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IL-21 and BAFF/BLyS Synergize in Stimulating Plasma Cell Differentiation from a Unique Population of Human Splenic Memory B Cells

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Both constitutive Ig secretion by long-lived plasma cells (PC) and the recurrent differentiation of memory (mem) B cells into PC contribute to the maintenance of serologic mem. However, the relative contribution of each is unknown. In this study, we describe a novel population of human postswitched mem B cells that rapidly differentiate into PC and thus contribute to serologic mem. These IgG+ B cells reside in the region of human spleen analogous to the murine marginal zone and have not previously been examined. These cells are highly responsive to IL-21 in the context of CD40 stimulation. Uniquely, IgG+ marginal zone analog B cells are extrinsically sensitive to the combination of IL-21 and B cell-activating factor belonging to the TNF family (BAFF/BLyS) that synergizes in the absence of further costimulation to induce up-regulation of B lymphocyte-induced maturation protein-1 and drive PC differentiation. Other cytokine combinations are not active in this regard. This is the first demonstration that this unique population of mem B cells can respond specifically and exclusively to IL-21 and BAFF/BLyS by differentiating into IgG-secreting PC, and thus contributing to serologic mem in an Ag-independent manner. The Journal of Immunology, 2007, 178: 2872–2882.

F ollowing vaccination, protective Abs (serologic memory (mem)3) persist for decades, and are maintained by the combination of Ab secretion from long-lived PC as well as recurrent differentiation of mem B cells into plasma cells (PC) (1). Although human blood mem B cells can differentiate into PC in response to various nonspecific stimuli and T cell-derived factors (1, 2), it is unclear whether a unique population of mem B cells contributes to serologic mem in humans, such as preplasma mem cells in mice (3). In humans, unlike mice, CD27+ mem B cells with somatically mutated Ig genes are found in the region of the spleen analogous to the murine marginal zone (MZ) (4–6). This region contains both IgD+CD27+ and IgD−CD27+ mem B cells. In contrast, in mice, MZ cells are largely naive, long-lived, nonrecirculating cells that respond rapidly to T-independent type 2 Ags (TI-2) (7). Little is known regarding the role of human MZ analog (MZA) B cells in protective immunity, but studies have suggested that IgD+ MZA B cells may be involved in responses to T-independent Ags (8), and these cells have been suggested to be similar to circulating blood IgD−CD27+ mem B cells (9). However, the phenotypic and functional characteristics of IgD− MZA B cells have not been analyzed, and their potential role in maintaining serologic mem has not been assessed.

IL-21 belongs to a family of cytokines that bind receptors that signal through the common γ receptor. IL-21 is produced by activated T cells or spontaneously by CXCR5+ follicular helper T cells, and contributes to responses of T cells, B cells and NK cells, each of which express the IL-21R (10–12). Previously, we and others have demonstrated that IL-21 has the ability to induce class switch recombination (CSR) and PC differentiation from both mouse splenic B cells and human blood B cells by costimulating up-regulation of activation-induced cytokine deaminase (AID) and B lymphocyte-induced maturation protein-1 (BLIMP-1) (13–16). Although the combination of IL-2, IL-10, and anti-CD40 has the ability to stimulate PC differentiation from human mem B cells (17, 18), IL-21 coactivation is unique in its ability to induce CSR and PC differentiation from both naïve and mem peripheral blood B cells (15). The observations that helper T cells within secondary lymphoid organs constitutively produce IL-21 (11) and overproduction of IL-21 results in hypergammaglobulinemia and autoimmunity (14, 19), make IL-21 a prime candidate cytokine to be involved in the regulation of serologic mem.

Whereas IL-21 is clearly one of the major T cell-derived factors involved in humoral immune responses, dendritic cell (DC)-derived factors are also known to play a role. B cell-activating factor belonging to the TNF family (BAFF/BLyS) belongs to the TNF family and binds three receptors, BAFF/BLyS receptor (BAFFR), transmembrane activator and calcium modulator cyclophilin ligand interaction (TACI), and B cell maturation Ag (BCMA) (20). BAFF/BLyS is known to be a B cell survival factor, and overexpression of BAFF/BLyS results in hypergammaglobulinemia as well as autoimmunity (20). It is therefore another prime candidate to contribute to serologic mem. In humans, DC can induce PC differentiation (21–23), and BAFF/BLyS and a proliferation-inducing ligand (APRIL) expressed by activated DC may induce CSR (24). Importantly, the relative contribution of T cell- and DC-derived factors in protective immunity is not well understood.
In this study, we report a novel population of human splenic mem B cells poised to differentiate into PC following encounter with T cell-derived and DC-derived soluble factors. These IgG⁺ B cells reside in the region of human spleen analogous to the murine MZ and are exquisitely sensitive to IL-21. Notably, the combination of IL-21 and BAFF/BLYS synergizes in the absence of further costimulation to induce BLIMP-1 expression and PC differentiation from IgG⁺ human MZA B cells. The ability of IgG⁺ MZA B cells to respond to the combination of IL-21 and BAFF/BLYS demonstrates that these cells are uniquely receptive to Ag-independent signals from activated T cells and DC and are capable of rapid differentiation into PC, thereby replenishing serologic mem in a bystander fashion.

