Innate Immunity and Human B Cell Clonal Expansion: Effects on the Recirculating B2 Subpopulation

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Foci of autoantigen-specific B lymphocytes in nonlymphoid tissues have been associated with development of autoimmune disease. To better understand the genesis of such ectopic lymphoid tissue, this study investigated whether several B cell-tropic innate immune system molecules, known to be elevated in response to inflammatory stimuli, can cooperate in fostering the T cell-independent clonal expansion of mature human B2 cells under conditions of limiting BCR engagement. Notable synergy was observed between BCR coligation with the C3dg-binding CD21/CD19 costimulatory complex, B cell-activating factor belonging to the TNF family (BAFF), and IL-4 in generating B cell progeny with sustained CD86 and DR expression. The synergy was observed over a wide range of BCR:ligand affinities and involved: 1) cooperative effects at promoting early cell cycle progression and viability; 2) BCR:CD21 coligation-promoted increases in BAFF receptors that were highly regulated by IL-4; 3) reciprocal effects of IL-4 and BAFF at dampening daughter cell apoptosis typical of stimulation by BCR:CD21 and either cytokine alone; and 4) BAFF-sustained expression of antiapoptotic Mcl-1 within replicating lymphoblasts. The results suggest that significant clonal proliferation of recirculating B2 cells occurs upon limited binding to C3dg-coated Ag in an inflammatory in vivo milieu containing both BAFF and IL-4. When rare autoantigen-presenting B cells undergo such expansions, both B cell and T cell autoimmunity may be promoted. The Journal of Immunology, 2005, 175: 6143–6154.
the innate immune system. A greater understanding of how diverse costimuli cooperate in promoting B cell clonal expansion following limiting BCR engagement is important and may suggest new approaches to aborting autoreactive B cell activation in autoimmune disease.

We report here a notable synergy between low levels of surrogate C3dg-bound Ag, IL-4, and BAFF in promoting the in vitro clonal expansion of recirculating human follicular (B2) lymphocytes. The proliferating daughter cells expressed high levels of CD86 and class II MHC, molecules important in B cell APC function for T cells. Efforts to unravel the mechanism(s) for synergy indicate that all the costimuli are essential for optimal progression through the initial cell cycle. IL-4R and CD21/CD19-assisted BCR signaling are most important in promoting early cell cycles, both through influences on viability and cell cycle progression and in preparing B cells for additional rounds of replication. BAFF, in contrast, has a prolonged influence on the clonal expansion process, at least in part, through sustaining the viability of proliferating lymphoblasts. The data suggest that the integrated effects of these innate costimuli on recirculating human B2 cells exposed to limiting Ag is important for early B cell clonal replication in an inflammatory setting.

Materials and Methods

mAb:dextran (dex) conjugates

Synthesis and properties of the soluble mAb:dex conjugates were described previously (22, 38). Briefly, high molecular mass dextran (modified to contain amino groups and iodoadetacylated) was linked to S-acythiolated mAb through stable thioester bonds. Each dex molecule was covalently linked to two distinct mAb, i.e., an anti-human IgM mAb of low, intermediate or high affinity (or IgG1 control mAb) and THB-5 anti-human CD21 mAb (or IgG2a control mAb). The three IgG1 anti-IgM mAbs used to construct the conjugates are all specific for proximal (or identical) epitopes on the Cμ2 domain; their Fab’ affinities range from $K_a = 2 \times 10^5$ M$^{-1}$ (mAb P24), $K_a = 2 \times 10^7$ M$^{-1}$ (mAb Mu53), and $K_a = 5 \times 10^9$ M$^{-1}$ (mAb HB57 (DA4.4)) (22). The soluble conjugates have been determined to have 10–12 anti-IgM mAb (or control mAb) and 10–12 anti-CD21 mAb (or control mAb) per dex molecule (22).

Cytokines and additional culture reagents

Recombinant human BAFF was produced as described (39), BAFF and recombinant human IL-4 (R&D Systems) were used at optimal concentrations of 50 and 5 ng/ml, respectively. Transmembrane activator and CAML interacting protein (TACI) (Alexis Biochemicals) or control human IgM mAb was used at supraoptimal concentrations (2.5–5 μg/ml) with slightly suboptimal BAFF (5 ng/ml). Neutralizing goat anti-human IL-4 IgG (R&D Systems) was added at 5 μg/ml.

