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Human Invariant NKT Cell Subsets Differentially Promote Differentiation, Antibody Production, and T Cell Stimulation by B Cells In Vitro

Shijuan Grace Zeng,*1 Yasmeen G. Ghnewa,* Vincent P. O’Reilly,* Victoria G. Lyons,* Ann Atzberger,*2 Andrew E. Hogan,† Mark A. Exley,‡ and Derek G. Doherty*†

Invariant NK T (iNKT) cells can provide help for B cell activation and Ab production. Because B cells are also capable of cytokine production, Ag presentation, and T cell activation, we hypothesized that iNKT cells will also influence these activities. Furthermore, subsets of iNKT cells based on CD4 and CD8 expression that have distinct functional activities may differentially affect B cell functions. We investigated the effects of coculturing expanded human CD4+, CD8α−, and CD4+CD8α− double-negative (DN) iNKT cells with autologous peripheral B cells in vitro. All iNKT cell subsets induced IgM, IgA, and IgG release by B cells without needing the iNKT cell agonist ligand α-galactosylceramide. Additionally, CD4+ iNKT cells induced expansions of cells with phenotypes of regulatory B cells. When cocultured with α-galactosylceramide–pulsed B cells, CD4+ and DN iNKT cells secreted Th1 and Th2 cytokines but at 10–1000-fold lower levels than when cultured with dendritic cells. CD4+ iNKT cells reciprocally phenotypes of regulatory B cells. When cocultured with

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Abbreviations used in this article: Breg, regulatory B cell; CBA, cytometric bead array; DC, dendritic cell; DN, double-negative; GC, α-galactosylceramide; FMO, fluorescence minus one (flow cytometry control); iDC, immature dendritic cell; iNKT, invariant NKT; MFI, mean fluorescence intensity; MZB, marginal zone B cell; PD-1, programmed cell death-1; PPD, purified protein derivative of tuberculin; SEB, Staphylococcal enterotoxin B; T Follic, follicular Th.

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CD8αβ (double-negative [DN]), and CD4-CD8αβ (CD8α+) iNKT cells (11, 13, 26). CD4+ iNKT cells release the most Th2 cytokines, and CD8αβ and DN iNKT cells predominantly exhibit Th1 phenotypes and cytotoxic activity (11, 13, 27). To date, two studies (28, 29) have examined the relative contributions of human iNKT cells subsets to B cell help and found that both CD4+ and CD4- iNKT cells similarly induced B cell proliferation, but CD4+ iNKT cells induced higher levels of Ab production.

In addition to their roles in Ab production, B cells are potent APCs that can prime CD4+ T cells without the participation of DC or macrophages (30). Similar to DC, B cells can produce both Th1- and Th2-type cytokines and can be polarized toward one or the other subset subsequent to interaction with CD4+ Th1 or Th2 cells (31). The unique abilities of iNKT cells to selectively secrete 6B11, Th1, Th2, Th17, or regulatory T cell cytokines (10–13) and to induce DC maturation (7, 8, 32) led us to hypothesize that iNKT cells may exert stimulatory or regulatory control over Ag presentation and T cell activation by B cells. In this study, we have examined the outcomes of culturing human peripheral B cells with expanded autologous iNKT cells or sorted CD4+, CD8α+, and DN iNKT cell subsets in vitro in the absence or presence of α-GC. We show that the iNKT cell subsets differentially induce phenotypic differentiation, Ab secretion, and T cell stimulation by B cells. We also show that CD4+ iNKT cells promote the development of cells with phenotypes of regulatory B (Breg) cells and the production of IL-10 by some B cells. Thus, CD4+, CD8α+, and DN iNKT cells can differentially promote T cell or Ab responses via their interactions with B cells. This observation has implications for iNKT cell-based therapies for cancer and autoimmune disease, which may require the selective targeting of functionally distinct subsets of iNKT cells.

Materials and Methods

A bs and flow cytometry

Fluorochrome-conjugated mAbs specific for human CD1d, CD3, CD4, CD5, CD8α, CD19, CD20, CD22, CD24, CD27, CD38, CD40, CD58, CD69, CD80, CD83, CD86, CD95, CD107a, HLA-DR, IFN-γ, IL-4, IL-10, IL-13, IL-21, the TCR Vα24 and Vβ11 chains, the CD3δ of the iNKT cell TCR (6B11), CXCR5, programmed cell death-1 (PD-1), and isotype control mAbs were purchased from Immunotools (Friesoythe, Germany), eBioscience (Hatfield, U.K.), BioLegend (San Diego, CA), and BD Biosciences (Oxford, U.K.). A total of 10⁶ cells were labeled with mAbs and analyzed using a CyAN ADP flow cytometer (Beckman Coulter, High Wycombe, U.K.). Data were analyzed with the Summit v4.3 software (DakoCytomation) and FlowJo v7.6 (Tree Star). PBMC were isolated from blood samples obtained from healthy donors or from buffy coat packs (kindly provided by the Irish Blood Transfusion Service) by density gradient centrifugation over Lymphoprep (Nycomed Pharma, Oslo, Norway). B cells were purified by magnetic bead sorting using CD19 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and purity of B cells was determined to be >99% by flow cytometric analysis of CD20 expression. Enriched B cells were cryopreserved or maintained in iNKT cell medium, which consisted of RPMI 1640 containing 0.05 mM L-glutamine, 10% v/v HyClone FBS (Thermo-Scientific, Logan, UT), 0.02 M HEPES buffer, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B Fungizone, 1× MEM nonessential amino acids, and 0.05 mM 2-ME (Life Technologies BRL, Paisley, U.K.).