Materials and Methods

Isolation of human B cells

All human studies have been approved by the Warren G. Magnuson Clinical Center Institutional Review Board, and informed consent was obtained according to the Declaration of Helsinki. Spleens were obtained from organ donors. For total CD19⁺ splenic cultures, B cells were isolated by positively selecting B cells with anti-CD19 magnetic beads (Miltenyi Biotec). Briefly, splenic cells were incubated at 10⁷ cells/ml in staining buffer with mAbs CD27, and isolated with a MoFlo cell sorter (DakoCytomation). Briefly, B cells were isolated by expression of CD19, CD21, CD23, and IgG or CD19, CD23, and total MZA; CD19⁺ CD27⁺ CD23⁻ or CD19⁺ CD21⁺/CD23⁻ CD27⁻ and total MZA; CD19⁺ CD27⁺ CD23⁻ or CD19⁺ CD21⁺/CD23⁻ CD27⁻ B cells. To distinguish IgG from the IgG⁺ MZA subpopulation (and exclude CD27⁻ CD21⁻ low CD23⁻ conventional mem B cells and PC), IgG⁺ or IgG⁻ MZA were sorted as CD19⁺ CD23⁻ CD21⁺/CD23⁻ Igd⁺ or CD19⁺ CD23⁻ CD21⁺/CD23⁻ IgG⁺ subsets. Cells isolated using CD21 and CD23 and CD27 and CD23 responded similarly. Preparations were typically >98% pure. Cells sorted with CD21-FITC, CD23-PE, CD19-PerCP-Cy5.5, and IgG-allylalkylocycocyanin were analyzed after 5–12 days of culture by staining with IgG-FITC, CD19-PerCP-Cy5.5, and CD38-allylalkylocycocyanin to distinguish IgG⁺ CD38⁺ mem PC. Carryover of mAb used for isolation was observed in the other channels.

Cell culture

Purified B cells were cultured at 1 × 10⁶ cells/ml in either 1 ml in 24-well culture plates or 100 µl in 96-well round-bottom culture plates. The cells were incubated with a combination of human IL-21 (100 ng/ml; BioSource International) with either 1 µg/ml anti-human CD40 (R&D Systems), 5 µg/ml anti-IgM (Jackson ImmunoResearch Laboratories), or both. In some experiments, the cells were incubated with a combination of human IL-2 (100 U/ml; Roche), human IL-4 (100 ng/ml; R&D Systems), human IL-10 (25 ng/ml; R&D Systems), and human IL-6 (400 ng/ml; R&D Systems). All cytokines and stimuli were added at the initiation of culture. Human BAFF/BLYS was added at 200 ng/ml, and APRIL was used at 500 ng/ml (both obtained from R&D Systems).

Flow cytometry

Four-color flow cytometry was performed using a FACSCalibur (BD Biosciences). Briefly, all cells were harvested from 96-well cultures and stained for 30 min on ice with a combination of mAbs. The combination of anti-IgD-FITC, anti-CD27-PE, anti-CD19-PerCP-Cy5.5, and anti-CD38-allylalkylocyanin (clone HB7) was routinely used (BD Biosciences). In addition anti-CD21-FITC (Immunotech) with anti-CD23-allylalkylocyanin or anti-CD23-PE with anti-CD23-allylalkylocyanin or anti-CD21-PE with anti-CD23-allylalkylocyanin or anti-CD19-PerCP-Cy5.5 were used with PE-conjugated Abs to IgD, IgG, CD27, CD95, B220, CXCRC4 and allylalkylocyanin conjugated to CD38 and CD44 or FITC-conjugated to CD45RA (all obtained from BD Biosciences), CXCR5-PE (R&D Systems), IgM-PE (Serotec), CD148-AF647 (BioSource International) and anti-BCMA-biotin, anti-TACI-biotin or goat anti-BAFFR and goat IgG-biotin control was used (R&D Systems) followed by streptavidin-PE or anti-goat-FITC. Viable cells were identified by gating on lymphocytes and cells were analyzed immediately. All samples were collected for 30 s, and as a result the density of the dot plots reveals relative cell numbers. In some experiments, to obtain total B cell numbers more accurately, AccuCount Particles (Spherotech) were added and total cell numbers were determined per the manufacturer’s instructions.

FIGURE 1. Human spleen MZA B cells are IgM⁺or⁺CD19⁺CD27⁺ CD21⁻lowCD23⁻ Ki-67⁺. Serial cryosections of human spleen were doubled stained with anti-IgD-HRP (red) and either anti-CD19, CD27, CD21, IgM, CD1c, CD3, CD23, and Ki-67 mAbs (blue). Magnification shown is ×200 and bar shows 60 µm. Staining is representative of 15 spleens. Arrows show central arterioles.

