

# Cognate interaction with iNKT cells expands IL-10–producing B regulatory cells

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**Successful induction of B-cell activation and memory depends on help from CD4<sup>+</sup> T cells. Invariant natural killer T (iNKT) cells (glycolipid-specific, CD1d-restricted innate lymphocytes) provide both cognate (direct) and noncognate (indirect) helper signals to enhance B-cell responses. Both forms of iNKT-cell help induce primary humoral immune responses, but only noncognate iNKT-cell help drives humoral memory and plasma cells. Here, we show that iNKT cognate help for B cells is fundamentally different from the help provided by conventional CD4<sup>+</sup> T cells. Cognate iNKT-cell help drives an early, unsustained germinal center B-cell expansion, less reduction of T follicular regulatory cells, an expansion of marginal zone B cells, and early increases in regulatory IL-10–producing B-cell numbers compared with noncognate activation. These results are consistent with a mechanism whereby iNKT cells preferentially provide an innate form of help that does not generate humoral memory and has important implications for the application of glycolipid molecules as vaccine adjuvants.**

iNKT cells | marginal zone B cells | B regulatory cells | iNKT follicular helper cells | IL-10

Most successful human vaccines are effective because they induce long-term humoral memory. Establishing B-cell memory depends on a nuanced interplay between B, T, stromal, and antigen-presenting cells (APCs) with antigen in specialized germinal center structures within the spleen (1). We and others have recently found that, in addition to conventional CD4<sup>+</sup> T helper cells, invariant natural killer T (iNKT) cells can also provide help to B cells (2–4). iNKT cells are innate, memory-like T cells with a limited T-cell receptor (TcR) repertoire that recognizes CD1d-presented glycolipids. iNKT cells are rapidly activated by the marine sponge-derived glycolipid  $\alpha$ -galactosylceramide ( $\alpha$ GalCer) (5, 6). iNKT cells that specifically provide help to B cells, termed iNKT T follicular helper (iNKT<sub>TFH</sub>) cells (7), express many of the same surface proteins and transcription factors that identify CD4<sup>+</sup> T follicular helper (T<sub>FH</sub>) cells, including CXCR5, PD-1, ICOS, and BCL6, and are found in germinal centers (7). iNKT cells providing either cognate or noncognate help to B cells enhance primary, antigen-specific antibody production (8) through production of IFN- $\gamma$ , IL-21, and B cell-activating factor (BAFF), but only during cognate help do the cytokines come exclusively from iNKT cells (2, 9, 10). iNKT cells activated by nitrophenyl (NP)-haptened  $\alpha$ GalCer are induced to provide cognate help to NP-specific B cells (2). iNKT cells activated by  $\alpha$ GalCer plus a haptened protein [NP-keyhole limpet hemocyanin (KLH)] induce noncognate help by licensing dendritic cells (DCs), recruiting conventional T<sub>FH</sub> cells, and indirectly enhancing NP-specific B cells (2–4).

Germinal center B cells rely on cognate interactions with T<sub>FH</sub> cells, including binding of MHC class II, CD40L, ICOS, and B7.1 or B7.2 on B cells with their counterpart receptors on T<sub>FH</sub> cells, to progress through germinal centers and differentiate into antibody-secreting plasma cells (PCs) or memory cells (11–13). T<sub>FH</sub> cells secrete IL-4, IFN- $\gamma$ , and IL-21, which contribute to isotype class switching, germinal center maturation (14–17), and reciprocal regulation of T<sub>FH</sub> cells (18). However, what specifically steers

B cells to differentiate into PCs, germinal center cells, or memory B cells remains unknown (19). Immune regulators PD-L1, PD-L2, and PD-1 were recently found to control germinal center B-cell death and PC development (20). PD-L1 is constitutively expressed on T and B cells, but PD-L2 is exclusively and inducibly expressed on APCs, such as macrophages, DCs, and B cells (21). PD-1 is expressed on T and iNKT cells (22) and key for moderating T<sub>FH</sub> cell development and function, including selection and survival in the germinal center (23). PD-1 is a negative regulator that dampens TcR signals (24–26). PD-1 up-regulation is evidence of continuous TcR triggering by cognate antigen (27) and can be a sign of T-cell exhaustion (28), but the many roles of PD-1 in germinal center responses remain to be completely defined. PD-1-deficient mice have increased T<sub>FH</sub> cells, and interrupting the interaction between PD-1 and its ligand(s) has led to increased humoral immune responses (29, 30). However, others found that inhibiting PD-1 signaling reduces DC and PC responses (20, 31). Both of these conclusions are complicated by the fact that PD-1 is highly expressed by CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>+</sup> T follicular regulatory (T<sub>FR</sub>) cells, which (32) express Foxp3<sup>+</sup> and inhibit germinal center responses (33–35). At the same time, IL-2 has been found to signal through CD25 to negatively regulate bcl-6 expression and inhibit T<sub>FH</sub> cell development (36). This effect was mediated independently of Treg cells, relying instead on indirect up-regulation of CD25 on T cells.