Generation of monococyte-derived DC

Monocytes were enriched to >90% purity from PBMC by positive selection using CD14 microbeads (Miltenyi Biotec). The monocytes were allowed to differentiate into immature DC (iDC) by culturing them for 6 d in the presence of GM-CSF and IL-4 as described previously (32). Flow cytometry was used to verify that differentiation into iDC had taken place and cells expressed HLA-DR and CD11c but not CD14.

Generation of iNKT cell lines

iNKT cells were enriched from PBMC by staining with a PE-conjugated anti-iNKT cell mAb (clone 6B11) followed by positive selection of the PE-positive cells by magnetic bead separation (Miltenyi Biotec). In later experiments, anti-iNKT cell microbeads were used. Enriched iNKT cells were then purified to >99% purity by flow cytometric sorting of CD3+ Vα24+ Vβ11+ cells using a MoFlo XDP Cell Sorter (Beckman Coulter). Sorted iNKT cells were cultured in iNKT cell medium and expanded by one of two methods described previously (13, 32). In the first method, iNKT cells were subjected to a single stimulation with plate-bound anti-CD3 (HIT3A) mAb (BD Biosciences) in the absence of irradiated feeder cells and cultured in the presence of rIL-2. In the second method, iNKT cells were stimulated with PHA in the presence of irradiated allogeneic PBMC and IL-2. Purity and phenotype of iNKT cell lines were assessed by flow cytometry after staining the cells with mAbs specific for CD3, 6B11, CD4, and CD8. Both methods resulted in 750–1000-fold enrichment of iNKT cells and yielded cell lines of which >98% displayed 6B11+CD3+ phenotypes (Fig. 1A). iNKT cell lines were phenotyped for expression of CD4 and CD8α (Fig. 1B) and means of 20 ± 21, 68 ± 26, and 11 ± 8% of 6B11+ cells were CD4+, DN and CD8α+, respectively (Fig. 1C). Expanded iNKT cell lines were further sorted into CD4+, CD8+, and DN cell subsets by MoFlo (Beckman Coulter) cell sorting.

Coculture of B cells with iNKT cells

B cells were cocultured with iNKT or as controls non-iNKT cells (total PBMC expanded with anti-CD3 mAb or PHA and IL-2 as done with iNKT cells) for 3 or 10 d at 1:1 ratios in 96-well round-bottom plates (Corning Life Sciences) at cell densities of 10⁶ cells/ml. The following stimulators or blockers were added: 100 ng/ml α-GC (Funakoshi, Tokyo, Japan), 10 ng/ml PMA, and 1 μg/ml ionomycin (both from Sigma-Aldrich) and 10 μg/ml each of anti-CD1d, anti-CD40, anti-CD154, anti–IL-4, and anti–IL-13 mAb. Before adding to cultures, α-GC was subjected to heat, sonication, and vortexing as described previously (13). Supernatants were harvested and frozen at −20°C until they were analyzed for cytokine

FIGURE 1. Expansion of iNKT cell subsets. (A) Flow cytometric analysis of CD3 and the Vα24o18 TCR (6B11) expression by freshly isolated PBMC and a 6-wk-old iNKT cell line. Plots are representative of iNKT lines generated from 10 healthy donors. (B) Flow cytometric analysis of CD4 and CD8 expression by expanded iNKT cells after electronically gating on CD3+6B11+ cells. (C) Mean proportions of CD4+, DN, and CD8+ iNKT cell subsets found in seven iNKT cell lines.
and Ig production. Cells were recovered for phenotypic analysis by flow cytometry or for use as stimulators of T cells.

**Analysis of cytokine and Ab secretion**

Cytometric bead array (CBA) kits were used to quantify the levels of cytokines and IgGs in supernatants from the B–iNKT cell cocultures, according to the manufacturer’s instructions (BD Biosciences). The cytokines assayed were IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, and IL-13. The IgGs assayed for were IgA, IgM, total IgG, IgG1, IgG2, and IgE. Flow cytometric data were generated using a CyAn ADP flow cytometer (Beckman Coulter) and geometric means of the individual bead populations were analyzed using Summit v4.3 software (DakoCytomation). GraphPad Prism v5.0 (GraphPad Software, La Jolla, CA) was used to draw standard curves and obtain sample concentration values.

**Analysis of intracellular cytokine production by iNKT cell and B cell subsets**

Total B cells were cocultured for 3 d in medium alone or with equal numbers of sorted CD4+ iNKT cells in the absence or presence of α-GC as described above. Menominsin (0.05 mM; Sigma-Aldrich) was added for the last 4 h to promote intracellular accumulation of cytokines. Cells were then washed and stained for cell surface expression of 6B11, CD19, CD1d, CD5, CD24, and CD38 and intracellular expression of IFN-γ, IL-10, IL-4, and IL-21 using fluorochrome-conjugated mAb obtained from BD Biosciences or eBioscience and analyzed by flow cytometry (13).

