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CD1d and CD1c Expression in Human B Cells Is Regulated by Activation and Retinoic Acid Receptor Signaling

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B cell activation and Ab production in response to protein Ags requires presentation of peptides for recruitment of T cell help. We and others have recently demonstrated that B cells can also acquire innate help by presenting lipid Ags via CD1d to NKT cells. Given the newfound contribution of NKT cells to humoral immunity, we sought to identify the pathways that regulate CD1 molecule expression in human B cells. We show that ex vivo, activated and memory B cells expressed lower levels of CD1d compared with resting, naive, and marginal zone-like B cells. In vitro, CD1d was downregulated by all forms of B cell activation, leaving a narrow temporal window in which B cells could activate NKT cells. CD1c expression and function also decreased following activation by CD40L alone, whereas activation via the BCR significantly upregulated CD1c, particularly on marginal zone-like B cells. We found that the CD40L-induced downregulation of CD1d and CD1c correlated with diminished expression of retinoic acid receptor α (RARα) response genes, an effect that was reversed by RARα agonists. However, BCR-induced upregulation of CD1c was independent of the RAR pathway. Our findings that both CD1d and CD1c are upregulated by RARα signaling in human B cells is distinct from effects reported in dendritic cells, in which CD1c is inversely downregulated. One functional consequence of CD1d upregulation by retinoic acid was NKT cell cytotoxicity toward B cells. These results are central to our understanding of how CD1-restricted T cells may control humoral immunity.

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lipid-specific Abs, whereas presentation of lipid Ags acquired via LDLR-mediated uptake may serve as an adjuvant to promote production of Abs to any Ag.

Mice express only CD1d, but humans have a greater diversity of CD1 genes (CD1a–d) that have all been shown to present lipids to CD1-restricted T cells. In addition to CD1d, human B cells also express CD1c, which is highly expressed on the human counterpart to murineMZ B cells, referred to as MZ-like B cells (11). In contrast to mice, human MZ-like B cells recirculate through the periphery and reside in other secondary lymphoid tissues, including the lymph nodes and tonsils. MZ-like B cells are IgM+/IgD− and express the memory marker CD27 (12). The purpose of CD1c on these B cells and the role of CD1c-restricted T cells have yet to be determined. The regulation of CD1c expression is likely to be an important aspect of B cell physiology.

Relatively little is known about the regulation of CD1 molecules on human APCs. In DCs, CD1a, b, and c (type 1 CD1s) are upregulated during differentiation from monocytes. In contrast, CD1d (a type 2 CD1) is unaltered or downregulated (22). The peroxisome proliferator-activated receptor γ (PPARγ) pathway, acting through the production of all-trans retinoic acid (ATRA) and the RARs, has been shown to control CD1 expression, upregulating CD1d while downregulating the type 1 CD1 molecules (23–25). Thus, it appears that, in DCs, CD1d and type 1 CD1s are regulated in an inverse manner. Of interest, the RARs pathway has also been shown to exert an important influence on B cell physiology and humoral immunity (26), as well as on certain T cell populations (27–30). Given the growing body of research indicating the impact of B–NKT cell interactions on immunity and disease, we sought to examine the pathways that regulate CD1d and CD1c expression on human B cells.

Materials and Methods

Chemicals and ligands

The following chemicals were prepared as 1000× stock solutions in DMSO: AM580 (Biomol), Retinol and ATRA (Sigma), rosiglitazone (Cayman Chemical Company), GW9662 (Alexis Biochemicals), and AGN139109 (AGN) (Santa Cruz Biotechnology).

Cells

The CD1d-restricted NKT cell line MO (>99% CD4 positive, >95% Vo24/Vj11), the NKT cell clone BM2a.3, and the CD1c-restricted T cell clone CD1-1 (kindly provided by Dr. Branch Moody, Harvard Medical School, Brigham and Women’s Hospital, Boston, MA) were used as previously described (6, 31). Additional NKT cells (CD4/195) were expanded from donor PBMCs with γGalCer-CD1d–tetramer sorting (>99% CD4 positive, >97% γGalCer-CD1d–tetramer positive) and expansion with γGalCer. Tonsils were obtained from patients undergoing tonsillectomy at BC Children’s Hospital, with ethics approval obtained from the University of British Columbia, Vancouver, BC, Canada. Tonsils were disrupted mechanically, and lymphocytes were isolated by ficoll. B cells were purified by magnetic selection (yielding >98% CD19+ve cells) using a cell enrichment kit (Stemcell Technologies). Monocytes were purified from PBMCs following Ficoll separation of blood from healthy donors, using CD14 Microbeads (Miltenyi Biotec). CD14+ cells were then cultured for 5 d in the presence of 100 ng/ml GM-CSF (Genscript) and 60 ng/ml IL-4 (eBioscience).