Determination of Ig levels

Secreted Ig in the culture supernatant was quantitated by ELISA. Briefly, 96-well flat-bottom Nunc-Brand Immuno Plates (Nalge Nunc International) were coated with 5 µg/ml either affinity-purified goat anti-human IgM or goat anti-human IgG-Fc (Bethyl Laboratories) overnight at 4°C. Wells were then washed and blocked with a 0.2% BSA/PBS, and then titered culture supernatant was added and incubated overnight at 4°C. Bound Ig was detected with 0.5 µg/ml alkaline phosphatase (AP)-conjugated goat anti-human IgM or IgG (Bethyl Laboratories) and developed with p-nitrophenyl phosphate tablets (Sigma-Aldrich). Specific absorbance was measured and OD quantified at 410 nm by a Powerwave X 96-well plate reader (Bio-Tek Instruments).

B cell proliferation

To assess proliferative responses of cultured cells, 10⁵ purified B cells were cultured as described above in 96-well round-bottom plates. After 3 days of culture, [²H]thymidine (37 Kd/well) was added to the cultures for an additional 16 h. Thymidine uptake was measured using a liquid scintillation counter.

Real-time quantitative PCR

Purified B cell populations were stimulated as described above. After 3 days in culture, RNA was isolated using the RNeasy mini kit (Qiagen). Reverse transcription reactions were prepared using the SuperScript One-Step PCR System with platinum Taq polymerase and ROX reference dye (Invitrogen Life Technologies). Fifty nanograms of isolated RNA was added per reaction with 1.2 mM MgSO₄, TaqMan Assays on Demand Gene expression primer/probe sets (Applied Biosystems) were used for BLIMP-1.
(Hs00153357_m1), AID (Hs00221068_m1), Bim (Hs00197982_m1), and β-2 microglobulin (β-2M) (Hs99999907_m1). Final concentrations were 1.8 μM for primers and 0.5 μM for probes. RT-PCR was performed using the ABI Prism 7700 Sequence Detection System, and cycle conditions and relative quantification were completed as described by the manufacturer’s instructions (Applied Biosystems). mRNA expression for each gene was calculated using the comparative cycle threshold method with efficiency calculations and with all mRNA levels normalized to β-2M.

FIGURE 2. MZA B cells are composed of both pre- and postswitched mem B cells. A, Serial frozen tissue sections of human spleen imaged by confocal microscopy are shown at ×200. Single staining with triple overlays of CD27 (green), IgM (red), and IgD (blue) or CD3 (green; n = 12), IgM (red), and CD11c (blue; n = 6), or CD27 (green), IgM (red), and LYVE-1 (blue; n = 6) are shown. Arrows indicate the central arterioles. B, High-power (×400) triple staining shows CD11c− (red) cells located within the MZA area, as well as IgM+ (blue) cells. Note the many IgD− IgM− CD27+ B cells in this region. Bar shows 50 μm.

FIGURE 3. MZA B cells express a unique set of markers. Freshly obtained splenic cells were stained for expression of CD19, CD21, and CD23. Expression of various Ags in FO (CD21+/CD23−), MZA cells (CD21hi/CD23+), and CD21−/CD23− cells was determined as indicated. Numbers represent mean fluorescence intensity of the total population, and numbers in parentheses represent mean fluorescence intensity of the positive population. Data are representative results for 1 of 6 similar experiments for analysis of the total Ab profile and 11 experiments for analysis of CD27, IgD, IgM, IgG, and CD38 by populations defined by CD21/CD23 expression. The mean frequency of CD21+ CD23+ FO cells was 22%, range 1–38%, and the mean frequency of CD21hi/CD23+ MZA cells was 34%, range 11–74% (n = 11).
**Immunohistochemistry (IHC)**

Tissue samples for IHC were embedded in Tissue-Tek OCT compound (Miles). Six-micrometer-thick sections were cut, fixed in acetone, and blocked with 1.5% horse serum diluted in PBS/1% BSA. Abs used (diluted in PBS/1% BSA) were anti-human CD3-Alexa Fluor (AF) 647, CD11c-AF647, IgD-AF546, and IgM-Pacific Blue (BD Pharmingen). All Abs were directly conjugated using the AF mAb Labeling Kits (Invitrogen Life Technologies) according to the manufacturer’s instructions. Rabbit anti-human lymphatic endothelial hyaluronan receptor (LYVE)-1 Abs (Abcam) were detected with goat anti-rabbit IgG-AF546 (Invitrogen Life Technologies). Cross-talk-free confocal images were obtained on a Zeiss LSM 510 equipped with either a 405-, 543-, and 633-nm lasers. Figures were prepared using Adobe Photoshop (Adobe Systems). Because of uneven brightness of the three fluorochrome-labeled Abs, visual insistence was increased to the same extent of all three fluorochromes to delineate the distribution of the individual markers in the single-color panels. For colored substrate IHC, sections were first treated with 20% acetic acid to inactivate endogenous AP activity and 0.03% H2O2 in PBS to inactivate endogenous AP activity. Sections were then washed in PBS, and nonspecific binding was blocked with 1.5% horse serum (diluted in PBS/1% BSA) before incubation with mAbs appropriately diluted in PBS/1% BSA. Mouse mAbs directed against human CD3, CD27, IgM, Ki-67 (BD Pharmingen), CD19, CD24, CD38 (Caltag Laboratories), CD21, CD23 (Leinco), and CD1c (a gift from Dr. M. Brenner, Harvard Medical School, Boston, MA) were detected using the Vectastain ELITE ABC kit (mouse IgG-biotin then avidin-AP) and the Vector Blue AP substrate (Vector Laboratories). Mouse anti-human IgD-HRP (Southern Biotech) was detected using Vector NovaRED substrate (Vector Laboratories).