## Significance

**Invariant natural killer T (iNKT) cells can facilitate B-cell responses by enhancing helper signals from protein-specific T cells or independently induce a B-cell developmental program; however, key differences in humoral memory after iNKT-cell help remain unclear. We determined that, unlike protein-specific T-cell help, cognate iNKT-cell help expands a large number of IL-10–producing B10 regulatory cells. These findings have broad implications for the types of B cells that may be generated when synthetic iNKT glycolipid ligands are used as vaccine adjuvants and also, outline a direct means to elicit B regulatory cells, which are increasingly being pursued as immunotherapeutic targets for protection against autoimmune disease.**

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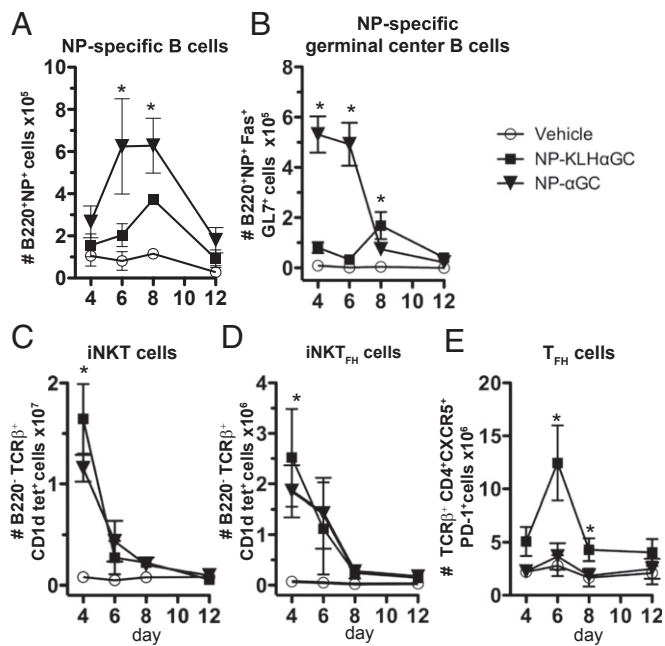
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**Fig. 1.** Noncognate iNKT-cell help induces more T<sub>FH</sub> cells and later development of germinal center B cells than cognate help. Splenocytes from C57BL/6 mice immunized with vehicle [PBS/0.1% (wt/vol) BSA], 100 μg NP-KLH plus 0.5 μg αGalCer (αGC), or 0.5 μg NPαGalCer (NPαGC) were assessed by FACS analysis for numbers of (A) NP-specific B cells, (B) NP-specific germinal center B cells, (C) iNKT cells, (D) iNKT<sub>FH</sub> cells, or (E) T<sub>FH</sub> cells. All cells were measured at days 4, 6, 8, and 12 after immunization. Each data point is a representative of two to four experiments ( $n = 8\text{--}21$  mice per group for days 4–8) or one experiment ( $n = 4$  mice per group for day 12). \* $P \leq 0.05$ .

It remains to be investigated how many of these mechanisms apply to B cells receiving iNKT-cell help. We have found that the primary IgG and IgM antibody titers are similarly induced by cognate and noncognate iNKT-cell help and depend on IL-21, BAFF, IFN- $\gamma$ , CD40L, and ICOS engagement. However, only noncognate iNKT-cell help induces antigen-specific humoral memory (7, 9). These results are relevant to human vaccine development given the increasing interest in  $\alpha$ GalCer as an adjuvant. We found that iNKT<sub>FH</sub> cells expand with similar kinetics when providing cognate or noncognate help, but conventional T<sub>FH</sub> cells only expand when protein is present during noncognate help. Clearly, T<sub>FH</sub> cells provide unique humoral memory signals that are not provided by iNKT<sub>FH</sub> cells, but the nature of these signals remains unknown. We show here that germinal center B cells multiply more rapidly after cognate iNKT-cell help than after noncognate help, but they are not sustained and undergo death before completing productive maturation beyond the germinal center. At the same time, we detected more protein-specific T<sub>FR</sub> cells after cognate iNKT-cell help than after noncognate iNKT-cell help, consistent with a regulatory milieu. Cognate iNKT-cell help also preferentially expands marginal zone (MZ) B cells and drives greater numbers of IL-10<sup>+</sup> regulatory B10 cells than noncognate activation. These data support the notion that innate, cognate iNKT-cell help induces a rapid, primary antibody response by innate B cells with an increased presence of T<sub>FR</sub>. However, this innate response can be overridden by inflammatory responses provided by adaptive peptide-specific T cells to drive a B memory response. These results will inform future efforts to develop  $\alpha$ GalCer as a vaccine adjuvant.

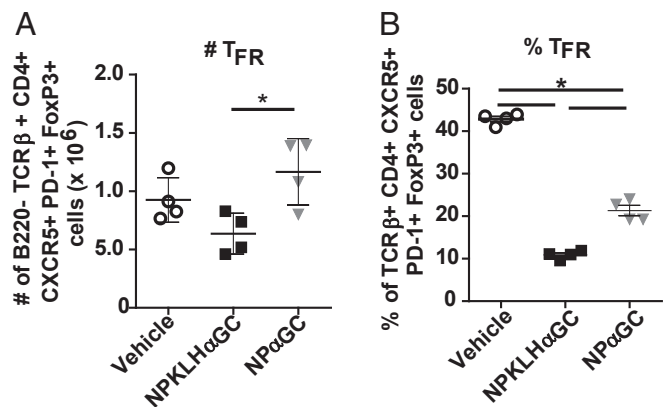
## Results

**Noncognate iNKT Cell Help Induces Higher Numbers of T<sub>FH</sub> Cells than Cognate iNKT-Cell Help.** Noncognate iNKT-cell help for B cells drives a greater humoral memory response than cognate iNKT-