Proliferation/lactate dehydrogenase assays

B cells were cultured in 96-well plates with NKt cells (5 × 105 cells/well of each in triplicate) in media (RPMI 1640 plus 10% FBS) alone, media with γGalCer, or media containing AM580 (10 nm) plus indicated doses of γGalCer. Control wells were stimulated with PHA, anti-CD3, or Staphylococcus aureus Cowan I bacteria (SAC) with or without AM580. On day 4, wells from each treatment were pooled, counted, and stained, as indicated below. Lactate dehydrogenase (LDH) was measured (day 2) in the supernatant using a CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) according to the manufacturer’s protocol. Where indicated, anti-CD1d [12 μg/ml, clone 12.1.1.1 (32)] blocking, or isotype control, Abs (32.3G11) were used.

NKT cell assays

Purified B lymphocytes were cultured as indicated, in either media alone, or stimulated with anti-Ig (10 μg/ml; Southern Biotech, Birmingham, AL) or with 5 × 108 of heat-killed SAC or with CD40L provided by a monolayer of irradiated (3000 rads) CD40L-expressing L cells. B lymphocytes were washed and cultured with T cells (5 × 103 cells/well of each in triplicate in 96-well plates) overnight in media and lipid Ags γGalCer or mannoseyl-B1-β-phosphorycoketide (MPM), kindly provided by Dr. Huib Ovaa (Netherlands Cancer Institute, Amsterdam, The Netherlands) and Dr. Branch Moody, respectively. The next day, culture supernatants were harvested, and T cell IFN-γ production was measured by ELISA (6).

Flow cytometry

Monoclonal anti-CD86, -CD19, -CD3, -CD107a, -CD209, -IgD, -CD1a, -CD1b, and -CD1d were purchased from BD Pharmingen. Anti-CD1c and anti-CD27 were purchased from Miltenyi Biotec and Biolegend, respectively. Isotype-matched Abs were used as controls, and 7-aminactinomycin D (BD Biosciences) was used to gate-out dead cells. For proliferation studies, cells were stained with anti-CD3 and anti-CD19 Abs, and fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences) according to the manufacturer’s instructions. Anti–Ki-67 Ab (Dako CytoTech, Glostrup, Denmark) was used to detect proliferating cells. CD107a staining was performed overnight in the presence of brefeldin A, as described (33). Aldehyde dehydrogenase (ALDH) activity was determined using the ALDEFLUOR staining kit (Stemcell Technologies) according to the manufacturer’s instructions. Flow cytometry was performed on FACSCalibur (BD Biosciences) or FACSARia (BD Biosciences), and data were analyzed using FlowJo (Tree Star, Ashland, OR).

Real-time quantitative RT-PCR

Total RNA (1–4 μg), isolated using TRizol (Invitrogen), was reverse transcribed into cDNA, using Superscript II according to the manufacturer’s instructions (Invitrogen). Quantitative PCR was performed in triplicate on a 7500 Fast Real-Time PCR System (Applied Biosystems) using TaqMan PCR Master Mix (Applied Biosystems), according to the manufacturer’s instructions. For each probe/primer, water was substituted for cDNA in a reaction as a control. The threshold PCR cycle number (Ct), corresponding to the point of exponential amplification, was used to obtain quantitative values. The relative mRNA levels in each sample were normalized to their corresponding cyclophilin A content. The fold change, relative to control, was determined with the following formula: 2−ΔΔCt, where ΔΔCt = [ΔCt (test sample) − ΔCt (control sample)]; and ΔCt = Ct (target gene) − Ct (cyclophilin A).

Statistical analysis

Data were analyzed with paired Student t test or ANOVA (Tukey post hoc test), using Excel or Prism software.

Results

Upon activation, B cells upregulate MHC-II, MHC-I, and additional costimulatory molecules, which makes them highly potent APCs. In addition, we recently demonstrated that activated B cells upregulate the LDLR. This upregulation enhances their ability to internalize and present apolipid Ags to NKT cells (6) and to recruit help, leading to Ab production. We wished to test whether CD1 molecule expression is likewise upregulated in response to B cell activation.

