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Enhanced IgA Class Switching in Marginal Zone and B1 B Cells Relative to Follicular/B2 B Cells

Denise A. Kaminski2 and Janet Stavnezer3

Mouse splenial marginal zone (MZ) B cells and B1 B cells enriched in the peritoneal cavity respond preferentially to T cell-independent Ags compared with follicular (FO)/B2 B cells. Despite the differential responses of B cell subsets to various stimuli, and despite the need for multiple stimuli to induce IgA class switching, the relative contribution of B cell subpopulations to IgA production is unknown. By culturing purified B cell populations, we find that MZ and peritoneal B1 cells switch more readily to IgA than do splenic FO or peritoneal B2 cells in BLyS/LPS/TGF-β. Addition of IL-4, IL-5, and anti-IgD dextran to the cultures enhances IgA switching in FO/B2 and MZ B cells to a similar frequency, but this treatment suppresses IgA class switching in B1 cells. Thus, IgA switching differs among purified B cell subsets, suggesting that individual B cell populations could contribute differentially to IgA expression in vivo, depending on available stimuli. The Journal of Immunology, 2006, 177: 6025–6029.

Humoral immune responses in mice are mediated by at least three subpopulations (subsets) of mature B cells. Follicular (FO)4 B2 B cells in the spleen, lymph nodes, and Peyer’s patches (PP) typically produce Abs in response to T cell-dependent Ags, whereas the nonfollicular B cells, such as those in the splenic marginal zone (MZ) and B1 B cells enriched in the peritoneal cavity (PerC) and pleural cavity, respond to challenge by T cell-independent Ags (1–4).

Adoptive transfer studies have shown that B1 cells contribute, in a T cell-independent manner, to at least half of the IgA plasma cells detected in the gut lamina propria (LP) (4, 5). IgA functions to neutralize toxins in the mucosa, inhibit Ag uptake, and inhibit bacterial adherence. Unlike other isotypes, IgA does not induce inflammation, which is optimal for its location in the mucosa (6–8). Despite the importance of IgA, which is the most abundantly produced isotype in the body, little is understood about how IgA class switching is regulated and which B cells perform this switch to produce IgA in vivo.

IgA class switching can be induced in cultured B cells stimulated with LPS and TGF-β (9). However, IgA switching is inefficient unless these cultures are treated with additional B cell activators such as IL-4, IL-5, and anti-IgD-conjugated to dextran (anti-Δ-Dex) (10). These reagents promote survival, proliferation, and differentiation responses that vary depending on the B cell subset under study. For example, MZ B cells proliferate and differentiate into plasma cells more rapidly than FO B cells in response to LPS (11, 12). FO B cells, which are characterized by higher expression of IgD, have a stronger proliferative response to anti-Δ-Dex with greater synergy between anti-Δ-Dex and IL-4 (12). The survival factor B lymphocyte stimulator (BLyS)/BAFF and its homologue APRIL can also promote IgA class switching (13). MZ B cells expand preferentially compared with FO B cells in BLyS transgenic mice (14) and are more profoundly reduced in blys−/− mice (15).

Despite these known differences in responses to B cell activators that enhance IgA class switching, it is unknown whether IgA class switching is regulated differentially among B cell subsets. We address this question by comparing the abilities of purified B cell subsets to switch to IgA in vitro. We show that when cultured under minimal stimulation conditions, purified splenic MZ B cells and PerC B1 cells switch to IgA at a higher frequency than do FO/B2 cells. This difference is specific to IgA, as it is not observed for the IgG isotypes tested. Surprisingly, B1 cell IgA switching is suppressed by IL-4/IL-5/anti-Δ-Dex, suggesting that in vivo, different B cell subsets may contribute to IgA production depending on the stimulus.