**Cytotoxicity assays**

Cytolytic degranulation by iNKT cells in response to B cells in the absence and presence of α-GC was examined by analysis of cell-surface CD107a expression. iNKT cells and B cells were cocultured for 4 h at 1:1 ratios in the presence of anti-CD107a PE-Cy7 mAb. Menominsin (2 μM) was added after 1 h to prevent proteolysis of the mAb conjugate upon reinternalization of CD107a. Frequencies of CD107a expression by iNKT subsets were determined by flow cytometry after electronically gating on CD4+, DN, and CD8α− subsets.

**Analysis of the capacity of B cells to drive T cell proliferation**

B cells were cocultured with equal numbers of autologous iNKT cells at densities of 10^6 cells of each type per milliliter in the presence or absence of 100 ng/ml α-GC for 3 d. B cells and iNKT cells were also cultured separately in medium alone as negative controls. The cocultured cells were harvested, washed, and examined for expression of CD40, CD69, CD80, CD83, CD86, and HLA-DR by flow cytometry or used as stimulators for autologous or allogeneic conventional T cells that were enriched by magnetic selection using CD3 microbeads (Miltenyi Biotec). The T cells were labeled using the CellTrace dye (eBioscience) and analyzed by flow cytometry (13).

**Statistical analyses**

Statistical analysis was performed using GraphPad Prism v5.0 (GraphPad). For comparison between two groups, the Mann–Whitney U test was used to compare unpaired data, and the Wilcoxon matched-pairs test was used to compare paired data. For comparison among three or more groups, the Kruskal–Wallis test was used to compare unpaired data, and the Friedman’s test was used to compare paired data. Dunn’s multiple comparison tests were performed post hoc to compare individual groups within an experiment. Two-way ANOVA with post hoc Bonferroni’s test was used to compare the effect of treatments.

**Results**

**CD1d is uniformly expressed across all B cell subsets**

PBMC from seven healthy donors were stained with mAbs specific for CD19 and CD1d and CD5, CD22, CD38, or CD27, which detect B-1, mature, plasma, and memory B cells, respectively (Fig. 2). CD1d expression by each B cell subset was determined by comparing the intensity of staining with anti-CD1d mAb with that of the corresponding FMO control, in which the anti-CD1d mAb was omitted (Fig. 2A). Up to 30% of circulating B cells expressed phenotypes associated with plasma cells, whereas 19–44% were B-1 cells and 12–31% were memory cells (Fig. 2B). CD1d was expressed at the cell surface of similar proportions (48–92%) of each B cell subset. Memory B cells displayed the highest proportion of CD1d expression (Fig. 2C), whereas the mean fluorescence intensity (MFI) of CD1d expression was also highest on this B cell subset. (Fig. 2D). One-way ANOVA showed no significant difference between the mean proportions of MFI of CD1d expression on each B cell subset. Whereas CD1d expression by B cells is reported to be downregulated by activation (33), we found that coculturing with iNKT cells did not affect the level of CD1d expression (Fig. 2E).

**CD4+, CD5−, DN and CD8α− iNKT cells can induce secretion of IgG, IgA, and IgM, but not IgE, by B cells**

iNKT cells can provide help to B cells for the production and secretion of Abs in vivo (18–24). We investigated whether sorted subsets of CD4+, DN, and CD8α− iNKT cells differed in their capacity to induce Ab production. Initially, B cells were cultured with total iNKT cells or non-iNKT cells in the absence of added Ag and, and cell supernatants were removed after 3 d (data not shown) or 10 d (Fig. 3A) and assayed for Ab production by multiplex CBA analysis. Relative to B cells cultured alone, there was increased production of IgA and IgM (p < 0.05) after 10 d of culture with iNKT cells and of total IgG (p < 0.01), IgM, and IgA (p < 0.05) after 10 d of B cell coculture with iNKT (Fig. 3A). In contrast, non-iNKT cells did not induce the release of these Abs by the same B cells. No IgE was detected in any of the stimulations or cocultures (data not shown). When sorted subsets of CD4+, DN, and CD8α− iNKT cells were cultured for 10 d with B cells, all three subsets induced IgM, IgA, and IgG production (Fig. 3B). Surprisingly, the addition of α-GC to the cocultures did not result in enhanced Ab production. The activation of B cells in the absence of α-GC may thus be due to the presence of a self-glycolipid presented by CD1d on the B cell.