Human B cells

Tonsils from 2- to 15-year-old donors, obtained by elective tonsillectomy, were used according to institutional review board guidelines. Spleens were obtained from National Disease Research Interchange and Cooperative Human Tissue Network after removal for trauma and processed into single-cell suspensions; cells were frozen at −150°C until purification just before culture. Tonsil follicular (B2) cells were purified as described (40). The cells were ≥90% IgM+ with ≤2% CD3+ or CD16+ cells and, when assayed, were ≥97% CD19+ and CD21+, and nearly all IgD+ and CD23+. For spleen B cells, T cell-depleted suspensions were subjected to magnetic bead negative selection by incubation with a mixture of FITC anti-CD43 (plus FITC anti-IgG (BD Pharmingen) plus FITC anti-IgA (Anclenc in some experiments) followed by anti-FITC and anti-CD27 magnetic beads (Miltenyi Biotec). This depleted non-B cells and B1 cells (CD3+ and switched and subepithelial (marginal zone-like) memory B cells (CD27+). Occasionally, cells were treated with FITC-anti-CD14+ and FITC-CD14- mAbs followed by anti-FITC magnetic beads to deplete contaminating dendritic cells and monocytes, with no notable difference in results. Peripheral blood B cells were obtained by Ficoll-Hypaque centrifugation and negative selection with a B cell isolation kit (Miltenyi Biotec).

Culture conditions

Cells were cultured in an enriched medium containing RPMI 1640 + 15% FCS, 5 × 10⁻³ M 2-ME, 50 μg/ml gentamicin, 40 μg/ml aprotin, 1 mM sodium pyruvate, 1/20% nonessential amino acids, and 20 mM HEPES. Cultures were established in 96-well microtiter plates (1×10⁶ cells/200-μl culture) or in 24 plates (1×10⁶ cells/2 ml).

Assays for B cell DNA synthesis, viability, and division

B cells were pulsed with [³H]thymidine from 45 to 68 h and harvested as described (22); mean cpm ± SEM of triplicate cultures is shown. To assess viability, cells were harvested, washed, fixed with 1% paraformaldehyde, and analyzed by flow cytometry. Apoptotic cells and viable cells were distinguished by changes in forward and side scatter (40). To assess clonal replication, cultures of CSFE-labeled cells were harvested on day 7 and processed as above, and increasing division number was determined by decreasing CSFE fluorescence (41).

Cell staining

Intracellular Ficol-Hypaque selected viable cells from day 6–7 cultures of CSFE-labeled cells were washed; fixed in electron microscopy grade formaldehyde (2% in PBS, pH 7.2) for 10 min at room temperature; washed and incubated for 15 min in PBS-HEPES containing 50% human AB serum with 0.1% saponin (assay buffer); and incubated at room temperature for 15 min. Mouse mAb were then added (0.5 μg Ab–10⁶ cells/50 μl): anti-Bcl-2 mAb (DAKO), anti-Bcl-x (H-5; Santa Cruz Biotechnology), and anti-Mcl-1 (BD Pharmingen). After 20 min, cells were washed and incubated with R-PE-conjugated goat (F(ab’)², anti-mouse IgM (H + L), preabsorbed to remove Abs reactive with human Ig (Southern Biotechnology). In some experiments, permeabilized cells were stained with PE-anti-active caspase 3 or PE-IGE control (BD Biosciences). Stained cells were washed, refixed with 1% paraformaldehyde, and assayed by two-color flow cytometry. Surface cultured B cells were evaluated for BR3 (BAFF-R), TACI, and B cell maturation Ag (BCMA) expression by indirect immunofluorescent staining using biotinylated Ab to hBAFF-R-Fc (clone 9-1); ITACI-IgG (clone A1G11.4 or CD47.4) and hBCMA-Fc (C4E2.2-1) and biotinylated mouse IgG1 control, or biotinylated BAFF, followed by SAv-CyChrome (BD Biosciences) and flow cytometric analysis. Expression of other surface molecules was assessed with PE- or CyChrome-conjugated Abs to CD86, CD27, CD20, CD40, CD38, and HLA-DR (BD Biosciences) and IgM (Southern Biotechnology).