Materials and Methods

Flow cytometry and B cell culture

Sorted purified splenic B cells were prepared by CD43 depletion with magnetic beads (Miltenyi Biotec) followed by staining with anti-B220 allophycocyanin (RA3-6B2; Caltag Laboratories), anti-CD23 PE (2G8; Southern Biotechnology Associates), and anti-CD21 FITC (7G6; BD Pharmingen) for cell sorting using a FACS Vantage cell sorter (BD Pharmingen). Goat anti-mouse IgM FITC (F(ab′)2), rat anti-mouse IgD Alexa Fluor 647 (clone 11-26), a gift from Dr. J. F. Kearney (University of Alabama at Birmingham, AL), and rat anti-mouse Mac-1 PE (clone M1/70; BD Pharmingen) were used for sorting PerC cells. Cells were stimulated on day 0 with flag-tagged human BLyS (100 ng/ml; affinity purified from transfected Chinese hamster ovary cells), LPS (50 µg/ml; Sigma-Aldrich), TGF-β1 (2 µg/ml; R&D Systems), IL-4 (800 U/ml), a gift from Dr. W. Paul (National Institute of Allergy and Infectious Diseases, Bethesda, MD), IL-5 (1.5 µg/ml; BD Pharmingen), and anti-Δ-Dex (0.3 µg/ml), a gift from Dr. C. Snapper (Uniformed Services University, Bethesda, MD). IL-4 was used for IgG1 stimulation at 2400 U/ml. Splenic B cells were plated at 10^6/ml and PerC cells were plated at 5 × 10^7/ml. Flow cytometry to measure switched isotypes on cultured cells was performed using PE-conjugated goat anti-mouse F(ab′)2 against IgA, IgG2b, IgG3, and IgG1. For each sample, 50,000–75,000 events were acquired for analysis. The frequency of switched cells was determined by gating on PE+ IgM+ events, as double-positive events are detectable in similar samples from class switching-defective aid−/− mice (our unpublished data).
Results

*MZ* B cells switch preferentially to IgA

To examine whether IgA class switching is differentially regulated among B cell subsets, splenic B220⁺ lymphocytes from C57BL/6 mice were purified by FACS into the CD21<sup>low</sup>CD23<sup>high</sup> FO and CD21<sup>high</sup>CD23<sup>low</sup> MZ B cell populations (Fig. 1A). The purified cells were stimulated for 4 days, and then assayed for IgA expression by flow cytometry. When cultured with BLYS/LPS, ≤0.06% of the cells from either population expressed surface IgA (Fig. 1B). In BLYS/LPS/TGF-β cultures, the frequency of IgA⁺ FO B cells increased to 0.61 ± 0.21%, whereas IgA class switching in the MZ B cell cultures increased to 2.4 ± 0.40% (Fig. 1, B and C). Because MZ B cells proliferate earlier than FO B cells in response to LPS in culture (11), we asked whether the FO B cells would switch to IgA as efficiently as MZ B cells at a later time point, and found they did not (Fig. 1C). This result suggests that the kinetics of B cell proliferation are unlikely to be responsible for the difference in IgA class switching. When IL-4/IL-5/anti-IL-10 antibodies were added (10), the two B cell subsets switched to IgA at a similar high frequency between days 3 and 5 of culture (Fig. 1B and unpublished data). Therefore, when appropriately stimulated, splenic MZ and FO B cells can switch to IgA equally well; however, if stimulation is limited, MZ B cells are more proficient.

Class switching to all isotypes occurs by H chain DNA recombination that requires activation-induced cytidine deaminase (AID) (16) and germline transcription through the corresponding switch region DNA (17). This germline transcription likely facilitates the DNA lesion-inducing activity of AID by: 1) promoting chromatin accessibility, 2) AID recruitment via RNA Pol II, and 3) generation of a ssDNA target for AID (17–19). Transcription through the H chain α region is initiated by B cell activation and TGF-β signaling (20).

To explore the mechanism of greater IgA switching in MZ compared with FO B cells in BLYS/LPS/TGF-β cultures, we examined steady-state germline α RNA and found at least 10-fold higher levels of these transcripts in MZ compared with FO B cells (Fig. 2B). This result suggests that splenic MZ B cells are more poised than FO B cells to initiate this molecular event that promotes IgA class switching. FO B cells stimulated with BLYS/LPS/TGF-β/IL-4/IL-5/anti-β-Dex served as a positive control because we have previously shown that in total splenic B cell cultures, this mixture of reagents increases germline α RNA levels compared with BLYS/LPS/TGF-β alone (D. A. Kaminski and J. Stavnezer, submitted for publication).

MZ B cells have a modestly higher (≈2-fold) level of *aid* mRNA than FO B cells when stimulated with BLYS/LPS/TGF-β (Fig. 2B), consistent with a previous study showing higher *aid* mRNA expression in LPS-stimulated MZ than in FO B cells (21). To determine whether this higher *aid* expression results in a higher frequency of class switching in MZ B cells in general, we examined IgG2b expression, which can also be induced by LPS with or without TGF-β (22, 23). Fig. 3A shows that the frequency of IgG2b⁺ B cells in BLYS/LPS/TGF-β cultures is similar between MZ and FO B cells at all time points tested. Thus, class switch recombination activities (e.g., sufficient cell division, AID, and DNA repair proteins) are readily available in cultured FO B cells. For each subset, and at all time points, the frequency of IgG2b⁺ cells in the TGF-β cultures are 2- to 10-fold higher than those induced by BLYS/LPS alone. Therefore, poor IgA induction in FO B cells in these same cultures is not due to an inadequate response to TGF-β. Furthermore, switching to IgG3 and IgG1 (induced by their appropriate stimuli) is also similar between the MZ and FO B cell cultures (Fig. 3, B and C). These results suggest that the previous report of more IgG3 secretion by MZ B cells than by FO B cells (12) is due to their rapid plasma cell differentiation (11) rather than preferential switching to IgG3. Collectively, we find that MZ and FO B cell subsets undergo class switching to IgG with similar efficiency.
PerC B1 cells switch preferentially to IgA