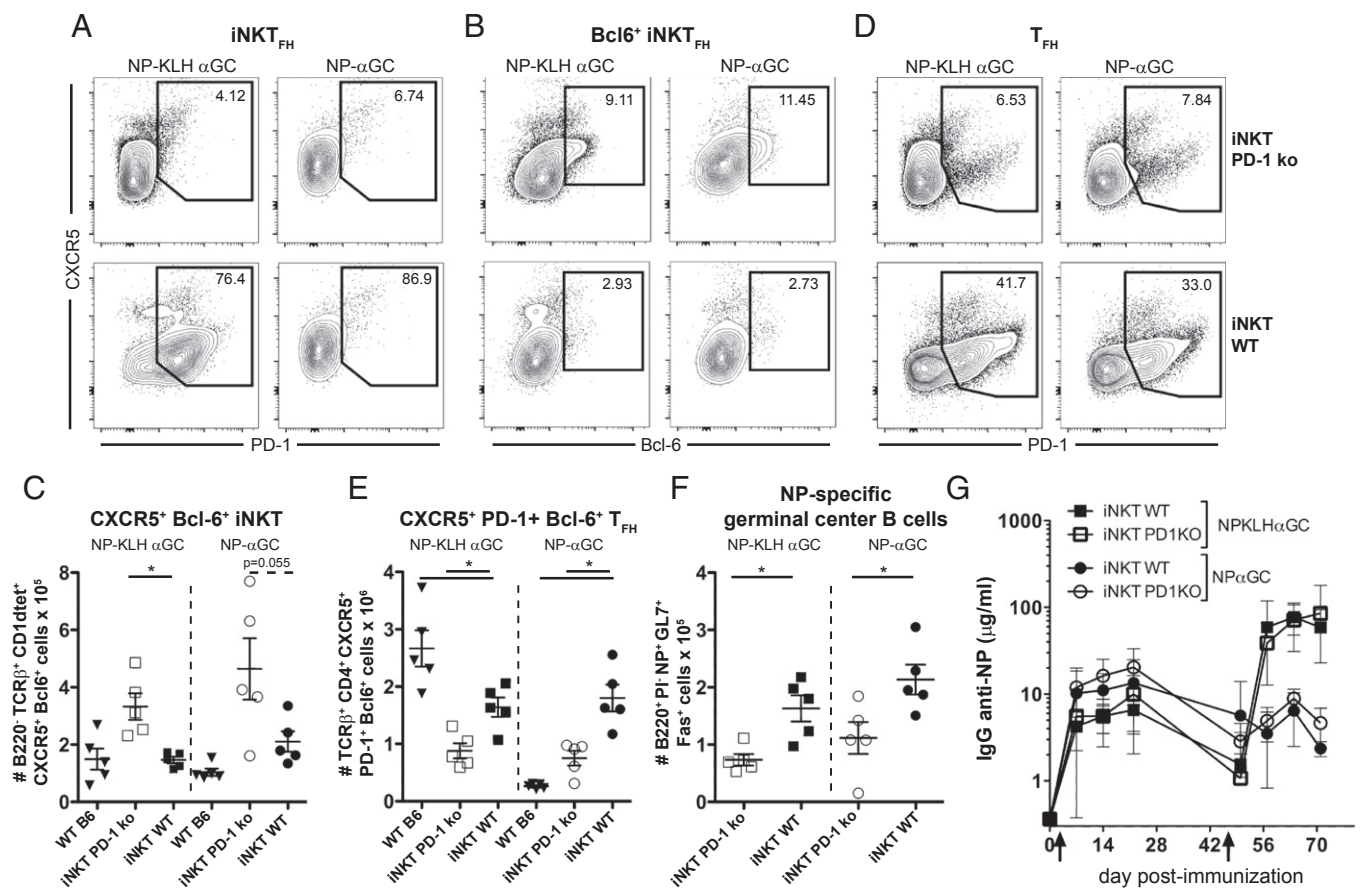
cell help (9). To determine the relative contribution of cellular subsets to these two types of responses, we immunized mice with NP-KLH plus lipid ( $\alpha$ GalCer) or NP $\alpha$ GalCer and monitored expansion of germinal center B, iNKT, iNKT<sub>FH</sub>, or T<sub>FH</sub> cells. The number of NP-specific B cells gradually increased from day 4 to day 8, but NP-specific B-cell numbers were significantly higher after immunization with NP $\alpha$ GalCer compared with NP-KLH $\alpha$ GalCer at day 6 and 8 (Fig. 1A). Cognate iNKT-cell help induced a dramatic early expansion of NP-specific germinal center B cells (B220<sup>+</sup>NP<sup>+</sup>Fas<sup>+</sup>GL7<sup>+</sup>) at days 4 and 6, whereas noncognate help resulted in a later, modest response with increases in germinal center B-cell numbers evident at days 8 and 12 (Fig. 1B). Cognate iNKT cell antigen does not induce B-cell memory, and therefore, higher numbers of NP-specific B and early germinal center B cells were unexpected. The precipitous drop in germinal center B-cell numbers 6 d after cognate antigen immunization coincides with an increase in apoptotic NP-specific B cells (Fig. S1).