To investigate the requirements for cell–cell contact and for CD1d and cytokines in iNKT cell–mediated B cell help for Ab production, B cells were cultured alone or with equal numbers of total iNKT cells for 10 d together or separated using transwell plates and in the absence or presence of blocking Abs against CD1d, IL-4, IL-13, CD40, or CD154. Supernatants from the cocultures were removed and assayed for IgG, IgM, and IgA release. When B cells and iNKT cells were separated in transwell plates or when blocking Abs against CD1d were added to the iNKT–B cell cocultures, Ab secretion was inhibited (see Fig. 3C for IgA). When anti-IL-4, anti-IL-13 (Fig. 3C), anti-CD40, or anti-CD154 mAbs (Fig. 3D) were added to the cultures, there was no inhibition of IgG, IgM, or IgA release, and the anti-CD40 mAb may have a weak agonistic effect on B cell activation. Therefore, all three subsets of human iNKT cells can provide B cell help for Ab production by a mechanism that requires cell contact and CD1d but not α-GC and does not appear to require CD40–CD154 interactions or Th2 cytokine secretion.

**CD4+ iNKT cells induce the expansion of unswitched memory and CD1d+CDS+ cells**

Total B cells were cultured for 3 or 10 d in medium alone or with equal numbers of expanded total, CD4+, CD5−, or DN iNKT cells or non-iNKT cells (PBMC expanded by anti-CD3 mAb and IL-2). Changes in the percentages of naive (CD27−IgD−), unswitched memory (CD27+IgD−), switched memory (CD27+IgD+), and
CD27-memory (CD27+IgD−) B cells and two putative Breg cell subsets (CD1dthCD5+ and CD24thCD38hi) (34, 35) were analyzed by flow cytometry. The expression of the T FH (CXCR5+PD-1+) phenotype (36, 37) by the iNKT cells was also examined. Total and CD4+ iNKT cells, but not CD8+ nor DN iNKT cells, in the presence of α-GC induced modest expansions of unswitched memory B cells (28–46%) after 10 d with concomitant reductions in naive B cells (35–22%) by a mechanism that required cell–cell contact (data not shown). However, the numbers of switched and CD27-memory B cells were unaffected. Sorted CD4+ iNKT cells also induced significant expansions of CD1dthCD5+ B cells (Fig. 4A, 4B). In contrast, CD8+ and DN iNKT cells induced upregulation of CD5 but not CD1d on B cells. Induction of CD1dthCD5+ B cells by CD4+ iNKT cells required cell–cell contact but not prior activation of the iNKT cells with α-GC. CD4+ iNKT cells in the presence of α-GC also induced a moderate expansion of CD24thCD38hi B cells (Fig. 4B). These results provide evidence that resting CD4+ iNKT cells may induce the differentiation of Breg cells in vitro.

Although iNKT cells provided B cell help for Ab secretion (Fig. 3), <2% of CD4+, CD8α+, or DN expressed the CXCR5+PD-1+ phenotype found on T FH cells and murine iNKT cells (36, 37). The presence of B cells or α-GC did not significantly alter the frequencies of iNKT cells that express T FH phenotypes (Fig. 4C).

B cells present α-GC to CD4+ and DN iNKT cells resulting in the release of low levels of IFN-γ, TNF-α, IL-4, IL-5, IL-10, and IL-13. The ability of B cells to present α-GC to iNKT cells resulting in cytokine release was investigated by coculturing B cells alone or with autologous sorted CD4+, DN, or CD8α+ iNKT cells in the absence or presence of α-GC. Cell supernatants were removed after 3 d and assayed for cytokine levels by multiplex CBA.
analysis. We found that coculturing B cells with any of the iNKT cell subsets in the absence of α-GC did not lead to significant increases in IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-10, or IL-13 release compared with B cells, iNKT cells, or non-iNKT cells cultured alone. When α-GC was present, low levels of IFN-γ, TNF-α, IL-4, IL-5, and IL-13, but not IL-2 nor IL-10, were released by the cocultures of B cells with CD4+ or DN iNKT cells (Fig. 5A). Although CD4+ iNKT cells induced the expansion of cells with phenotypes associated with Breg cells (Fig. 4), no IL-10 was detected in the supernatants of these cocultures. When monocyte-derived DC were used as APCs for α-GC, all subsets of iNKT cells released the above cytokines, including IL-10, with 100–1000-fold more IFN-γ and TNF-α and 10–100-fold more IL-4, IL-5, and IL-13 released compared with when B cells were used as APCs (Fig. 5B). It is unlikely that the low amounts of cytokines produced by cocultures of B cells and iNKT cells are due to contaminating monocytes or DC, because the B cells were enriched to purities of >99.5%, as shown by flow cytometric analysis of CD19 and CD20 expression. These results suggest that B cells can present α-GC to iNKT cells but are 10–1000 times less efficient than DC at stimulating cytokine production by the cells. To determine the cellular sources of these cytokines and further investigate if the CD1d<sup>hi</sup>CD5<sup>+</sup> B cells that were induced by CD4+ iNKT cells have cytokine profiles typical of Breg cells, total B cells were cultured for 3 d in medium alone or with equal numbers of expanded CD4<sup>+</sup>, CD8<sup>+</sup>, or DN iNKT cells in the absence or presence of α-GC. (A) Representative flow cytometry dot plots showing isotype control mAb staining and FMO for CD1d mAb staining (left panel) and CD1d and CD5 expression (right panel) by gated CD19<sup>+</sup> cells. (B) Mean (± SEM) percentages of B cells that expressed CD1d<sup>hi</sup>CD5<sup>+</sup> (left panel) or CD24<sup>hi</sup>CD38<sup>hi</sup> (right panel) phenotypes. *p < 0.05. (C) Mean (± SEM) percentages of iNKT cells that expressed CXCR5<sup>+</sup>PD-1<sup>+</sup> T<sub>FH</sub> phenotypes.