Studies using mouse PerC chimeras suggest that half of serum IgA and half of IgA plasma cells in the intestinal LP are derived from B1 cells (4, 5). To examine whether B1 cells, like splenic MZ B cells, have a predisposition to switch to IgA, peritoneal lavage cells were sort-purified into the B1 (IgMhighIgDlowMac-1low) and B2 (IgM lowIgDhighMac-1neg) subsets (Fig. 4A). After 4 days of stimulation with BLyS/LPS, very few IgA+ cells were detected (Fig. 4C), despite a previous report that B1 cells constitutively express germline α RNA (24). After addition of TGF-β, purified B1 cells switched to IgA at an ~8-fold higher frequency than did B2 cells (Fig. 4, B and C). Although considerable variation was noted, it is striking that some samples achieved 10% and 15% IgA+ cell levels, values much higher than ever observed with splenic B cells under these minimal stimulation conditions (D. A. Kaminski and J. Stavnezer, submitted for publication) (Fig. 1C). These data suggest that like MZ B cells, B1 cells have a greater propensity to switch to IgA in response to minimal stimulation compared with B2 cells. In contrast to either splenic B cell subset (MZ or FO), and in contrast to PerC B2 cells, switching to IgA by PerC B1 cells was not enhanced by adding IL-4/IL-5/anti-Dex to the BLyS/LPS/TGF-β culture (Fig. 4C). Instead, IgA class switching was suppressed by 4.8-fold. This observation suggests that the signaling pathways for class switching differ between MZ and B1 cells.

In marked contrast to both splenic B cells and PerC B2 cells, PerC B1 cells switched poorly to IgG2b under all conditions tested, including BLyS/LPS/TGF-β (compare Fig. 4, B and C, with Fig. 3A). Therefore, as in the case of MZ B cells, enhanced IgA switching in B1 cells appears to be specific to that isotype.

Discussion

We show here that splenic MZ B cells undergo IgA class switching at a higher frequency than FO B cells when cultured with BLyS/LPS/TGF-β, but IL-4/IL-5/anti-Dex enhance IgA class switching to the same frequency in each population. BLyS/LPS/TGF-β also induce a higher frequency of IgA class switching in PerC B1 compared with B2 cells; however, IL-4/IL-5/anti-Dex suppress IgA in B1 cultures, instead of enhancing it. Although IgG2b class switching is highly efficient in splenic MZ and FO B cells, and nearly as efficient in PerC B2 cells, IgG2b class switching in B1 cells is particularly poor. These observations indicate that nonfollicular B cell subsets have a specific propensity to switch to IgA under minimal conditions, and that individual B cell subsets respond differentially to B cell activators to either enhance or suppress IgA class switching.

PerC B1 cells switch preferentially to IgA

Our finding that FO B cells, which constitute more than half of splenic B cells, are particularly poor at IgA switching may explain why IgA is difficult to induce when total splenic B cells are cultured with LPS/TGF-β with or without BLyS. A predisposition by MZ B cells to switch to IgA may explain why early (3- to 5-day) dextran-specific Ab responses in the spleen after i.v. challenge are dominated by IgM and IgA, as these cells respond rapidly to such T cell-independent Ags that have repetitive carbohydrate epitopes (25).

Few nonfollicular B cells, as defined by an IgMhighIgDlow phenotype, are detected in lymph nodes and PP (26). The enrichment of FO-type B cells in the PP, where constitutive germinal centers are detectable (26), suggests, together with our results, that the relatively frequent IgA class switching that occurs in PP (24) results from an abundance of B cell activation signals from Ags, T cells, TLR agonists, and dendritic cells, rather than the presence of IgA-committed B cells (27).

However, mice that lack PP can still produce normal levels of mucosal IgA (30–32). This PP-independent IgA may be derived...
from at least two sources. One is the B cells in the isolated lymphoid follicles of the gut LP, that, like those of the lymph nodes and PP, show characteristics of B2/FO B cells, ~10% of which have a germinal center phenotype (33). The second source is the well-documented contribution of B1 cells to mucosal IgA production. Studies using mouse PerC chimeras suggest that half of serum IgM and half of IgA plasma cells in the intestinal LP are derived from B1 cells (4, 5). In situ switching to IgA from one or both of these sources could explain the ability to detect molecular evidence of \( \mu \)-to-\( \alpha \) recombination directly in the LP (28). Our results showing that B1 cells switch readily to IgA, but only under minimal stimulation, support the concept that these cells may be a preferential source of this isotype in T cell-independent responses.