NP-KLH $\alpha$ GalCer immunization induced slightly more iNKT (B220<sup>+</sup>TCRβ<sup>+</sup>CD1d tet<sup>+</sup>) and iNKT<sub>FH</sub> (B220<sup>+</sup>TCRβ<sup>+</sup>CD1d tet<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup>) cells than NP $\alpha$ GalCer at day 4 (Fig. 1C and D). The most dramatic difference was the significant expansion of T<sub>FH</sub> cells (B220<sup>+</sup>TCRβ<sup>+</sup>CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup>) over the entire NP-KLH $\alpha$ GalCer kinetic course, with a peak at day 6, whereas NP $\alpha$ GalCer induced no detectable increase in T<sub>FH</sub> cells during the entire monitoring period (Fig. 1E). Thus, T<sub>FH</sub> cells only expanded in the presence of protein antigen (plus  $\alpha$ GalCer). To consider other factors that influence the survival and function of antigen-specific B cells, we measured expression of BAFF by iNKT, iNKT<sub>FH</sub>, and T<sub>FH</sub> cells after immunization. NP-KLH $\alpha$ GalCer and NP $\alpha$ GalCer induced equal BAFF expression by iNKT, iNKT<sub>FH</sub>, and T<sub>FH</sub> cells at day 4, 6, or 8 (Fig. S2A–C). As shown for T<sub>FH</sub> cells (37), BAFF was preferentially expressed by the iNKT<sub>FH</sub> cell subset but was not different between the two immunization groups (Fig. S2D and E). These results indicate that cognate help drives an early, abortive germinal center B-cell response, but only noncognate iNKT-cell help leads to expansion of T<sub>FH</sub> cells and a mature germinal center B-cell response.

**Cognate iNKT-Cell Help Retains More T<sub>FR</sub> Cells.** We next investigated the possibility that humoral memory fails to develop after NP $\alpha$ GalCer immunization because of the actions of a regulatory population of cells. T<sub>FR</sub> cells are CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup>; express Foxp3, Bcl-6, and Blimp-1; and function to inhibit the germinal center response (33–35). We detected a significant decrease in percentage and number of FoxP3<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup> T<sub>FR</sub> cells at



**Fig. 2.** Cognate iNKT-cell help induces more T<sub>FR</sub> cells than noncognate iNKT-cell help. Splenocytes from C57BL/6 mice immunized with vehicle [PBS/0.1% (wt/vol) BSA], 100 μg NP-KLH plus 0.5 μg  $\alpha$ GalCer (αGC), or 0.5 μg NP $\alpha$ GalCer (NPαGC) were assessed at day 6 by FACS analysis for (A) numbers or (B) percentages of B220<sup>+</sup>TCRβ<sup>+</sup>CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup>FoxP3<sup>+</sup> T<sub>FR</sub> cells (representative of three experiments;  $n = 4$  mice per group). \* $P \leq 0.05$ .



**Fig. 3.** Selective elimination of PD-1 on iNKT cells increases CXCR5<sup>+</sup>Bcl6<sup>+</sup> iNKT<sub>FH</sub> cells but does not rescue humoral memory. Eight days after i.v. immunization of iNKT PD-1 KO or iNKT WT mixed BM chimeras with 0.5 μg NPαGalCer (NPαGC) or 100 μg NP-KLH plus 0.5 μg αGalCer (αGC), splenocytes were labeled for FACS to determine numbers of (A) CXCR5<sup>+</sup>PD-1<sup>+</sup> iNKT cells, (B and C) CXCR5<sup>+</sup>Bcl6<sup>+</sup> iNKT cells, (D and E) CXCR5<sup>+</sup>PD-1<sup>+</sup>Bcl6<sup>+</sup> T cells, and (F) NP-specific germinal center B cells (representative of three experiments; *n* = 5 mice per group). \**P* ≤ 0.05. (G) ELISA also detected 4-hydroxy-5-iodo-3-NP (NIP) -specific IgG in sera from iNKT PD-1 KO or iNKT WT mixed BM chimeras immunized i.v. with 100 μg NP-KLH plus 0.5 μg αGC or 0.5 μg NPαGC on day 0 followed by an i.v. boost of 100 μg NP-KLH or 0.5 μg NPαGC on day 50 (representative of two experiments; *n* = 4–6 mice per group). \**P* ≤ 0.05.

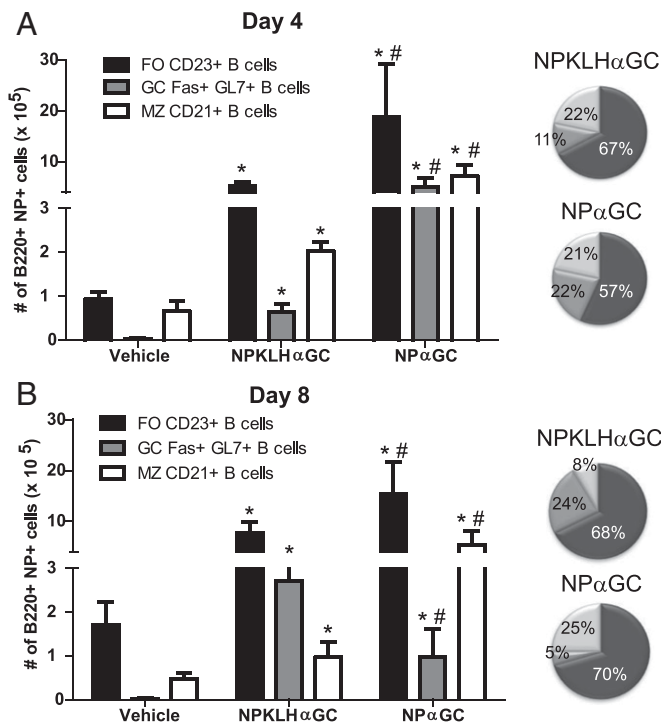
6 d after NP-KLHαGalCer compared with the vehicle control; however, NPαGalCer induced a significantly smaller decrease in percentage and no decrease in the number of T<sub>FR</sub> cells after NPαGalCer immunization (Fig. 2 and Fig. S3A). There were also no differences in Treg, iNKT<sub>FR</sub> (natural killer T follicular regulatory cells), or iNKTreg cell numbers after both immunizations (Fig. S3A and B). PD-1-PD-L1 interactions negatively regulate T<sub>FR</sub> cell numbers, and as a result, PD-1-deficient mice have more T<sub>FR</sub> cells (32). We evaluated B-cell PD-L1 and PD-L2 expression and found that cognate iNKT-cell help for NPαGalCer fails to up-regulate PD-L1 and PD-L2 on germinal center B cells at day 4, whereas noncognate help does up-regulate early germinal center B-cell PD-L1 and PD-L2 expression after NP-KLHαGalCer immunization (Fig. S3C and D). PD-L1 and PD-L2 up-regulation during noncognate iNKT help likely inhibits T<sub>FR</sub> cells and facilitates expansion of NP-specific germinal center B-cell numbers, which support the humoral memory induced by noncognate iNKT-cell help.