FIGURE 4. CD4<sup>+</sup> iNKT cells induce the expansion of CD1d<sup>hi</sup>CD5<sup>+</sup> and CD24<sup>hi</sup>CD38<sup>hi</sup> B cells. B cells from seven donors were cultured for 3 d in medium alone or with equal numbers of expanded CD4<sup>+</sup>, CD8<sup>+</sup>, or DN iNKT cells in the absence or presence of α-GC. (A) Representative flow cytometry dot plots showing isotype control mAb staining and FMO for CD1d mAb staining (left panel) and CD1d and CD5 expression (right panel) by gated CD19<sup>+</sup> cells. (B) Mean (± SEM) percentages of B cells that expressed CD1d<sup>hi</sup>CD5<sup>+</sup> (left panel) or CD24<sup>hi</sup>CD38<sup>hi</sup> (right panel) phenotypes. *p < 0.05. (C) Mean (± SEM) percentages of iNKT cells that expressed CXCR5<sup>+</sup>PD-1<sup>+</sup> T<sub>FH</sub> phenotypes.
expression in response to B cells, whether or not α-GC was present. Thus, it is likely that DN iNKT cells can kill autologous B cells.

iNKT cells induce expression of activation and costimulatory markers by B cells

To determine if iNKT cells can induce maturation of B cells into cells with APC phenotypes, total B cells were cultured for 3 or 10 d in medium alone, with PMA and ionomycin or with equal numbers of expanded iNKT cells or non-iNKT cells. Changes in cell-surface expression of CD40, CD83, CD86, CD69, CD80, and HLA-DR by total B cells and naive, unswitched memory, switched memory, CD27+ memory B cells, and CD1dhiCD5+ and CD24hiCD38hi B cells were analyzed by flow cytometry. We observed increased expression of CD40 (p < 0.05), CD95 (p < 0.01), CD86 (p < 0.05), and CD83 (not significant), but not CD80 nor HLA-DR on total B cells after 3 d of coincubation with total iNKT cells (Fig. 7A). After 10 d of coincubation with iNKT cells, these markers were not expressed at significantly higher levels than on B cells cultured alone. When non-iNKT cells were substituted for iNKT cells, the levels of the above-mentioned markers were similar to those on B cells cultured alone (Fig. 7A). CD40 and CD86 were upregulated on naïve, unswitched memory, switched memory, CD27+ memory B cells, and the two putative Breg cell subsets (CD1dhiCD5+ and CD24hiCD38hi) (data not shown).

When sorted CD4+*, DN, and CD8+ iNKT cells were cultured with the B cells, only the CD4+ subset was found to significantly
and CD8+ iNKT cell subsets differ in their abilities to do so and in presence of PMA and ionomycin (PMA/I) and a culture in medium alone or with autologous B cells in the absence and presence of PMA and ionomycin (PMA/I) and α-GC.

induce CD40 and CD86 expression, and this occurred only when α-GC was present. CD8+ and DN iNKT cell subsets also weakly induced CD86 expression, but whereas CD4+ and DN iNKT cells required the presence of α-GC to do so, CD8+ iNKT cells did not (Fig. 7B). Therefore, iNKT cells induce the expression of activation and costimulatory molecules by B cells, but the CD4+, DN, and CD8+ iNKT cell subsets differ in their abilities to do so and in their requirements for ligand activation.

The requirements for cell–cell contact, CD1d, and cytokines in iNKT cell–mediated upregulation of CD40, CD83, and CD86 expression by B cells were tested using transwell plates and in the absence or presence of blocking mAbs against CD1d, IL-4, and IL-13. When B cells and iNKT cells were separated in transwell plates or when anti-CD1d blocking Abs were added to the iNKT–B cell cocultures, the upregulation of all three markers was reduced (Fig. 7C). Blocking IL-13 resulted in moderate inhibition of CD86 expression only, but blocking IL-4 had no effect on the expression of any of these markers.

iNKT cells prevent the induction of T cell proliferation by B cells

The effect of total iNKT cells on the ability of B cells to promote proliferation of autologous and allogeneic T cells was investigated using the CellTrace Violet Cell Proliferation assay (Invitrogen). B cells that were cultured in medium alone were able to induce proliferation of autologous T cells in the absence of Ag and to a greater degree in the presence of PPD, SEB, or PHA (Fig. 8). However, B cells that were precultured with iNKT cells were greatly reduced in their ability to induce T cell proliferation. Non-specific and PPD-stimulated T cell proliferation was abrogated by the presence of iNKT cells, whereas SEB-specific T cell proliferation was not. Allogeneic T cell proliferation was also abrogated by iNKT cells. This inhibition of B cell–stimulated T cell proliferation occurred whether or not α-GC was present in the iNKT–B cell cocultures.