**Statistics**

Statistics were performed using a one-tail, two-sample (assuming unequal variances) t test with p value (*, p < 0.05; **, p < 0.01; ***, p < 0.005) compared with cultures with no cytokine for each group.

**Results**

Human spleen MZ-like areas contain a novel mem B cell subset

Initially, we analyzed the organization of human splenic B cell compartments. Unlike mouse spleen, in which B cell follicles form around T cell areas, the white pulp of human spleen consisted largely of CD19+ B cell aggregates with small patches of CD3+ T cells outside this region (Figs. 1 and 2) (4, 5, 9). The CD19+ B cell regions were composed of a central follicle containing IgD+ IgM+ B cells that was often associated with central arterioles (arrows). In the spleens examined (n = 15), ~10% of FO areas included germinal centers that were Bcl-6+ and contained Ki-67+ proliferating lymphocytes, CD21high follicular DC (FDC), and a few CD3+ T cells (Fig. 1 and data not shown). Surrounding the follicle was a zone of CD21+ CD23+ Ki-67+ CD27− CD1c− B cells (Fig. 1) that was located in a region analogous to the murine MZ. A portion of these MZA B cells were

**FIGURE 4.** Pre- and postsort analysis of splenic B cell subpopulations. A. Splenic B cells were analyzed based on expression of CD19, CD38, IgD, CD21, CD23, and CD27. Cells were sorted based on expression of CD19, CD27, and CD23. FO B cells were identified as CD19+CD23−CD27+ and MZA B cells, and a small percentage of mem B cells were identified as CD19+CD23+CD27−. Pre- and postsort analysis of expression of CD23 and CD27 is shown as well as cell surface phenotype of presorted CD19+ B cells: CD19 and CD38; IgD and CD38; and CD21 and CD23. Pre- and postsort analysis of splenic B cell subpopulations. B. Splenic B cells were analyzed for expression of IgD, CD38, CD21, CD23, and IgG. Cells were sorted based on expression of CD19, CD21, CD23, and IgG. FO B cells were identified as CD19+CD23−CD21− IgG−, and total MZA B cells were identified as CD19+CD23−CD21high IgG+. CD21highCD23− MZA or IgG−CD21highCD23− MZA B cells are also shown. Pre- and postsort analysis is shown as well as IgD vs CD38 cell surface phenotype of presorted cells. Pre-sorted spleens contained 0.3% PC on day 0 (IgD−/CD38high boxed cells). Postsort analysis shows a maximum of 0.7% PC in IgG+ MZA B cells, as determined by the frequency of IgG+CD21+CD23+ B cells.
IgM$^-$IgD$^-$$^{high}$ and the remainder were IgM$^-$IgD$^-$, presumably postswitched B cells (Fig. 2)(4, 9, 25, 26). No marginal sinus was present in this region (8, 26, 27), nor is the region vascularized by LYVE-1-expressing endothelium, which is present in the red pulp (Fig. 2)(28). Importantly, the MZA contained CD3$^+$ T cells as well as CD11c$^+$ DC, which were concentrated at the edge of the CD27$^+$ mem B cell zone (Fig. 2). This collection of mem B cells surrounding the FO area was not noted in tonsil or lymph nodes (data not shown).

Based on the immunohistology, splenic FO B cells were identified as CD19$^+$CD21$^-$CD23$^+$ cells and mem MZA B cells were CD19$^+$CD21$^+$CD23$^-$ cells. Flow cytometry was used to characterize these B cell subpopulations more completely. FO B cells were found to be small and CD21$^+$CD23$^+$CD27$^+$IgD$^+$IgM$^+$IgG$^-$$^{low}$PC were boxied. B, Proliferation (day 3), PC number (day 5), and secreted IgG and IgM (day 7–10) were also determined. MZA B cell responsiveness was assessed only in nil and anti-CD40-stimulated cultures. IgM production by all subpopulations was assessed only in nil and anti-CD40-activated cultures. Data are from one representative experiment (FO, n = 8; MZA/mem, n = 3; MZA, n = 1).

**FIGURE 5.** MZA are hyperresponsive to IL-21 co-stimulation compared with FO B cells. A and B, Splenic FO and MZA/mem B cells (sorted based on expression of CD23 and CD27), or CD21$^{high}$ MZA B cells (sorted based on high expression of CD21 from a spleen with no CD21$^{high}$CD23$^+$ B cells) were assessed for CD19, IgD, and CD38 expression after 7 days of stimulation as indicated (A). IgD$^-$$^{high}$CD38$^{high}$PC are boxied. B, Proliferation (day 3), PC number (day 5), and secreted IgG and IgM (day 7–10) were also determined. MZA B cell responsiveness was assessed only in nil and anti-CD40-stimulated cultures. Data are from one representative experiment (FO, n = 8; MZA/mem, n = 3; MZA, n = 1).