**Selective Increases in iNKT<sub>FH</sub> Cells Do Not Rescue Humoral Memory.** NPαGalCer immunization induces iNKT<sub>FH</sub> cells but not T<sub>FH</sub> cells, and retains T<sub>FR</sub> cells, a combination that may not support memory B-cell development. To determine if one critical aspect of this unsupportive environment is the limiting number of iNKT helper cells, we used mixed bone marrow (BM) chimeras to artificially increase the number of available splenic iNKT<sub>FH</sub> cells.

The precise role of PD-1 on T<sub>FH</sub> cells is complex, but at least one report found that PD-1-deficient mice have more T<sub>FH</sub> cells

and enhanced humoral immunity (29). We hypothesized that this mechanism may also regulate iNKT<sub>FH</sub> cell numbers. To increase iNKT<sub>FH</sub> cells, we generated mixed BM chimeras, in which PD-1 was selectively missing on iNKT cells. Irradiated Jα18-deficient (iNKT cell-deficient) hosts were reconstituted with a mixture of 25% PD-1-deficient BM and 75% Jα18-deficient BM (iNKT PD-1KO chimeras). Control chimeras with PD-1-sufficient iNKT cells were created by reconstituting Jα18-deficient hosts with 25% C57BL/6J WT BM mixed with 75% Jα18-deficient BM (iNKT WT chimeras). To control for inconsistencies caused by irradiation, we immunized unmanipulated C57BL/6 mice in parallel and noted that the WT BM chimeras generated at least as many iNKT<sub>FH</sub> cells as the unmanipulated WT B6 mice (Fig. 3C). iNKT PD-1KO chimeras showed no detectable PD-1 on their iNKT<sub>FH</sub> cells (Fig. 3A), but as expected, significantly more CXCR5<sup>+</sup>Bcl6<sup>+</sup> iNKT<sub>FH</sub> cells were generated than in the iNKT WT chimeras (Fig. 3B and C). These data confirm that PD-1 plays the same inhibitory role in iNKT<sub>FH</sub> cell differentiation as it does for T<sub>FH</sub> cells (38). Unexpectedly, the number of NP-specific germinal center B and T<sub>FH</sub> cells after both cognate and noncognate immunization is reduced compared with that in WT chimeras (Fig. 3D–F). Furthermore, despite increased iNKT<sub>FH</sub> cell numbers, iNKT PD-1KO chimeras showed no increase in primary or memory anti-NP IgG antibody over WT chimeras (Fig. 3G). Thus, PD-1 signaling on iNKT cells negatively regulates iNKT<sub>FH</sub> cell numbers.

To verify that these results were not a caveat of the PD-1 system, we altered iNKT<sub>FH</sub> cell numbers using a complementary



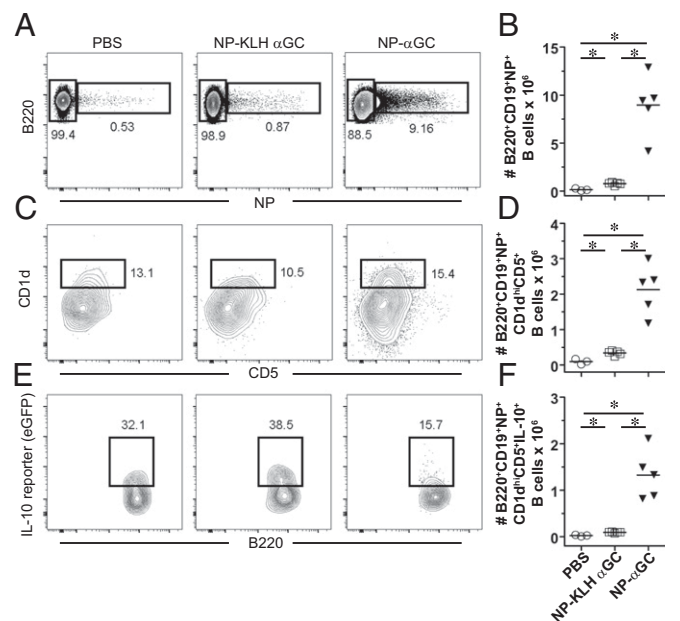
**Fig. 4.** Cognate iNKT-cell help preferentially expands innate B-cell populations. Splenocytes from C57BL/6 mice immunized with vehicle [PBS/0.1% (wt/vol) BSA], 100  $\mu$ g NP-KLH plus 0.5  $\mu$ g  $\alpha$ GalCer ( $\alpha$ GC), or 0.5  $\mu$ g NP $\alpha$ GalCer (NP $\alpha$ GC) were assessed by FACS analysis for numbers of follicular (FO) B220<sup>+</sup>NP<sup>+</sup>CD21<sup>-</sup>CD23<sup>+</sup> B cells, B220<sup>+</sup>NP<sup>+</sup>Fas<sup>+</sup>GL7<sup>+</sup> NP-specific germinal center (GC Fas<sup>+</sup>GL7<sup>+</sup>) B cells, and MZ B220<sup>+</sup>NP<sup>+</sup>CD23<sup>lo</sup>CD21<sup>+</sup> B cells at days (A) 4 and (B) 8 postimmunization. Pie charts show B-cell subsets as percentages of total B cells in the spleen (A) 4 and (B) 8 d after immunization with NP $\alpha$ GC or NP-KLH plus  $\alpha$ GC (representative of two experiments;  $n = 8$ –10 mice per group). \* $P \leq 0.05$  to vehicle control; # $P \leq 0.05$  to NP-KLH $\alpha$ GC.