Discussion

iNKT cells provide cognate and noncognate help for lipid-reactive and protein-reactive B cells. They are required for the generation of protective Ab responses against some murine pathogens (18–22), and they dramatically augment Ab responses to coadministered Ags in vivo (18–22). These findings have led to interest in iNKT cells and their ligands as adjuvants for the development of vaccines and immunotherapies (38, 39). However, despite their name, iNKT cells are heterogeneous and multifunctional in nature. Human CD4+, CD8α+, and DN iNKT cell subsets differ in their pattern of cytokine production. CD4+ iNKT cells produce IFN-γ, TNF-α, and Th1 cytokines such as IL-12. CD8α+ iNKT cells produce IFN-γ and TNF-α, whereas CD8β− iNKT cells produce IFN-γ and TNF-α. These variable Th cell cytokine profiles prompted us to compare the relative abilities of CD4+, CD8α+, and DN iNKT cell subsets to provide help for B cell differentiation and Ab production in vitro. We also have investigated the influence iNKT cell subsets...
absence or presence of Ag, induced IgM production by B cells as stimulators of sorted autologous or allogeneic conventional CD3+ T cells and iNKT cells were also cultured separately in medium alone as negative controls. The cocultured cells were harvested, washed, and used as stimulators of sorted autologous or allogeneic conventional CD3+ T cells that were labeled using the CellTrace Violet (Invitrogen). The T cells were cultured with the B/iNKT cells at a ratio of 3:1 with medium only, 10 μg/ml PPD, 1 μg/ml SEB, or 5 μg/ml PHA. Proliferation of the T cells was assayed by flow cytometric examination of dilution of the CellTrace dye after 6 d in culture. Plots are representative of three independent experiments.

FIGURE 8. iNKT cells inhibit the induction of T cell proliferation by B cells. B cells were cocultured with equal numbers of autologous iNKT cells in the absence or presence of 100 ng/ml of α-GC for 3 d. B cells and iNKT cells were also cultured separately in medium alone as negative controls. The cocultured cells were harvested, washed, and used as stimulators of sorted autologous or allogeneic conventional CD3+ T cells that were labeled using the CellTrace Violet (Invitrogen). The T cells were cultured with the B/iNKT cells at a ratio of 3:1 with medium only, 10 μg/ml PPD, 1 μg/ml SEB, or 5 μg/ml PHA. Proliferation of the T cells was assayed by flow cytometric examination of dilution of the CellTrace dye after 6 d in culture. Plots are representative of three independent experiments.

have on cytokine production, Ag presentation, and T cell activation by B cells.

Two previous in vitro coculture studies of B cells with fresh (29) and expanded (28) human iNKT cells and on murine iNKT cells (40) have shown that iNKT cells can induce Ab production by human B cells in vitro. We found that all subsets of expanded iNKT cell lines induced the secretion of IgG (both IgG1 and IgG2), IgA, and IgM by autologous B cells. Also observed by Galli and coworkers (28), we found that IgE was not induced by any of the three iNKT cell subsets. This may reflect a need for conventional CD4+ T cells, which are required for iNKT cell–mediated IgE production by murine B cells (41). iNKT cell–mediated help for B cells required cell–cell contact and CD1d, but did not appear to involve CD40–CD154 interaction, and it was not inhibited by neutralizing Abs against IL-4 or IL-13. These findings compare and contrast with those from in vivo studies in mice immunized with α-GC and protein Ag, which showed that iNKT cell–mediated B cell help required B cell expression of CD1d (21, 24, 42) and CD40 (24, 37) but did not require IL-4 (24). iNKT cell–mediated enhancement of Ab responses in vivo also required DC but did not require the expression of CD1d by DC (43). Another difference between these in vivo models of α-GC–mediated B cell help and our system is that α-GC was not required for iNKT cell–mediated B cell help in vitro. Galli and coworkers (28) also found that human CD4+ iNKT cells, in the absence or presence of Ag, induced IgM production by B cells in vitro. Our findings confirm that the direct interaction between iNKT cells and B cells is sufficient to stimulate Ab production in vitro and that exogenous Ag is not required.

The ability of iNKT cells to provide B cell help for Ab production prompted us to examine whether these iNKT cells express CXCR5+PD-1+ phenotypes of Tfh cells or produce IL-21, attributes that are associated with murine iNKT cells stimulated in vivo with α-GC (25, 36, 37). We found that up to 2% of expanded iNKT cells expressed Tfh phenotypes or released IL-21, and this frequency was not increased by coculturing them with B cells in the absence or presence of α-GC. Similar proportions of iNKT cells displaying Tfh phenotypes were found in mice immunized with Ag and α-GC (36, 37).

The ability of iNKT cells to induce Ab secretion by B cells highlights them as potential targets for therapeutic boosting of Ab responses in vaccines and infections or inhibition of pathogenic Ab production in autoimmune and allergic disease. Devera and coworkers (42) exploited iNKT help to B cells to enhance and sustain neutralizing Ab responses toward the Bacillus anthracis lethal toxin, which led to sustained survival and good health in mice challenged with the toxin. However, because of the multiple effector and immunoregulatory activities of iNKT cells, a complete understanding of the mechanisms underlying the interaction between iNKT cells and B cells is essential to predict or program the outcomes in vivo.