MZA B cells brightly express B220 and CD45RA that are normally expressed by naive blood B cells and are usually down-regulated when CD27 is expressed (29). In addition, CD95 that is normally expressed by mem B cells is only modestly up-regulated by MZA B cells. However, CXCR4, the receptor for CXCL12, is expressed at a considerably lower density on MZA B cells compared with conventional splenic mem B cells (Fig. 3). CD148 expression, which has previously been shown to be associated with human MZA B cells (4), was not detected by immunohistology (data not shown) but by flow cytometry, and was found to be expressed by IgD$^-$$^{low}$IgM$^+$MZA B cells (data not shown). Moreover, MZA B cells were CD80$^-$$^{low}$CD86$^-$$^{low}$, and routinely CD25$^-$$^{low}$HLA-DR $^+$ (data not shown). IL-21R was expressed at low density by all splenic populations (data not shown). The third population of B cells was CD21$^-$$^{low}$CD23$^+$ and comprised a mixture of CD27$^+$PC, CD27$^+$mem B cells, and CD27$^-$transitional B cells. Taken together, the data demonstrate that MZA B cells have a distinct phenotype compared with other splenic B cell subpopulations or other mem B cell populations.

**MZA B cells are hyperresponsive to IL-21 coactivation**

To elucidate whether IL-21 differentially impacts the responsiveness of human B cell subpopulations, splenic B cells were initially

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sorted into CD19⁺CD27⁺CD23⁺ naive FO as well as CD19⁺ CD27⁺CD23⁻ mem MZA, which includes a small population of conventional mem B cells based on expression of CD19, CD23, and CD27 (Fig. 4A). Fig. 4A indicates the phenotype of the splenic B cells analyzed and the results of the postsort analysis. Comparison with Fig. 3 indicates the heterogeneity between spleen samples. Although the same B cell populations are found in all spleens examined, the relative proportions of the various populations differ considerably. The sorted B cell subpopulations were assessed for their functional responsiveness.

As previously described for murine splenic and human blood B cells (14, 15), the majority of naive FO B cells were killed by the combination of BCR and IL-21 stimulation (Fig. 5A). In contrast, the combination of anti-CD40 and IL-21 induced FO B cells to differentiate into CD21highIgD⁺IgM⁺ PC. This response was enhanced by engagement of the BCR (Fig. 5A). Compared with FO B cells, PC differentiation was significantly greater when either CD21low/high MZA/mem B cells were stimulated with anti-CD40 and IL-21, and BCR engagement did not enhance this response (Fig. 5A). Similar results were obtained when MZA splenic B cells were sorted based on high expression of CD21 that excluded the CD21⁻low conventional mem B cell population (Fig. 5A). Furthermore, MZA/mem and MZA B cells generated significantly more PC and produced greater amounts of Ig than FO B cells in response to IL-21 costimulation, although all populations manifested extensive proliferation (Fig. 5B). Finally, MZA B cells not only responded more robustly than FO B cells to IL-21 costimulation but also more rapidly. By day 5, MZA B cells costimulated with IL-21, anti-IgM, and anti-CD40 generated significant numbers of PC (16,329 and 21,900 PC for day 5 and day 7, respectively), whereas FO B cells stimulated under identical conditions required a longer incubation for the differentiation of substantial numbers of PC (1,501 and 7,715 PC for day 5 and day 7, respectively). At all time points examined, the number of PC generated from FO B cells was substantially less than that from MZA/mem or MZA B cells.

Because the CD21high MZA B cell population contains both preswitch IgMhighIgD⁺⁺ and postswitch IgG⁺ mem B cells, CD19⁺CD21highCD23⁻ splenic B cells were sorted into IgG⁺ or IgG⁻ cells (as shown in Fig. 4B) to assess their function. Both IgG⁺ and IgG⁻ MZA B cells were induced to differentiate into PC and produce IgG following stimulation with IL-21 and anti-CD40 or anti-CD40 and anti-IgM (Fig. 6, A and B), demonstrating that IL-21 coactivation induces both CSR and PC differentiation. IL-21 costimulation of IgG⁺ MZA B cells induced considerable levels of IgM, especially when both CD40 and the BCR were engaged, as well as low levels of IgG (Fig. 6B). Importantly, postswitched IgG⁺ MZA B cells generated significantly more PC and secreted much greater amounts of IgG in response to IL-21 costimulation compared with IgG⁻ MZA and FO B cells (Fig. 6B). Notably, in
all populations. IL-21- and anti-CD40 costimulation induced AID and BLIMP-1 up-regulation (Fig. 6B), although human MZA B cells have previously been reported not to express AID in situ (30). Finally, IL-21 alone induced IgG⁺ MZA B cells to produce low levels of IgG (mean 435 ng/ml; n = 9) and significantly up-regulates BLIMP-1, but not AID, expression (Fig. 6B). These results
indicate that IgG⁺ MZA B cells are responsive to IL-21 in the absence of further costimulation.