approach. PD-1 impairs IL-2 signaling through the CD25 receptor (39), which normally favors blimp-1 over bcl-6 expression and T effector over T<sub>FH</sub> cell development (36). In this context, CD25 negatively regulates T<sub>FH</sub> cell development, and therefore, we hypothesized that CD25 would negatively regulate iNKT<sub>FH</sub> cell development. We created mixed BM chimeras as described above, in which CD25 was selectively absent from iNKT cells to increase iNKT<sub>FH</sub> cell numbers during the antigen response. As predicted, iNKT<sub>FH</sub> cells expanded preferentially in iNKT CD25KO BM chimeras compared with controls (Fig. S4 A and B); however, humoral memory remained undetectable after cognate immunization (Fig. S4C). The iNKT CD25KO chimera results are consistent with the iNKT PD-1KO chimera data and confirm that increasing iNKT<sub>FH</sub> cell numbers does not enhance humoral memory. Thus, the lack of humoral memory after NP $\alpha$ GalCer is not a result of insufficient iNKT<sub>FH</sub> cells but instead, suggests that iNKT<sub>FH</sub> cells provide fundamentally different help from conventional T<sub>FH</sub> cells.

**Cognate iNKT-Cell Help Expands Innate B-Cell Populations.** We next considered that iNKT helper cells may partner with innate B cells that do not support humoral memory. Cognate antigen immunization localizes iNKT cells in the MZ (40), and MZ B cells express the highest levels of CD1d of any immune cell (41); therefore, we examined the influence of iNKT-cell help on MZ B cells. We found that mice receiving either NP $\alpha$ GalCer or NP-KLH $\alpha$ GalCer had expanded MZ B-cell populations at day 4, but only mice receiving NP $\alpha$ GalCer still had an expanded MZ B-cell population at day 8 (Fig. 4). Thus, cognate iNKT-cell help sustains expansion of MZ

B cells, but noncognate iNKT-cell help favors a more transient MZ B-cell expansion and a later germinal center B-cell response.

Because cognate iNKT-cell help favors expansion of CD1d<sup>hi</sup> MZ B cells in the spleen, we evaluated the effect of iNKT activation on a related splenic innate, regulatory B-cell population (IL-10-producing B10 cells), which is also CD1d<sup>hi</sup>. NP $\alpha$ GalCer induced more NP-specific B10 cells, and higher numbers of those cells were IL-10<sup>+</sup> 4 d after immunization than after NP-KLH $\alpha$ GalCer (Fig. 5). We obtained these results using VertX IL-10 reporter mice, which accurately report IL-10 production using a bicistronic GFP reporter downstream of the IL-10 gene (42). LPS immunization confirms the reliability of these mice, inducing significantly more IL-10-producing B cells over vehicle-immunized mice after 2 days (Fig. S5). NP $\alpha$ GalCer immunization induced a higher number of NP-specific B cells (Fig. 5 A and B), a higher number of CD5<sup>+</sup>CD1d<sup>+</sup> B cells (Fig. 5 C and D), and a higher number of IL-10-producing B10 cells in VertX mice than NP-KLH $\alpha$ GalCer (Fig. 5 E and F). We also confirmed these results using intracellular FACS staining for IL-10 protein (Fig. S6 A–F). However, cognate iNKT-cell help does not preferentially induce IL-10-secreting B cells, because the percentage of IL-10<sup>+</sup> B cells expanded in the antigen-specific CD5<sup>+</sup>CD1d<sup>+</sup> B regulatory cell populations is actually slightly lower in NP $\alpha$ GalCer-immunized mice than in NP-KLH $\alpha$ GalCer immunized mice (Fig. S6G). Because of the large expansion of antigen-specific B cells, the consequence of cognate iNKT-cell help is still an expanded regulatory B-cell population in the spleen. The innate B10 cells engaged by cognate interactions with iNKT cells may go on to become PCs, but whether they go on to develop into long-term memory B cells is unknown (43). This innate B-cell trait may explain the fundamentally different memory outcomes after cognate and noncognate iNKT-cell help.