Activation of human iNKT cells requires the presence of immature but not mature B cells (44). However, we found that CD1d is expressed at comparable levels by mature, plasma, memory, and B-1 B cells, suggesting that all B cell subsets can present glycolipids to iNKT cells. CD4+ iNKT cells were found to promote expansions of unswitched (IgD+) memory B cells in a contact-dependent manner, resulting in decreased frequencies of naive B cells. Despite inducing Ab release by B cells, none of the iNKT cell subsets induced significant expansions of switched (IgD−) memory B cells or the expression of IgM or IgG by any memory B cell subset. This suggests that iNKT cells promote Ab release by class-switched memory B cells rather than inducing class switching. Thus, it appears that iNKT cells may interact differently with naive and memory B cells, promoting differentiation of naive B cells into unswitched memory cells that are not required for iNKT cell activation while promoting differentiation of switched memory B cells into Ab-secreting plasma cells. Future experiments involving stimulation of B cells with iNKT cell subsets followed by detection of specific Ab-secreting memory B cells are required to confirm that iNKT cells can restimulate memory B cells.

We investigated if iNKT cells could induce the expansion of putative Breg cells. The CD1dhiCD5+ B cell phenotype defines a subset of murine B cells that downregulate immune responses via secretion of IL-10 and inhibit the development of autoimmune disease (34, 45, 46). In humans, an IL-10–producing B cell population that inhibits Th1 cell differentiation resides in the CD24hiCD38+ B cell compartment, and this subset is impaired in patients with systemic lupus erythematosus (35, 47). The majority of human CD1dhiCD5+ B cells are reported to be contained in the CD24hiCD38hi B cell subset (35); therefore, we investigated both subsets as putative Breg cells. We found that coculturing B cells with total iNKT cells did not significantly affect CD1d expression. However, CD4+ iNKT cells induced the expansion of a population of CD1dhiCD5+ B cells by a mechanism that required cell–cell contact but not activation of the iNKT cells with α-GC. CD4+ iNKT cells in the presence of α-GC also induced a moderate expansion of CD24hiCD38hi B cells. Although, we did not detect IL-10 in the supernatants of cocultures of CD4+ iNKT cells with
B cells using multiplex CBA analysis, up to 1% of the B cells in these cultures expressed intracellular IL-10. IL-10 production is a hallmark feature of Breg cells (34, 35, 48–50). We were unable to show convincingly that this IL-10 was produced by the CD1d\(^+\) CD5\(^+\) subset of B cells; however, our data indicate that CD4\(^+\) iNKT cells induced IL-10 production by some B cells, suggesting that they promote Breg cell differentiation. We also found that up to 1 ng/ml of IL-10 was released by cocultures of DC with all subsets of iNKT cells, suggesting that iNKT cells can induce regulatory DC as well as B cells.

DC can present α-GC to iNKT cells resulting in the rapid secretion of Th1 and Th2 cytokines in vivo and in vitro (7, 8, 13, 32) (Fig. 5B). However, experiments aimed at demonstrating that B cells can similarly present α-GC and induce cytokine production by iNKT cells have been conflicting. Bialecki and coworkers (51) reported no IFN-γ or IL-4 production by cocultures of murine iNKT cells and marginal zone B (MZB) cells presenting α-GC in vitro, but both cytokines were produced when DC were added. Two other studies demonstrated weak Th2 (IL-4 and IL-13) production by murine iNKT cells after stimulation with α-GC–pulsed total B cells (52) or MZB cells (53) in vitro and in vivo, and these cytokine profiles were skewed toward Th1 when DC were present. We found that when α-GC–pulsed human B cells were cultured with CD4\(^+\) or DN iNKT cells, but not CD8\(^+\) iNKT cells, IFN-γ, TNF-α, IL-4, IL-5, and IL-13 were secreted into the supernatants. Using intracellular cytokine staining and flow cytometry, we showed that iNKT cells are the main source of these cytokines. However, the amounts of cytokines released were 10–1000-fold lower than those when DC were used as APC for α-GC. Glycolipid presentation by B cells appears to be required for optimal activation of iNKT cells by DC, because removal of MZB cells reduced iNKT cell activation by murine spleen cells pulsed with α-GC in vitro (51), and human PBMC depleted of B cells failed to support iNKT cell expansion and cytokine release in response to α-GC (44). In contrast, Bezbradica and coworkers (52) reported that B cells suppressed murine DC-mediated iNKT cell activation in vivo. Collectively, these findings support the view that B cells can present glycolipid Ags to iNKT cells, but rather than being potent stimulators of cytokine secretion, B cells modulate cytokine production by iNKT cells activated by DC. Reciprocally, iNKT cells can induce cytokine production by DC (7, 8, 32) and by small proportions of B cells.