BAFF/BLyS does not promote naive FO B cell responsiveness to IL-21

FO B cells respond poorly to the combination of anti-CD40 and IL-21 and died when stimulated with anti-IgM and IL-21. Therefore, we sought to determine whether provision of a survival factor might enhance the responsiveness of FO B cells to IL-21 signal. BAFF/BLyS is known to promote the survival of certain human B cell subsets (20, 31). We therefore addressed whether BAFF/BLyS could promote responsiveness to IL-21. As shown in Fig. 7A and B, BAFF/BLyS clearly increased survival of total splenic CD19⁺ B cells and FO B cells, that were maintained without other stimuli or in the presence of anti-IgM.

However, BAFF/BLyS did not increase the responsiveness of these cells following stimulation with anti-CD40 in the presence of IL-21 (Fig. 7, A and B), but did marginally increase the number of PC generated from FO B cells stimulated with IL-21, anti-IgM, and anti-CD40 (Fig. 7C). Although BAFF/BLyS increased survival of FO B cells in the absence of IL-21, it did not significantly (p = 0.20) decrease the death of FO B cells stimulated by anti-IgM and IL-21 (Fig. 7B). Because Bim has been implicated in IL-21-mediated cell death in mice (32) and BAFF/BLyS has been shown to down-regulate Bim (33), we examined the impact of BAFF/BLyS on Bim expression by splenic B cells stimulated with IL-21. As expected from the functional results, neither IL-21 nor BAFF/BLyS affected expression of Bim in total splenic CD19⁺ B cells or purified FO B cells alone or after stimulation with anti-IgM (Fig. 7D and data not shown).

IL-21 and BAFF/BLyS synergize to induce PC differentiation from IgG⁺ MZA B cells

Although BAFF/BLyS did not increase the responsiveness of FO B cells to IL-21, strikingly, the combination of IL-21 and BAFF/BLyS in the absence of other stimulation or with anti-IgM induced PC differentiation from CD19⁺ splenic B cells (Fig. 8). Moreover, the combination of IL-21 and BAFF/BLyS also induced significant Ig production from total splenic CD19⁺ B cells regardless of the addition of anti-IgM (Fig. 8). IL-21 and BAFF/BLyS-induced PC differentiation of CD19⁺ B cells was preceded by induction of BLIMP-1 mRNA (Fig. 8). In contrast, AID mRNA was not induced by the combination of IL-21 and BAFF/BLyS, but rather was noted following stimulation with IL-21 and anti-IgM with or without BAFF/BLyS (Fig. 8). Finally, BAFF/BLyS did not increase the responsiveness of CD19⁺ B cells to IL-21 and anti-CD40 with or without anti-IgM, nor did it induce PC differentiation from B cells costimulated with anti-CD40 (Fig. 8).

Because BAFF/BLyS had minimal effect on the responsiveness of naive FO B cells, we examined the effect of this cytokine on
CD23⁺CD27⁺ MZA B cells that contained small numbers of CD23⁻CD27⁺ conventional mem B cells. We found that culture with BAFF/BLyS alone increased the survival of both FO and MZA/mem B cells, but only induced minimal PC differentiation (Fig. 9A). Culture with IL-21 alone had no significant effect on B cell survival, and only small numbers of PC were induced from MZA/mem B cells, but only induced minimal PC differentiation with BAFF/BLyS alone increased the survival of both FO and MZA splenic B cells were sorted based on high expression of CD21, which excluded the CD21low conventional mem B cell population. When MZA B cells were purified from conventional mem B cells and subdivided into pre- and postswitched B cells, culture with IL-21 alone, but not BAFF/BLyS was again noted to induce low levels of PC differentiation and IgG production from IgG⁺ MZA B cells (Fig. 9, B and C). Notably, however, IL-21 synergized with BAFF/BLyS resulting in substantial PC differentiation and IgG production from IgG⁺ MZA B cells in the absence of further costimulation (Fig. 9, B and C). In contrast, only minimal numbers of PC and minimal IgM or IgG secretion were generated from IgG⁻ MZA B cells stimulated with the combination of IL-21 and BAFF/BLyS (Fig. 9, B and C). Moreover, compared with IgG⁺ MZA B cells, CD27⁺CD21low⁻CD23⁻ IgG⁻ conventional splenic mem B cells responded less well to IL-21 and BAFF/BLyS or IL-21 and anti-CD40 with regard to both PC differentiation and IgG production (Fig. 10 and data not shown).

The ability of IL-21 and BAFF/BLyS to induce PC differentiation from IgG⁺ MZA B cells was not related to sorting with anti-IgG, and thus signaling through the BCR, because IL-21 and BAFF/BLyS induced IgG production from total splenic B cells and total MZA B cells not purified with anti-IgG (Figs. 8 and 9). Moreover, costimulation of IgG⁺ (IgMhigh) MZA B cells with IL-21, BAFF/BLyS, and anti-IgM did not induce large numbers of PC (Fig. 10).