**Fig. 5.** Cognate iNKT-cell help expands antigen-specific IL-10<sup>+</sup> B10 B cells. FACS labeling identified expanded populations of B220<sup>+</sup>CD19<sup>+</sup>CD1d<sup>+</sup>CD5<sup>+</sup>IL-10<sup>+</sup> cells using VertX IL-10 reporter mice 4 d after immunization with vehicle [PBS/0.1% (wt/vol) BSA], 100  $\mu$ g NP-KLH plus 0.5  $\mu$ g  $\alpha$ GalCer ( $\alpha$ GC), or 0.5  $\mu$ g NP $\alpha$ GalCer (NP $\alpha$ GC). FACS analysis revealed (A and B) B220<sup>+</sup>NP<sup>+</sup>, (C and D) B220<sup>+</sup>NP<sup>+</sup>CD1d<sup>+</sup>CD5<sup>+</sup>, and (E and F) B220<sup>+</sup>NP<sup>+</sup>CD1d<sup>+</sup>CD5<sup>+</sup>IL-10<sup>+</sup> subsets of cells. CD5 vs. CD1d gates were selected based on B220<sup>+</sup>NP<sup>-</sup> control samples as published (53) (representative of two experiments;  $n = 8$ –10 mice per group). \* $P \leq 0.05$ .

## Discussion

We set out to understand the differences in B-cell activation outcome after cognate and noncognate iNKT help. Clarifying the fundamental biology of iNKT and B-cell cooperation may allow us to use this type of help for vaccine development. We examined iNKT and B-cell kinetics after immunization with cognate or noncognate antigen. These studies revealed parallel increases in iNKT cell numbers but early differences in antigen-specific germinal center B cells. It seems that cognate glycolipid antigen induces a marked but unsustained germinal center B-cell expansion. Numbers of germinal center B cells induced by cognate glycolipid antigen drop precipitously at day 6, the same kinetics that de Vinuesa et al. (44) described for spontaneous involution of germinal centers after T-independent antigen immunization. Physiological germinal center maturation may depend on T-cell signals to prevent centrocytes from undergoing apoptosis (44). T-cell signals may rescue germinal center B cells from apoptosis and induce a proportion of germinal center B cells to differentiate into centroblasts or memory B cells. In the absence of appropriate T-cell signals, germinal center B cells fail to become centroblasts and will not produce memory B cells or PCs. Thus, premature involution of germinal centers and lack of memory B cells or PCs after cognate iNKT-cell help are consistent with a lack of T-cell signals to sustain germinal centers. Wermeling et al. (45) have reported that antigen-activated B cells significantly down-modulate CD1d expression on entry into the germinal center, and therefore, these signals may also need to be provided before entry into the germinal center.

In contrast, noncognate antigen induces a later, modestly sustained germinal center B-cell expansion that is consistent with germinal center B-cell expansion patterns after protein-specific  $T_{FH}$ -cell help. Furthermore, only noncognate immunization is able to induce expansion of peptide-specific  $T_{FH}$  cells. These results are consistent with the findings of others suggesting that  $T_{FH}$  cells are critical for iNKT $_{FH}$ -helped long-term humoral memory. For example, when  $CD4^+$  T cell-deficient MHC class II $^{-/-}$  mice are immunized with peptide plus  $\alpha$ GalCer, they form germinal centers and initiate a primary antibody response but do not maintain long-lasting secondary antibody (46). Thus,  $CD4^+$   $T_{FH}$  cells are the cells primarily responsible for noncognate humoral memory. However, these findings still beg the question of why iNKT cells are ineffective at driving B-cell memory on their own.

We first considered that cognate iNKT-cell help may fail to induce humoral memory because of a dominant regulatory population. We compared Treg,  $T_{FR}$ , iNKTreg, and iNKT $_{FR}$  cells and found that cognate iNKT-cell help does not reduce  $T_{FR}$  cells as much as noncognate iNKT-cell help does.  $T_{FR}$  cells suppress germinal center B-cell responses, including affinity maturation, IgG production, and PC differentiation by limiting the number of  $T_{FH}$  cells (32–34). The higher levels of  $T_{FR}$  cells remaining after cognate iNKT cell activation may contribute to a generally suppressive environment but may not be the only cellular mechanism responsible for the lack of humoral memory.

We next considered that the number of iNKT $_{FH}$  cells generated during cognate iNKT cell activation was inadequate to sustain germinal center B cells through memory B-cell development. To address this possibility, we assessed humoral memory in two sets of mixed BM chimeras with selectively increased iNKT $_{FH}$  cell numbers. This approach revealed that PD-1 and CD25 have the same inhibitory roles in iNKT $_{FH}$  cell development as have been reported for  $T_{FH}$  cells (38), because iNKT $_{FH}$  cell numbers were increased in mice selectively missing PD-1 or CD25 on only iNKT cells (23, 29, 38). However, increasing iNKT $_{FH}$  cell numbers did not improve the B-cell humoral memory outcome after cognate iNKT antigen immunization. It is also possible that removing PD-1 from iNKT cells has increased numbers of iNKT $_{FR}$  cells in the same way that  $T_{FR}$  cell numbers

are enhanced in PD-1-deficient mice (32). However, this caveat is not a concern in the CD25 BM chimeras, which impart their effect independent of PD-1, and they produce the same humoral outcome as the PD-1 chimeras in our studies. These two complementary BM chimeras suggest that the lack of humoral memory after cognate antigen immunization cannot be overcome by increasing iNKT $_{FH}$  cell numbers but rather, that B-cell memory is a unique product of  $T_{FH}$ -cell help.