Tonsil B cells were costimulated with Abs to the activation marker CD86 and to CD1a–d. In agreement with previous studies (34), B cells expressed CD1c and CD1d (Fig. 1A), but not CD1a or CD1b (not shown). Surprisingly, we found that CD86 expression on ex vivo B cells was not associated with CD1d expression. In fact, although the majority (68.5% ± 2.5; n = 7) of resting (CD86+) B cells were CD1d+, only a minority (34.5% ± 4.3; n = 7) of activated (CD86+) B cells were CD1d+ (Fig. 1A). CD1c, in contrast, was expressed on 42% (± 4.5; n = 6) of resting B cells, and on 32.8% (± 5.4; n = 5) of activated B cells, suggesting that CD1d molecules, and, to a lesser extent, CD1c molecules, are generally downregulated upon activation of B cells.
FIGURE 1. B cell activation downregulates CD1d and CD1c expression, diminishing lipid Ag-presenting function. A, Tonsil B cells stained ex vivo with Abs to CD19, CD1c, CD1d, and CD86. Representative dotplots (from at least five experiments) gated on CD19+ B cells showing CD1d and CD1c coexpression (top left), CD86 and CD1d expression (top middle), or CD86 and CD1c expression (top right). Representative dotplot showing CD19+ B cells gated on CD86hi- and CD86lo-expressing cells and corresponding histograms of CD86hi (dashed line) versus CD86lo (solid line). Unstained cells are shown in solid gray. B, Purified tonsil B cells were cultured on a monolayer of CD40L-expressing cells. Representative histograms (from a total of three experiments) showing CD1d and CD1c expression on unstimulated cells (ex vivo) and CD40L-activated cells on days 1, 3, and 5. C, NKT cells were cocultured with tonsil B cells ex vivo or B cells that had been activated with CD40L for 1, 3, or 5 d, with αGalCer as indicated. IFN-γ secretion was measured by ELISA. Results (means ± SEM) obtained from five independent experiments using B cells purified from individual tonsils are shown. D, CD1c-restricted T cells, CD8-1, were cocultured with B cells, as shown in C, together with the lipid Ag MPM as indicated. Graph shows mean IFN-γ secretion from one representative experiment of 2. For B–D, all B cell groups were processed on the same day to allow side-by-side comparisons.
To test whether CD1c and CD1d expression is downregulated upon B cell activation, B cells were stimulated with a variety of stimuli in vitro. Initially, we used CD40L stimulation to model conventional T cell help. The expression of CD86, MHC, and CD1c/d was then assayed after 1, 3, and 5 d of culture. As anticipated, CD86 and MHC-II expression on B cells was increased upon CD40L treatment (not shown). In contrast, expression of both CD1d and CD1c decreased over time. The downregulation of CD1d on CD40L-activated B cells was particularly dramatic, being essentially undetectable by day 5 (Fig. 1B).

We tested the ability of activated and ex vivo B cells to present lipid Ags to CD1d-restricted NKT cells and CD1c-restricted T cells, using the model lipid Ags αGalCer and MPM, respectively. Consistently, presentation of αGalCer to the NKT cell line MO, as assessed by IFN-γ release, was highest following 1 d of CD40L activation. However, longer periods of B cell activation resulted in a loss in the ability of B cells to stimulate NKT cells (Fig. 1C).

Similar results were observed using the NKT cell clone BM2a.3 and CD4(195) NKT cells expanded from donor PBMC (Supplemental Fig. 1). All three NKT cell lines tested produced IFN-γ and IL-4, in parallel. Next, the ability of B cells to present MPM to the CD1c-restricted T cell clone CD8-1 was tested (31). CD8-1 activation was highest after B cells had been stimulated for 1 d with CD40L. As with CD1d, longer periods of activation with CD40L decreased the capacity for CD1c-mediated Ag presentation. Previously, we observed that 18–24 h of CD40L activation enhanced B cell-presenting function compared with ex vivo B cells (6); however, as we show in this study, this capacity declines steadily thereafter.

In summary, CD40L stimulation, mimicking conventional T cell help (or bystander help in the absence of Ag presentation) results in a “window of opportunity” of enhanced B cell presentation to CD1-restricted T cells, compared with unstimulated or long-term stimulated B cells. This short temporal peak in the capacity to stimulate CD1-restricted cells is likely due to the combination of rising levels of activation-induced LDLR and costimulatory molecules, observed to be high in the 18- to 24-h period (6), and relatively high levels of CD1c/d that persist up to 18–24 h, prior to the decline that occurs thereafter.

We next asked whether B cells stimulated via their BCR would overcome the CD1c/d downregulation observed with CD40L activation. B cells were cultured on CD40L-expressing L cells in media alone or media supplemented with anti-Ig Abs or crude preparations of SAC. Although SAC contains many TLR ligands, the stimulatory activity found in SAC is primarily attributed to its high expression of surface protein A that can cross-link surface BCR, similar to what occurs with anti-Ig treatment (35–37). As shown previously, expression of both CD1c and CD1d was low or absent by day 4 of CD40L stimulation alone (Fig. 2A). The addition of anti-Ig or SAC treatment did not alter the loss of CD1d. In contrast, after addition of anti-Ig or SAC, a subset of the CD40L-activated B cells were CD1c positive. Next, we confirmed that the upregulated CD1c on anti-Ig/SAC-treated CD40L-activated B cells correlated with the functional ability to present MPM and activate CD8-1 cells. As expected, B cells activated with CD40L in combination with anti-Ig or SAC showed a greatly enhanced capacity to stimulate CD8-1 cells, compared with CD40L-activated B cells in the absence of anti-Ig or SAC treatment (Fig. 2B). Thus, elevated CD1c in these cells enhances their function as APCs.