IL-21 and BAFF/BLyS-induced PC differentiation was preceded by up-regulation of expression of BLIMP-1 mRNA in both populations of MZA B cells, although the magnitude of BLIMP-1 increase was greater in the IgG⁺ subpopulation (Fig. 9D). AID was not induced in IgG⁺ MZA B cells by the combination of IL-21 and BAFF/BLyS.

Despite the finding that the combination of IL-21 with BAFF/BLyS induced marked PC differentiation from IgG⁺ MZA B cells, BAFF/BLyS had little effect on PC differentiation of any splenic B cell populations stimulated with anti-CD40 and IL-21 with or without BCR engagement (Fig. 10). Importantly, BAFF/BLyS also did not induce PC differentiation from splenic B cell subsets stimulated with anti-CD40 or anti-IgM and anti-CD40 in the absence of IL-21 (Figs. 7 and 8).

Notably, the BAFFRs TACI and BAFFR were expressed at high density on MZA B cells (Fig. 3) (34). Because both BAFF/BLyS and APRIL bind TACI, we cultured IgG⁺ MZA B cells with APRIL and IL-21. In three independent experiments, APRIL and IL-21 induced only a fraction of the PC differentiation and Ig secretion (3, 5, and 33%) of cells stimulated by IL-21 and BAFF/BLyS. These data suggest that BAFFR (which does not bind APRIL) is likely to be the major receptor inducing PC differentiation and IgG production. However, signaling through TACI alone or combined engagement of both TACI and BAFFR may provide optimal signaling for PC differentiation.

Other cytokine combinations do not induce extensive PC differentiation of MZA B cells compared with IL-21 and BAFF/BLyS

The combination of IL-2 and IL-10 is a known inducer of PC from mem splenic B cells costimulated via CD40 (4). Moreover, as shown in Fig. 11, A and B, the ability of IL-2 and IL-10 alone to induce PC differentiation from CD21high MZA was significantly less than that of IL-21 and BAFF/BLyS. Notably, the combination of IL-21 and BAFF/BLyS induced comparable numbers of PC and levels of IgG secretion from MZA B cells as the combination of IL-2, IL-10, and anti-CD40 (Fig. 11B). Furthermore, we found that none of the other cytokine combinations we analyzed resulted in significant PC differentiation from total CD19⁺ splenic B cells (Fig. 11A). These data support the conclusion that the combination of IL-21 and BAFF/BLyS is unique in its ability to induce PC differentiation from MZA B cells.

Discussion

The current results indicate that a unique population of IgG⁺CD21highCD23low⁻CD27high mem B cells resides in the “MZ-like” structure of human spleen. Previously, these cells have not been examined (9, 35), although they clearly are an important component of the human MZA. Phenotypically, these postswitched IgG⁺ cells are very similar to preswitched MZA B cells,
differing only in BCR isotype and expression of CD148. Notably, however, this is the first demonstration that IgG⁺ MZA B cells are functionally distinct. A unique feature of these cells is their general hyperresponsiveness to IL-21 and their capacity to respond exclusively to the combination of IL-21 and BAFF/BLyS with rapid and substantial differentiation of PC. Neither of these functional activities can be explained by the density of IL-21R expression, which did not vary between pre- and postswitched MZA B cells.

In most circumstances, human B cells require engagement of CD40 and/or BCR to respond with significant PC differentiation. However, IgG⁺ MZA mem B cells are unique in requiring only signals from T cell-derived IL-21 and DC-derived BAFF/BLyS for this response. IL-21 is a well-known T cell-derived cytokine that can costimulate differentiation of human B cells into PC (15, 16), but previously has been shown to require activation signals induced through engagement of CD40 or CD40 and the BCR for this response. In humans, DC can induce PC differentiation (21–23), and BAFF/BLyS and APRIL expressed by activated DC also can contribute to CSR (24). Importantly, BAFF/BLyS shares certain signaling pathways with CD40 (36, 37). In the IgG⁺ MZA B cell population, BAFF/BLyS may mimic certain CD40 signaling capabilities, and therefore allow synergistic interactions between IL-21 and BAFF/BLyS. Support for this concept derives from the observation that BAFF/BLyS appears to have little effect on B cell responsiveness or survival when cells are also stimulated through CD40 or activated via anti-CD40 and IL-21, suggesting that overlapping signaling pathways with CD40 may explain its activity. BAFF/BLyS has also been shown to promote the survival, but not proliferation, of human splenic plasmablasts by preventing their apoptosis (31). Such a mechanism could contribute to the current results, although a number of findings make this possibility unlikely. First, stimulation with BAFF/BLyS did not induce PC differentiation or IgG production above background even when PC were present in the initial cultured cell population. Secondly, BAFF/BLyS and IL-21 increased the generation of PC in experiments in which IL-21 induced minimal PC differentiation alone. Thirdly, the addition of BAFF/BLyS to IL-21-stimulated cultures was essential for the induction of BLIMP-1 expression that is required for PC differentiation. In our experiments with sorted B cells, no PC were present in the initial culture. Thus, the combination of BAFF and IL-21 is unlikely to be effective merely by increasing survival of PC present in the initial culture. Finally, the combination of IL-21 and BAFF/BLyS led to a significant increase in the absolute number of PC that could not be explained by an influence on plasmablast apoptosis rather than precur-

