitroiodophenol (NIP) IgM antibodies in the absence of NKT cells or CD1d in vivo (19), whereas, in our system, substantial IgM responses could be observed even in the absence of CD1d, as is typical of T-independent type II antigens. BCR cross-linking is essential for efficient internalization of antigen and transport to endosomal compartments for CD1d loading and presentation, as shown in the response to nanoparticle-bound hen egg lysozyme (HEL) and αGalCer (20). With this particulate antigen, isotype switch was detected but the studies were not conducted beyond day 14 and the potential contribution of HEL-specific T cells elicited in the presence of αGalCer could not be eliminated. The distinct outcomes may also be influenced by changes in lipid uptake and presentation (33) or by distinct targeting of B-cell subsets such as B1-B or CD1d^{high} marginal zone B cells (30, 34–36). In any case, our results radically alter the previous conclusion that NKT cells were "timid" helpers and instead emphasize their superior efficiency at eliciting affinity-matured and isotype-switched antibodies, as well as long-term B-cell memory against clinically relevant microbial capsular PS. Further studies are needed, however, to determine the location of NKT–B-cell interactions and the origin of the B cells involved in the anti-PS response. Although it remains to be seen whether natural pneumococcal NKT ligands can also promote antibody responses, our results also have important potential implications on host–pathogen interactions and microbial strategies of immune evasions, as subtle changes in microbial lipids can have drastic consequences on CD1d-mediated presentation and activation of NKT cells (9, 37, 38). Furthermore, as liposomal nanoparticles are a well-established pharmaceutical method of antigen delivery in humans (39, 40), our study highlights the untapped potential of NKT cell help for B-cell vaccines, especially against T-independent antigens such as pneumococcal capsular PS.

Materials and Methods

Mice. C57BL/6.*Cd19-cre* (B6.129P2(C)-Cd19$^{tm1(cre)Cgn}$) and C57BL/6 mice were from The Jackson Laboratory. C57BL/6.*Cd11c-cre* (C57BL/6J-Tg (Itgax-cre,-EGFP)4097Ach/J) mice were obtained from Alexander Chervonsky at the University of Chicago. C57BL/6.*Cd1d*$^{fl/fl}$ mice were generated in our laboratory and were crossed to *Cd19-cre* and *Cd11c-cre* to obtain *Cd19* Δ/Δ and *Cd11c* Δ/Δ mice (25). Littermate controls included a mixture of *cre*$^{-/-}$, *cre*$^{+/-}$ *Cd1d*$^{fl/+}$, or *cre*$^{+/-}$ *Cd1d*$^{+/+}$ littermates. All animal experiments were conducted according to protocols approved by the Institutional Animal Care and Use Committee of the University of Chicago.

Flow Cytometry. Splenic lymphocytes were isolated by mincing and passing through a 70-μm nylon cell strainer (Falcon). Fluorochrome-labeled monoclonal antibodies against mouse CD3ε, PD-1, ICOS, CD1d, and CD19 were purchased from eBioscience or Biolegend. CXCR5 was detected using purified anti-CXCR5 (BD Pharmingen), followed by biotinylated anti-rat IgG (Jackson ImmunoResearch Laboratories) and APC-labeled streptavidin (Invitrogen). In CXCR5/PD-1 double staining experiments, cells were incubated with 100 μg/mL of unconjugated rat IgG2a-kappa (BD Pharmingen) after CXCR5 staining and before addition of the rat anti-mouse PD-1 antibody, to saturate free rat IgG binding sites in the biotinylated anti-rat anti-IgG antibody used to reveal anti-CXCR5. CD1d-PBS57 tetramers were obtained from the National Institute of Allergy and Infectious Diseases Tetramer Core Facility. Samples were analyzed on an LSR II or, after staining with CD1d-PBS57 tetramer and CD3ε, were sorted on a FACS Aria (BD Biosciences).

Lipid and Polysaccharide Antigens. The 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) was from AvantiPolar Lipids (850375C) and cholesterol from Sigma (47127-U). PBS57 was produced as described previously (41–43). For synthesis of PBS150, the diacylthioglycerol–tetrasaccharide conjugate was prepared via amide bond formation using the corresponding activated ester and amine. All of the lipids were dissolved in chloroform and stored at −20 °C at concentrations of 5–25 mg/mL.

Liposomes. For immunizations, a mixture containing DOPC, cholesterol, PBS150, and PBS57 (35/40/20/5 molar ratio) was dried under a stream of nitrogen to remove chloroform, then hydrated and subjected to freeze/thaw cycles to produce multilamellar vesicles, and finally sized by extrusion through a polycarbonate filter of 400-nm pore size (Whatman; 80028) in the Avanti miniextruder (AvantiPolar Lipids; 610000). The quality and size of these "pneumococcal" liposomes was monitored by cryoelectron microscopy. Liposomes were used immediately or within 1 wk of storage at 4 °C.

Anti-Polysaccharide Antibody Assay. DOPC/cholesterol liposomes containing 20% (molar ratio) PS were adsorbed onto 3- to 10-μM glass beads (Polysciences Inc.; 07666) and incubated with serial dilutions of immune or naïve sera before adding FITC-conjugated isotype-specific goat antibodies from Southern Biotech (anti-IgM, 1020–02; IgG1, 1070–02; IgG2c, 1079–02; and IgG3, 1100–02) and measuring mean fluorescence intensity by flow cytometry. Arbitrary units (AU) per milliliter were determined by reference to a standard hyperimmune serum pool, with the following reciprocal end-point

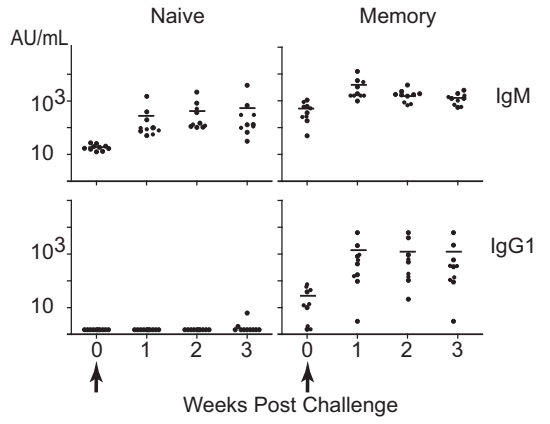

Fig. 6. Cognate NKT cell help promotes long-term B-cell memory. Mice immunized twice with pneumococcal liposomes (as in Fig. 1) and unimmunized, naïve littermates were reinjected 8 mo later with pneumococcal liposomes. The plots show individual titers of PS-specific IgM and IgG1 after challenge. Note that preimmunized mice had detectable residual PS-specific IgM and IgG1 antibodies before challenge. Results are representative of two independent experiments with a total of 20 mice.

titer equivalences for 1 AU/mL: IgM, 16; IgG1, 32; IgG2c, 64; and IgG3, 8). For estimation of affinity maturation, sera were tested against glass beads coated with liposomes containing 20% PBS150 (PS-20%) or 0.5% PBS150 (PS-0.5%) and the ratio of reciprocal titers was calculated as an estimate of affinity. Alternatively, anti-PS antibodies were measured by ELISA in microplates coated with 10 μg purified pneumococcal capsular PS serotype 14 or serotype 3 (ATCC).

Immunization. Mice were injected intramuscularly in the thigh with 20 μL of liposome solution containing the equivalent of 1 μg NKT lipid PBS57 and 4 μg polysaccharide PBS150.

Statistical Analysis. Different groups were compared using two tailed *t* test or Mann–Whitney test. $*P < 0.05$, $**P < 0.01$.

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