We then tested whether a similar effect occurred with anti-Ig or SAC treatment alone, thus mimicking a T-independent B cell response. To this end, B cells were cultured in media with or without SAC or anti-Ig. CD1c and CD1d expression was assessed on day 3, and B cell activation was confirmed by CD86 upregulation (not shown). Unlike the CD1c downregulation observed following CD40L stimulation, SAC and anti-Ig treatment significantly upregulated CD1c expression, compared with that in control cells (Fig. 2A, 2B). Of interest, CD1d was generally downregulated, as with CD40L stimulation, but most prominently on the CD1c-negative rather than the CD1c-positive population.

To determine whether CD1c and CD1d downregulation upon B cell activation was regulated at the level of transcription, B cells were cultured for 24 h in media only (resting) or were activated with CD40L, SAC, or anti-Ig. CD1c and CD1d mRNA expression was assessed by relative quantitative RT-PCR. CD1c and CD1d expression in CD40L-activated cells, compared with that in resting cells, was downregulated by ∼2-fold and 4-fold, respectively (Fig. 2E, 2F). That CD1c showed a smaller decrease in expression than did CD1d, by 24 h of CD40L activation, is consistent with the generally slower decline in surface CD1c expression seen in Fig. 1. Of note, cells activated for >5 d with CD40L showed ∼100-fold lower CD1d and CD1c expression than was found in ex vivo tonsil B cells (not shown). BCR cross-linking, mediated by anti-Ig or SAC, resulted in the upregulation of CD1c, but not CD1d, also consistent with our FACS data and functional results (Fig. 2E, 2F).

Together, these data suggest that stimulation of BCR signaling with anti-Ig or SAC (alone or in addition to CD40L stimulation) enhances CD1c, but not CD1d, Ag presentation, and that the observed changes in CD1c/d expression occurs at the level of transcription.

Human MZ-like B cells are known to express high levels of CD1c (11). Because CD1c appears to be expressed on a fraction of total tonsil B cells, we explored which B cell populations express the highest levels of CD1d and whether the observed CD1c upregulation following BCR cross-linking can be attributed to specific B cell subset(s). To this end, purified tonsil B cells were stained with Abs to IgD and CD27, to identify naïve (IgD+/CD27−), memory (IgD−/CD27+), and MZ-like B cells (IgD−/CD27+) (Fig. 3A). These cell subsets were sorted and stimulated separately in vitro with SAC for 3 d. CD1d and CD1c expression on unstimulated (ex vivo) and SAC-activated B cell subsets was then compared. We observed that ex vivo MZ-like B cells, compared with naive and memory B cells, express high levels of CD1d and display exceptionally high CD1c expression (Fig. 3A, 3B). Memory B cells, in contrast, showed the lowest expression levels of both CD1 molecules, whereas naïve B cells expressed relatively high CD1d but low CD1c levels. Interestingly, although CD1c expression is the highest on MZ-like B cells, we found that all B cell subsets significantly upregulated CD1c following SAC stimulation; however, upregulation was most striking on the MZ-like B cells (Fig. 3A, 3C).

Given that little is known about the factors regulating CD1 expression in B cells, we sought to identify the underlying mechanisms. Previous studies showed that the expression of CD1 molecules on monocyte-derived DCs is altered by exposure to synthetic and natural PPARγ ligands. Nagy et al. (24) demonstrated that PPARγ activation modulates CD1 expression via upregulation of vitamin A-metabolizing enzymes that amplify intracellular conversion of retinol (vitamin A) to ATRA. The buildup of intracellular ATRA activates RARα, which then acts at the transcriptional level to downregulate type I CD1 molecules (CD1a-c) and upregulate the type II CD1 molecule CD1d.

To explore whether CD1 levels in B cells are modulated in a similar manner, we used quantitative RT-PCR to determine the relative expression of RARα and TGM2, a known RARα target gene (38), in resting and activated cells, as in Fig. 2E, 2F. We found that RARα expression in anti-Ig and SAC-activated B cells
remained relatively similar to that in resting cells (not shown), but that the expression of its target gene, TGM2, was significantly downregulated (Fig. 4A), indicating that RARα transcriptional activity is decreased upon B cell activation, paralleling what is seen for CD1c and CD1d.