iNKT cells consolidate in the MZ and favor interactions with MZ B cells (40) which express a higher level of CD1d than all other APCs in the spleen (41), and iNKT cells cultured with MZ B cells proliferate more vigorously than iNKT cells mixed with follicular B cells (2). Given this previous work, we evaluated the possibility that activated iNKT cells preferentially expand innate MZ B cells. The MZ is comprised of innate B cells, such as MZ B, MZ-T2 B, and B10 cells, which have restricted repertoires, reduced activation thresholds, and alternative activation responses (47). These B cells all produce IL-10, an immunoregulatory cytokine, which contributes to development of Treg cells, induces expansion of more B regulatory cells, and inhibits activity of effector B cells (48). We found that cognate iNKT-cell help favored more sustained expansion of MZ B cells than noncognate iNKT-cell help, and it yields larger numbers of expanded antigen-specific CD1d $^+$ CD5 $^+$ IL-10 $^+$  B10 cells than noncognate iNKT-cell help. Thus, iNKT cells favor interaction with innate MZ-localized B-cell subsets, which may be unlikely or unable to develop into memory B cells. The ability or inability of antigen-specific IL-10-producing B cells to develop into memory B cells in this system will need to be more precisely addressed using mice engineered to visualize IL-10 $^+$  cell fate mapping.

It is intriguing to speculate that exclusive iNKT-cell help favors an innate B-cell response, because the two cells cooperate to generate an innate humoral response in advance of a more precise and evolved adaptive response. Recent data from Wingender and coworkers (49) have suggested that iNKT cells previously activated by  $\alpha$ GalCer become IL-10-producing iNKT10 cells. Thus, IL-10 from iNKT cells may dominate the response to expand MZB cells and maintain  $T_{FR}$  cells under conditions where there are no inflammatory cytokines from  $T_{FH}$  cells to override this regulatory environment and expand B effector cells and inhibit  $T_{FR}$  cells. It would also follow that innate iNKT cells then preferentially help innate B cells make an early plasmablast antibody response using primarily MZ B cells, which do not develop into a memory population unless accompanied by confirmatory signals from a conventional protein-specific helper response. These results may be one mechanism that allows for an extremely rapid but innate humoral response to bridge the gap between more traditional innate and adaptive responses.

## Materials and Methods

**Mice.** C57BL/6J WT mice, C57BL/6 Pcd1 KO mice (gift from Dario Vignali, St. Jude Children's Research Hospital, Memphis, TN) lacking programmed cell death-1 (50), VertX mice (gift from Markus Mohrs, Trudeau Institute, Saranac Lake, NY) (42), B6.129S4-Il2ra $^{tm1Dw}/J$  mice lacking CD25 (51), and  $\alpha$ 18-deficient mice lacking  $\alpha$ 18 $^+$  iNKT cells (52) (and a subset of  $CD4^+$  T cells) were housed and bred at the Trudeau Institute and the Karolinska Institutet. All live animal experimental protocols were approved by the Trudeau Institute or Karolinska Institutet Animal Care and Use Committee.

**Flow Cytometry.** Single-spleen cell suspensions were stained with mAbs for flow cytometry as listed in *SI Materials and Methods*. Antigen-specific B cells were identified with NP-allophycocyanin as published (9). iNKT cells were identified with mouse CD1d- $\alpha$ GalCer tetramers (PB557; NIH Tetramer Core Facility) conjugated to allophycocyanin or phycoerythrin. The Foxp3/Transcription Factor Staining Set (eBioscience) was used for intracellular staining of Foxp3, Bcl6, and IL-10. Samples were acquired on a FACSCanto II (BD) and analyzed with FlowJo software (TreeStar).

**Bone Marrow Chimeras.** Recipient C57BL/6  $\alpha$ 18-deficient mice were irradiated two times with 500 rad, with a 3- to 4-h rest between doses. Recipient mice were reconstituted with  $5\text{--}10 \times 10^6$  BM cells. Donor BM included 75%  $\alpha$ 18-deficient BM mixed with either 25% *Pdcd1* KO BM or 25% CD25 KO BM. Controls were reconstituted with 75%  $\alpha$ 18-deficient BM mixed with 25% C57BL/6 WT BM. These reconstitution BM mixtures resulted in mice in which predominantly iNKT cells were deficient in PD-1 or CD25 or all cells were PD-1- or CD25-sufficient. Reconstitution of iNKT, T, and B cells in the spleen was confirmed by flow cytometry after 8–10 wk.

**Antigens, Immunizations, and Serum Collections.** NP(25)-KLH (Biosearch Technologies), NP(27)-KLH (Biosearch Technologies), NP(30)-KLH (Biosearch Technologies), NP(33)-KLH (Biosearch Technologies), NP- $\alpha$ GalCer, or  $\alpha$ GalCer were administered i.v. Immunizations included 100  $\mu$ g protein suspended in PBS plus 0.1% (wt/vol) BSA or 0.5  $\mu$ g glycolipid antigen solubilized in  $\leq 0.1\%$  (vol/vol) DMSO and then, resuspended in PBS plus 0.1% (wt/vol)

BSA. Serum was collected submandibularly and stored at  $-20^\circ\text{C}$  until ELISA assessment.

**ELISA.** NP-specific IgG and IgM were detected in serum by heteroclitic 4-hydroxy-5-iodo-3-NP-specific ELISA as published (2) and are described in *SI Materials and Methods*.

**Statistics.** GraphPad PRISM5 software was used for two-tailed *t* tests for normally distributed datasets.

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