Because CD8\(^+\) and DN iNKT cells and, to a lesser degree, CD4\(^+\) iNKT cells are potent cytotoxic T cells capable of killing CD1d\(^+\) cells presenting α-GC (13), we investigated if these iNKT cell subsets could kill autologous B cells presenting this glycolipid. Interestingly, only the DN subset of iNKT cells degranulated in response to B cells, and this occurred whether α-GC was present or not. Thus, although iNKT cells can promote Ab production by B cells, DN iNKT cells may regulate this activity by killing the B cells.

iNKT cells can induce maturation of DC into APC that express costimulatory and adhesion molecules and can prime naïve conventional T cells (7, 8, 32). B cells are also professional APCs (30), and they express CD1d; therefore, we investigated the influence that iNKT cells and their subsets have on the APC function of B cells. Extending the findings of Kitamura and coworkers (54), we found that iNKT cells induced the expression of CD40, CD69, CD83, and CD86, but not CD80 nor HLA-DR, on B cells by a mechanism that was dependent on cell–cell contact and CD1d. Of the three iNKT cell subsets, CD4\(^+\) iNKT cells were the most potent inducers of costimulatory molecule expression by B cells. Blocking IL-13 resulted in moderate inhibition of CD86 expression, but blocking IL-4 had no effect on the expression of these markers by B cells. These phenotypic changes suggest that iNKT cells induce maturation of B cells into APCs. We found that B cells presented Ags, superantigens, and mitogens to T cells resulting in their proliferation. However, B cells that were pre-cultured with iNKT cells were greatly reduced in their ability to induce T cell proliferation. Nonspecific and PPD-stimulated T cell proliferation was abrogated by iNKT cells, whereas SEB-specific T cell proliferation was not. As SEB does not require intracellular processing to be presented on MHC, our results suggest that iNKT cells may inhibit intracellular Ag processing by B cells. Allogeneic T cell proliferation was also abrogated by iNKT cells. This inhibition of B cell–stimulated T cell proliferation occurred whether or not α-GC was present in the iNKT–B cell cocultures. These data are consistent with a model in which iNKT induce the differentiation of B cells into tolerogenic APC that inhibit conventional T cell activation. Subsets of B cells that are tolerogenic APCs have been described (55, 56), and although not shown in this study, it is possible that the induction of T cell proliferation by B cells may be inhibited by CD1d\(^+\)CD5\(^+\) Breg cells or by IL-10 released by other B cells.

CD4\(^+\) T cell help for B cells is a key requirement for the generation of Ab-secreting plasma cells and memory B cells. The interaction between the two cells generally takes place in secondary lymphoid organs, involves presentation of processed Ag by the B cell to the T cell, signaling through costimulatory molecules such as CD40, and the production of cytokines (1–3). Our study demonstrates that human B cells can present glycolipid Ag to iNKT cells, resulting in low-level Th1 and Th2 cytokine secretion in vitro. Reciprocally, iNKT cells can have diverse effects on B cells, depending on the iNKT cell subset, on whether glycolipid Ag is added and possibly on the differentiation status of the B cell. Firstly, CD4\(^+\), CD8\(^+\), and DN iNKT cells can all stimulate Ab production by B cells by a mechanism that requires CD1d but not exogenous glycolipid Ag nor CD40–CD154 interaction. Secondly, CD4\(^+\) iNKT cells in the presence of glycolipid Ag promote differentiation of naive B cells into memory cells, but they do not appear to promote Ig isotype switching by these cells. Thirdly, CD4\(^+\) iNKT cells in the absence of exogenous glycolipid Ag induce the expansions of B cells that exhibit phenotypes of Breg cells and B cells that produce IL-10, whereas DN iNKT cells in the absence of added glycolipid may kill autologous B cells. Finally, iNKT cells (and in particular the CD4\(^+\) subset) induce maturation of all B cell subsets into cells with APC phenotypes, but these APCs fail to stimulate proliferation of conventional T cells, as seen when untreated B cells are used. Collectively, these results indicate that the presence of exogenous α-GC is a major determinant of the outcome of B–iNKT cell interactions: in the presence of α-GC, B cells promote cytokine secretion by iNKT cells, which will boost T cell–mediated immunity, whereas in the absence of α-GC, iNKT cell activation by B cells results in Ab-mediated immune responses and suppression of T cell–mediated immunity. The dependence of these functional outcomes on the absence or presence of α-GC raises the question of how other glycolipid Ags will modify B cell responses to iNKT cells, and in this regard, several chemical analogs of α-GC that can skew cytokine responses of iNKT cells are being tested as potential immunomodulators for the treatment of disease (32, 57, 58). The diverse outcomes of iNKT–B cell interactions have important implications for therapy for autoimmune disease, in which induction of Breg cells or prevention of pathogenic Ab responses may be beneficial (34, 35, 45, 46, 48–50), and for cancer and infectious disease, in which T cell responses are required (4, 5). The presence of iNKT cell subsets with opposing roles in immune responses might explain why clinical trials involving these cells to
date have been unsuccessful, 38). Therapeutic manipulation of iNKT cells may necessarily require the sorting of iNKT cells into functionally-distinct subsets and/or selective activation of particular effector functions using customized glycolipid Ags.

Disclosures
M.A.E. has an equity relationship with NKTherapeutics, Inc. The other authors have no financial conflicts of interest.

References

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