Next, we directly tested whether PPARγ and RARα pathways regulate CD1d and CD1c expression. B cells were stimulated with CD40L in media alone, in media with the PPARγ agonist rosiglitazone, or with the RARα agonist ATRA. CD1d and CD1c expression on B cells decreased by day 4 of CD40L stimulation in...
media alone (Fig. 4B). However, ATRA-treated B cells expressed high surface levels of both CD1d and CD1c (Fig. 4B). As anticipated, the RAR\(\alpha\) inhibitor AGN blocked the ATRA-induced induction of CD1d and CD1c expression. In contrast, rosiglitazone-treated B cells did not induce CD1d or CD1c expression, resembling media-treated cells. Increasing rosiglitazone concentration 10-fold did not affect CD1 expression (data not shown), nor did culturing B cells in media containing human serum, a source of natural PPAR\(\gamma\) ligands (23, 39), in place of FBS. Neither treatment altered the expression of other surface molecules such as CD86, LDLR, CD1a/b, or MHC class II (data not shown).

As a positive control, we confirmed that DCs upregulated CD1d and downregulated CD1a in response to rosiglitazone, human serum, and AM580, as previously reported (23, 24, 39) (Supplemental Fig. 2). Moreover, the PPAR\(\gamma\) antagonist GW9662 blocked human serum-induced CD1a downregulation and CD1d upregulation.

To test whether RAR\(\alpha\) regulates CD1c expression downstream of BCR signaling, we dosed CD40L/SAC- and CD40L/Ig-treated B cells with AGN for 4 d. By day 4, activated B cells cotreated with SAC or anti-Ig expressed CD1c, but not CD1d (Fig. 4B). However, because AGN did not reduce CD1c expression, we postulate that BCR signaling maintains CD1c expression independently of the RAR\(\alpha\) pathway. Taken together, our data suggest that CD1d is regulated by the RAR\(\alpha\) pathway and that CD1c is regulated by both the BCR and RAR\(\alpha\) pathways.

ATRA, the active product of vitamin A, controls gene expression via RAR\(\alpha\) (Fig. 4C). The availability of ATRA is tightly regulated in cells and tissues by the balance of retinoic acid synthesis and metabolism. Vitamin A (retinol) is converted to ATRA by a multistep process that involves enzymes, including ALDH, (Fig. 4C), which are selectively expressed in only certain cell types. Given that ALDH activity is the primary rate-limiting step in ATRA production, we used a FACS-based assay to directly assess its synthesis in B cells. In this assay, specific ALDH activity is measured by detecting the conversion of a substrate to a fluorescent product and comparing it with background fluorescence in cells incubated in the presence of substrate and diethylaminobenzaldehyde (DEAB), an ALDH inhibitor. We found that, unlike control K562 cells, which showed high ALDH activity, ex vivo B cells and CD40L-activated B cells demonstrated similar low levels of specific ALDH activity, above cells treated with DEAB (Fig. 4D).

As our next step, we compared the ability of activated B cells to respond to retinol versus RAR\(\alpha\) ligands. CD40L-activated B cells were cultured for 24 h with rosiglitazone, retinol, ATRA, or AM580, a synthetic RAR\(\alpha\)-specific ligand. CD1c, CD1d, and TGM2 expression was assessed by RT-PCR. CD1c and CD1d expression in activated B cells, cultured in the presence of RAR ligands (AM580 and ATRA), increased by 8- to 12-fold and 100- to 150-fold, respectively, over cells grown in media alone (Fig. 4B). However, ATRA-treated B cells expressed high surface levels of both CD1d and CD1c (Fig. 4B). As anticipated, the RAR\(\alpha\) inhibitor AGN blocked the ATRA-induced induction of CD1d and CD1c expression. In contrast, rosiglitazone-treated B cells did not induce CD1d or CD1c expression, resembling media-treated cells. Increasing rosiglitazone concentration 10-fold did not affect CD1 expression (data not shown), nor did culturing B cells in media containing human serum, a source of natural PPAR\(\gamma\) ligands (23, 39), in place of FBS. Neither treatment altered the expression of other surface molecules such as CD86, LDLR, CD1a/b, or MHC class II (data not shown).

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In contrast, retinol did not elicit the same CD1c and CD1d upregulation. Rosiglitazone treatment also failed to induce CD1c and CD1d. TGM2 expression was tightly correlated with CD1d and CD1c expression. Together these findings indicate that B cells, like DCs, upregulate CD1d expression in response to RARα ligands. However, unlike DCs, B cells do not respond to PPARγ ligands. Given that activated B cells, like DCs, upregulate PPARγ expression