The white pulp area of both the murine and human spleen contains a population of B cells that surround the B cell FO area. In the mouse, these MZ B cells are largely nonmigratory, naive, unactivated B cells with the phenotype, IgD⁺ IgM<sup>hi</sup>CD21<sup>hi</sup>CD23<sup>low</sup> (7, 38). Murine MZ B cells are especially responsive to TI-2 with the capacity to differentiate rapidly into short-lived IgM-secreting PC, but manifest minimal CSR, somatic hypermutation, and generation of mem to TI-2 (7, 38). However, these cells can undergo CSR, somatic hypermutation, and PC differentiation in response to T-dependent Ags (39, 40). Moreover, in the rat, mem B cells with mutated Ig genes have been reported to migrate to the MZ following immunization (41). Importantly, the murine MZ is more than a collection of B cells, but rather an organized anatomic structure immediately outside the marginal sinus that contains a specific population of metallophilic macrophages (38). This architecture appears to contribute to the function of MZ B cells because it fosters the rapid entry of bacteria-containing DC that can facilitate MZ B cell differentiation in a BAFF/BLyS-dependent manner (42). The human MZA is considerably different than the murine MZ. There is no marginal sinus and no metallophilic macrophages, although resident stromal cells may contribute to migration, retention, and responsiveness of lymphocytes (28, 43). The peri-FO zone just outside of the MZA, however, is vascularized and contains sialoadhesin-expressing macrophages (43). Importantly, human MZA B cells are also considerably different than murine MZ B cells in that they are CD27<sup>hi</sup> mem B cells consisting of both pre- and postswitched cells (Refs. 4, 26, and this study). As opposed to the murine MZ, which are largely nonmigratory cells, human IgD⁺ MZA are thought to appear in the peripheral circulation (9). Despite the marked differences between murine MZ and human MZA there are certain similarities, including the surface phenotype (CD21<sup>hi</sup>CD23<sup>low</sup>/−) and the ability of murine MZ and human IgD⁺ CD27<sup>hi</sup> MZA B cells to respond to TI-2 (7). Finally, both populations can respond to BAFF/BLyS stimulation and appear to be hypersensitive to IL-21 costimulation (Ref. 42 and this study). Thus, in our preliminary studies, we found that murine MZ B cells respond robustly to IL-21 costimulation (data not shown). Interestingly, mice that acutely (IL-21 plasmid-injected) or constitutively (BXSB yaa mice) express high levels of IL-21 do not have MZ B cells (14, 44), perhaps because of IL-21-induced PC differentiation.

The postswitched human IgG⁺ MZA B cells have not previously been examined. These cells appear to reside at the outer edge of the MZA structure in a region interspersed with CD3⁺ T cells and CD11c⁺ DC. They are a polyclonal population with heavily mutated Ig genes (5, 6, 39). Importantly, one might assume that the MZA is a survival niche for these cells that reflect the humoral mem of the subject. The finding that they can vigorously respond to the combination of IL-21 and BAFF/BLyS suggests that any stimulus that can simultaneously induce production of these cytokines can enhance serologic mem polyclonally by causing rapid induction of PC differentiation from IgG⁺ MZA. Our previous finding that IL-21 induces differentiation of long-lived nondividing PC (15) suggests that the contribution of IL-21 and BAFF/BLyS to serologic mem may be persistent and not transient, as is seen with stimulation of serologic mem MZ B cells.

Importantly, we show that IgG⁺ MZA B cells are in close association with T cells and DC that are potentially capable of producing IL-21 and BAFF/BLyS, respectively. This proximity suggests that locally produced factors can stimulate IgG⁺ MZA B cells to differentiate into PC. Alternatively, because both IL-21 and BAFF/BLyS can be detected in the plasma in autoimmune disease (Ref. 45 and our unpublished results), MZA B cells may be poised to react in an endocrine manner to products of distantly activated T cells and DC. In humans, BAFF/BLyS has also been shown to be produced by FDC (46, 47). These and our results suggest that FO helper T cells (which spontaneously produce IL-21 (11), and BAFF/BLyS-expressing FDC could also play a role in driving the rapid differentiation of MZA mem B cell responses in an Ag-independent manner. These considerations raise the intriguing possibility that the induction of Ag-specific germinal center responses may lead to increased production of IL-21 from Ag-specific T cells and BAFF/BLyS from FDC and thereby drive polyclonal activation of MZA mem B cells in adjacent peri-FO areas. This possibility is consistent with two previous observations. First, the MZA region appears to be more prominent around follicles containing germinal centers (26), and second, specific immunization is frequently associated with polyclonal hyperglobulinemia in both mice and humans (48–50).

In summary, our results strongly suggest that human splenic IgG⁺ MZA B cells are a reservoir of cells that reflect the natural history of Ag exposure of the individual. Moreover, these IgG⁺ MZA B cells can respond to soluble factors from T cells and DC (or FDC) with rapid differentiation of PC and, therefore, are prime candidates to replenish serologic mem in an Ag-independent manner.
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Disclosures

The authors have no financial conflict of interest.

References