Another unique feature of B1 cells that we have discovered is that they switch very poorly to IgG2b under any condition tested. We have previously observed that purified IgG2b+ cells switch poorly to IgA when restimulated (D.A. Kaminski and J. Stavnezer, submitted for publication), suggesting that direct switching from IgM to IgA is favored over sequential switching from IgG2b. Therefore, B1 cells may be well suited for producing IgA because they are poised for this direct switching pathway, whereas B2 cells more readily switch instead to IgG2b. In support of this conclusion, the first cell lines isolated that could isotype-switch in culture (1.29\( \alpha \) and CH.12) were B1 cell-derived lymphomas that predominantly switch to IgA (34–36).

Sort-purified B1a cells (the CD5+ subpopulation of B1 cells) from the PerC were previously shown to switch to IgG1 3- to 10-fold less well than B2 cells in response to CD40L or LPS with IL-4/IL-5. Interestingly, germline \( \gamma \) RNA levels induced by IL-4 were similar in the B1a and B2 cells in these cultures (37), suggesting that a level of regulation other than germline transcription is responsible for the difference. These observations, together with our results, suggest that B1 cells undergo poor class switching in general, with the noted exception of IgA (which is still only 14% at most). This inefficient class switching is consistent with the fact that B1 cells are the source of natural serum IgM (38).

Serum and mucosal IgA, but not other isotypes, can be detected in \( \mu \)MT mice, which have a block in conventional B cell development due to a deletion in the transmembrane region of IgM (39). Thus IgA, but not other isotypes, can infrequently rescue B cell development in the absence of surface IgM expression. Because the constant region of the \( \alpha \) H chain is more similar to the constant region of \( \mu \) H chain in comparison with \( \gamma \) constant regions, one possible explanation for the isotype bias in \( \mu \)MT mice is that the IgA protein may be more successful than IgG at assembling a BCR capable of supporting further development. A second possible, and not mutually exclusive, explanation for the isotype bias in \( \mu \)MT mice is that some B cell progenitors in these mice occasionally class switch, and when they do, they switch preferentially to IgA, as we have found for B1 cells. Similar to B1 cell-derived IgA (4), \( \mu \)MT IgA reacts with commensal bacteria cell wall proteins, is T cell-independent, and requires peripheral lymphoid structures other than spleen (39). The ability of \( \mu \)MT bone marrow to reconstitute IgA in \( \text{rag}^{-/-} \) mice suggests that the source of \( \mu \)MT IgA is either B2 cells or the CD5+ (B1b) subpopulation of B1 cells, as B1a cells cannot be reconstituted with adult bone marrow (2). Similar to the variability noted in our cultures, the levels of IgA among the \( \mu \)MT mice ranged from undetectable up to levels found in normal C57BL/6 mice (39).

In summary, we find that nonfollicular MZ and B1 cells undergo class switching to IgA in minimal culture conditions at a higher frequency than FO/B2 cells. B1 cells are particularly unique in this aspect because the IgA switching observed in minimal conditions is suppressed by addition of stimuli that enhance IgA switching in other B cell subsets, and because B1 cells undergo poor IgG2b switching. The results suggest an organization of humoral immunity in which individual B cell populations contribute to IgA in response to different stimuli, which has important implications for mucosal homeostasis and vaccine development.

**FIGURE 4.** Class switching in cultures of purified PerC B1 and B2 cells. A, Peritoneal lavage cells were stained, and cells in the lymphoid gate were sort-purified as indicated. Profiles of Mac-1 expression (bottom) are shown for the B1 (IgM\( ^{\text{lo}} \)IgD\( ^{\text{hi}} \)) gate in the open histogram and B2 (IgM\( ^{\text{hi}} \)IgD\( ^{\text{lo}} \)) gate in the gray-filled histogram. B, Flow cytometry profiles showing IgA and IgG2b expression on the sort-cells from A cultured with BlyS/LPS/TGF-\( \beta \) for 4 days. C, Compiled data for IgA and IgG2b expression on PerC B cells after 4 days with (+) or without (−) the stimuli indicated. Symbols indicate B1 (□) and B2 (■) cells from individual mice within the same experiment.  #,  \( p = 0.053 \); *, \( p \leq 0.032 \) as determined by ANOVA for a mixed model.
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Disclosures
The authors have no financial conflict of interest.

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