**FIGURE 4.** Decreased CD1d and CD1c expression in CD40L-activated B cells correlates with lower RARα transcriptional activity that is augmented with exogenous RARα ligands. A, TGM2 transcript levels in tonsil B cells following 24-h culture in media (resting) or stimulation with CD40L, SAC, or anti-Ig. Expression levels were determined in triplicate by quantitative RT-PCR and normalized to cyclophilin A. Fold differences, relative to unstimulated (resting) cells, are shown and represent an average (mean ± SEM) of three individual tonsils. *Significant fold change versus resting cells (ANOVA). *p < 0.01. B, Purified B cells were cultured as indicated on CD40L-expressing cells for 4 d. Representative dotplots of CD1d and CD1c expression and average (mean ± SEM; n = 5) percent of CD1c-positive cells is shown. Significant differences are indicated; *p < 0.05, **p < 0.001, ANOVA. C, ATRA is synthesized from vitamin A (retinol) and regulates expression of genes by activating RARα. Relevant chemical inhibitors (DEAB and AGN) of the pathway are shown. D, Purified ex vivo B cells and CD40L-activated B cells were incubated for 1 h with ALDEFLUOR substrate, with or without the ALDH inhibitor DEAB. Representative histograms (from five individual tonsils) of specific ALDH activity (solid line) are shown relative to DEAB-treated cells (solid gray). K562 cells served as a positive control. E, CD40L-activated B cells (at ~1 wk on L cells) were cultured in media alone or treated with 100 nM retinol, retinoic acid, AM580, or 5 μM rosiglitazone. CD1c, CD1d, and TGM2 mRNA expression was determined by quantitative RT-PCR and normalized to cyclophilin A. Fold differences, compared with untreated (media) cells, are shown and represent an average (mean ± SEM) of three individual tonsils. *Significant fold change versus untreated cells (ANOVA). *p < 0.05.
[(40) and data not shown] argues against the possibility that low PPARγ levels are the reason for the apparent inability to induce retinol metabolism in B cells with PPARγ ligands, as seen in DCs; rather, this inability appears to be due to low levels of ATRA biosynthesis. Surprisingly, our finding that an RARα agonist positively regulated CD1c expression in B cells also contrasts with reports in DCs. In fact, CD1c expression, like that of other group I CD1s, has been shown to be downregulated by rosiglitazone and AM580 treatment in human DCs (24, 25).

Next, we tested the functional consequence of increased CD1d and CD1c expression on B cells. Purified B cells were stimulated with CD40L for 1, 3, and 5 d in media with or without AM580. As described above, CD1d and CD1c expression on B cells steadily decreased over 5 d of CD40L stimulation in media alone, whereas AM580 treatment increased the surface expression of both CD1d and CD1c by ~50-fold, compared with untreated cells (Fig. 5A), and typically 90–95% of the cells became CD1d and CD1c positive. Consistent with the elevation in CD1 expression, AM580-treated B cells showed an enhanced ability to stimulate both CD1c- and CD1d-restricted T cells over media-treated B cells (Fig. 5B, 5C).

Because NKT cells provide help for B cell responses, we postulated that enhanced CD1c/d would increase B cell lipid-presenting capacity and thereby promote B cell–NKT cell interactions, resulting in enhanced proliferation of both cell types (6). Purified B cells were cocultured with αGalCer and NKT cells in the presence or absence of AM580. On day 4, cells were counted, and then the fraction of CD19- and CD3-positive cells that stained for the nuclear proliferation marker Ki67 was determined by FACS (Fig. 6A). AM580 treatment significantly enhanced NKT and B cell proliferation, at low doses of αGalCer (<1 ng/ml), as shown by the increased number (Fig. 6F, 6G), percentage (Fig. 6B, 6C), and absolute number (Fig. 6D, 6E) of Ki67-expressing B and NKT cells. This trend was maintained for NKT cells at increasing doses of αGalCer (≥7.8 ng/ml) resulted in a dramatic downturn in B cell proliferation (Fig. 6B, 6D, 6F), but only in the presence of AM580.

To verify that the effect of AM580 on proliferation was dependent on CD1d-mediated presentation, rather than by other mechanisms, B cells and NKT cells were cocultured in media alone or in media with AM580, and then activated nonspecifically with

![Figure 5](http://www.jimmunol.org/)
anti-CD3, PHA, or SAC. B cell proliferation in the presence of AM580 was comparable to that in media when cells were stimulated with these control stimuli (Supplemental Fig. 3). Total Ki67hi NKT cell numbers were slightly increased in AM580-treated cocultures stimulated with PHA. However, this increase did not occur when NKT cells were cultured alone, indicating that AM580 does not directly promote NKT cell proliferation, but instead promotes B cell–NKT cell interactions.

**FIGURE 6.** AM580 treatment enhances NKT expansion. B cell proliferation is augmented at low doses of αGalCer; higher doses decrease B cell numbers. NKT cells and tonsil B cells were cultured with αGalCer (0–500 ng/ml) with (top) or without (bottom) AM580 (10 nM), as indicated. On day 4 of culture, cells were counted and stained with anti-CD19, anti-CD3, and anti-Ki67. A, Representative dotplots of CD19 and intracellular Ki67 expression on day 4 of culture. Average (means ± SEM) recoveries on day represented, as follows: percent of Ki67hi B cell and NKT cells (B, C); absolute Ki67hi B cell and NKT cell numbers (D, E); total B cell and NKT cell numbers (F, G). Cumulative data obtained from five individual tonsils from independent experiments. Significant differences, *p < 0.05, paired t test.
We investigated the mechanism that may account for our surprising observation that B cell numbers fell at increasing doses of αGalCer in the presence of AM580. Because NKT cells secrete IFN-γ and are cytotoxic (41, 42), we investigated the possibility that enhanced B cell Ag presentation due to AM580 was stimulating the cytotoxic activity of NKT cells. Cytotoxicity was determined by two methods. First, NKT cells were stained for CD107a, a marker transiently retained on the plasma membrane following degranulation of lytic lysosomes (33, 43). CD107a expression on NKT cells was moderately increased at high doses of αGalCer, and this increase was significantly augmented by AM580 (Fig. 7A, 7B). In fact, CD107a expression at 31 ng/ml of αGalCer in the presence of AM580 was similar to that observed at 500 ng/ml αGalCer in media only. Second, we quantified the extent of B cell lysis by measuring the release of intracellular LDH. Significantly higher B cell cytotoxicity was seen in cocultures treated with AM580 than in the presence of αGalCer only (Fig. 7B). This effect was CD1d dependent because CD1d-blocking Abs prevented LDH release and CD107a expression (Fig. 7A–C). To show that the cytotoxic effect is mediated by NKT cells, we determined that titrating the number of NKT cells resulted in more killing at higher E:T ratios (data not shown). We conclude that by upregulating CD1d expression, AM580 treatment promotes NKT cell help for B cell proliferation at low concentrations of lipid Ag; however, higher concentrations skew NKT cells toward cytotoxicity.

Discussion

B cell expression of Ag-presenting molecules likely plays a key role in determining specific B–T interactions. For instance, the upregulation of MHC-II upon B cell activation promotes the recruitment of CD4 T cell help. In this article, we report that CD1d and CD1c expression in human B cells is regulated by distinct and overlapping signals that define different functional outcomes. CD40L stimulation, modeling conventional T cell help, resulted in decreased CD1d and CD1c expression and function, leaving only a narrow temporal window in which lipid Ag presentation could occur. Whether the CD40L signaling pathway directly influences CD1 transcription or whether it acts by an indirect mechanism is unknown. The expression of TGM2, a bona fide RARα target gene, was also concurrently reduced in activated cells, suggesting that decreased RARα-mediated transcriptional regulation may account for CD1d/c downregulation. In support of this hypothesis, we found that RARα agonists upregulated both CD1s, indicating that RARα plays a role in regulating their expression in human B cells.

Our finding that B cells, unlike DCs, are probably not intrinsically capable of functionally significant ATRA biosynthesis, suggests that a critical source of ATRA for B cells is likely obtained from other cells, such as DCs in the local microenvironment of lymphoid tissues. In fact, nuclear PPARγ and higher TGM2 expression could be detected in perifollicular zones on interdigitating DCs of tonsil (23, 24), in proximity to neighboring B cells, as well as in a population of DCs in human GALT (24, 26). Therefore, dietary PPARγ ligands, such as the metabolites of polyunsaturated fatty acids (44), could presumably stimulate local DCs to release ATRA that would influence neighboring cells, including B cells.

Although expression of both CD1d and CD1c decreased with ongoing CD40L activation, we observed that CD1c expression levels decreased at a slower rate than CD1d levels (Figs. 1B, 2B). We postulate that CD1c expression may be regulated in vivo by BCR signaling. Indeed, it has been suggested that MZB cells maintain higher tonic BCR signaling than do follicular B cells, owing in part to their polyreactivity to ubiquitous and self Ags (13, 23).
45–47). For instance, mice that express a BCR transgene for which the specific Ag is absent develop follicular B cells, but not MZB cells (48), indicating that BCR signaling is required to generate the MZB compartment. Our data support and further those findings by indicating that continued signaling may also be important to maintain the CD1c^{hi} phenotype associated with human MZ-like B cells.

Activation of BCR with anti-Ig or SAC alone was sufficient to augment CD1c expression (Fig. 2A). Human B cells, which dominate the Ab response to encapsulated organisms, bear surface BCRs that use a relatively restricted subgroup of V_{H} chains. It is thus tempting to speculate that the BCR specificity to multimerized bacterial glycolipids Ags may specifically stimulate MZ-like B cells in vivo to upregulate CD1c for the purpose of enhancing their presentation. Furthermore, we found that surface CD1c was upregulated by 18–24 h following SAC treatment, potentially prompting rapid “innate” B cell and CD1c-restricted T cell responses.

Interestingly, unlike the concomitant induction of CD1d and CD1c expression we observed following activation of the RARs pathway, BCR cross-linking upregulated CD1c but downregulated CD1d. The expression of type 1 and type 2 CD1 molecules on other APCs has distinct and often inverse patterns. For instance, human monocytes initially express high levels of CD1d (type 2), but during the course of GM-CSF/IL-4–driven differentiation into immature DCs, type 1 CD1s (CD1a–c) are upregulated, whereas CD1d expression is reduced (22, 23). In contrast, culture of DCs in the presence of human serum, PPAR{gamma} ligands, or RAR agonists reverses this CD1 profile (23, 24, 39). It is unclear why CD1d and CD1c are differentially regulated. However, it has been hypothesized that type 1 CD1s have roles that are fundamentally different from those of CD1d, the former presenting lipids to diverse (and perhaps more conventional) T cells and the latter to more innate-like NKT cells with limited diversity and characteristic self-reactivity (22)—although it has also been suggested that self-reactivity is a common feature to all CD1-restricted T cells (49). Nevertheless, given that MZB cells are also inherently self-reactive (45–47), it is possible that downregulation of CD1d following BCR cross-linking would decrease the potential for presentation of self-lipid Ags and NKT-cell help, yet allow them to elicit help from CD1c-restricted T cells.

Mouse studies have served as an in vivo model for studying human B–NKT cell interactions; however, important differences exist between the species. Unlike human CD1d, murine CD1d broadly traverses all endocytic compartments, presumably allowing for access to a greater array of lipid Ags and replacing the function of the missing group I CD1s found in other species. CD1d associated with mouse MZB cells may have replaced CD1c expression associated with this population in humans and other nonrodent animals (50). Of note, we found that mouse B cells do not upregulate CD1d in response to RARs agonists, or downregulate CD1d upon CD40L activation, indicating that these genes are also regulated differently in these species. This finding is perhaps not surprising, given that the upstream promoter regions of human and mouse CD1d are not conserved (51).

In addition to their role in responses to T-independent Ags, evidence indicates that murine and human MZB cells also participate in T-dependent immune responses (11, 13). Whether CD1-restricted T cells directly participate in cognate T–B interactions, like conventional Th cells, is not known. NKT cells have been identified in the MZ of the spleen (52). Previous studies have shown that B cells can acquire lipid Ags by a BCR-independent pathway, which implies that NKT cell–B cell interactions are not entirely dependent on shared Ag specificity (1, 2, 6). Therefore, the inherent self-reactivity associated with CD1-restricted T cells and MZ-like B cells means that their responses need to be regulated to avoid autoimmunity. Perhaps the potential for self-reactivity is controlled by limiting the time during which activated B cells can elicit NKT cell help. We found that CD1d and CD1c are downregulated following CD40L activation and propose that this establishes a “window of opportunity” that limits the amount of help from CD1-restricted T cells.

NKT cells have been shown to have regulatory roles in vivo. A recent report indicates that exposure to apoptotic bodies, a trigger for autoantibody production that can cause systemic lupus erythematosus in susceptible animals, activates NKT cells (53). This NKT cell activation, and shift toward anti-inflammatory cytokine production, was revealed to be critical to suppressing the expansion of autoreactive B cells. It is known, however, that NKT cells express FasL and perforin in vivo and that they are cytotoxic (41, 42). Therefore, NKT cells may regulate B cell responses through their ability to kill. We have shown that short-term activated B cells have a greater capacity to present lipid Ag and stimulate NKT cells than do resting B cells. However, when CD1d downregulation was prevented with AM580 treatment, higher {alpha}GalCer concentrations promoted NKT cell cytotoxicity and significantly reduced B cell numbers. Although our studies used NKT cell lines, which could arguably be skewed toward more INF-γ release and killing than autologous primary NKT cells, our data suggest that CD1 downregulation following B cell activation may be necessary to avoid NKT cell killing. Consistent with this notion, Wingender et al. (21) report that NKT cell cytotoxicity in vivo directly correlates with target cell CD1d expression. In addition, another recent study reported that NKT cells selectively inhibit autoreactive B cells that express more CD1d, yet in turn activate and help nonautoreactive B cells expressing lower levels of CD1d (54). It is therefore possible that natural sources of ATRA in vivo may be involved in promoting CD1d upregulation and NKT-mediated regulation of the B cell compartment.

In conclusion, we have found that multiple pathways affect CD1c/d expression in B cells. We propose that CD40L, BCR, and RARs are important physiological regulators of CD1c/d expression in B cells. Our data provide candidate mechanisms by which these pathways influence not only the potential for interactions with CD1-restricted T cells but also how these could affect possible outcomes (i.e., help versus killing) in vivo. These pathways may also be exploited therapeutically for designing strategies aimed at enhancing humoral immunity, as well as controlling B cell-mediated immune diseases or B cell malignancies.

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Disclosures
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