Western blotting

Techniques were as described (40) with the exception that lysates were prepared with M-PER lysis solution (Pierce) + 1 mM EDTA + protease inhibitor mixture (Roche); additional blotting was performed with anti-Mcl-1 (BD Pharmingen); and HRP-conjugated goat anti-mouse IgG1 (H chain specific) (Southern Biotech, Inc) was used. Bcl-2 and Mcl-1 were detected with ECL (Amersham Pharmacia Biotech) and Bcl-xL with Super Signal West Femto Maximum Sensitivity Substrate (Pierce). Within each experiment, an equivalent amount of protein was loaded into lanes of a 15% SDS-PAGE gel. Band densitometric intensity was compared with a catalase-loading control, as described (40).

Results

BAFF, IL-4, and anti-IgM:anti-CD21:dex (surrogate for C3dg-Ag) exhibit synergy in promoting S phase entry of human B2 cells

The threshold lowering effect of exposing BCR-triggered B2 cells to multiple innate immune system costimuli was examined with a set of well-defined anti-IgM:dex conjugates whose Fab binding sites have either low, intermediate, or high affinity for human Cμ2 (Materials and Methods and Refs. 22, 24, 38, 40, and 42). As discussed elsewhere, the moderate multivalency of these high molecular mass soluble surrogates for Ag simulates the organized presentation of epitopes on many foreign and self antigenic substrates (22, 38, 40). The impact of concomitant C3dg engagement with CD21 was assessed with bispecific anti-IgM:anti-CD21:dex conjugates (Materials and Methods; Ref. 22) as surrogates for C3dg-bound Ag. Cultures of highly purified IgM⁺IgD⁺CD27neg B2 cells from tonsil, spleen, or B cells from peripheral blood were stimulated with the above mAb:dex conjugates at a dose (0.01 μg/ml) shown elsewhere to be limiting for S phase entry (22, 38).
both with and without IL-4 and/or BAFF. \[^{[3]}H\]Thymidine uptake was assessed after a day 2–day 3 pulse.

Although IL-4 and CD21 ligand exhibited synergy in promoting BCR-triggered DNA synthesis, consistent with our previous findings (22, 42), no such synergy was noted between BAFF and CD21 (Fig. 1A). Indeed, at higher BCR affinity, CD21 coligation with BCR in the presence of BAFF typically resulted in slightly lesser DNA synthesis than BCR engagement alone plus BAFF. Quite importantly, however, BCR:CD21 stimulation in the presence of both BAFF and IL-4 elicited a synergistic response that was significantly greater than the additive responses of each of the former combinations of stimuli (Fig. 1A). This synergy was consistently observed regardless of the source of B2 cells, i.e., tonsil, spleen, or peripheral blood. With the full repertoire of ancillary stimuli, a highly significant response was elicited even with low affinity BCR:CD21 engagement (stimulation index 14 ± 3 in cultures with both IL-4 + BAFF vs 7 ± 2 and 1.2 ± 0.3 in cultures with only IL-4 or BAFF, respectively (Fig. 1A).

**BCR:CD21 coligation, IL-4 and BAFF exhibit synergy in promoting the extended replication of human B2 cells**

BCR:CD21-stimulated cultures containing both cytokines exhibited considerably greater cell cluster size beginning at day 3 (Fig. 1B). Because this corresponds with the first evidence of B cell replication (data not shown), it appeared likely that the combined innate immune system stimuli might promote clonal expansion. This was examined by evaluating CFSE-labeled B2 cells for division after 6–7 days of culture with diverse stimuli. As shown by the representative experiment in Fig. 2A, cultures triggered by BCR:CD21 ligands in the presence of both IL-4 and BAFF generated significantly greater numbers of viable daughter cells (incrementally lower CFSE fluorescence (41); Fig. 2B) after 1 week of culture than did similarly triggered cultures supplemented with IL-4 alone (or with BAFF alone; Fig. 2C). At the limiting dose of moderately multivalent BCR ligand used, little replication occurred without added cytokine (Fig. 2C). Coengagement of BCR with the CD21/CD19 complex was important for optimal cytokine-driven responses because significantly lesser replication was noted in cultures with monospecific anti-IgM:dex (Fig. 2C). Importantly, although daughter cell yield directly correlated with affinity for BCR, with all the costimuli present, even low affinity BCR engagement could reproducibly trigger ≥4 replications in a minority of B cells (Fig. 2C).

In separate experiments (data not shown), the absolute recovery of cells was measured through standardization with a known quantity of beads added to the recovered cells analyzed by flow cytometry (43). In a representative experiment, ~70% of the input cells were recovered from IgG control:dex cultures with no significant division, and optimally stimulated cultures (high affinity BCR: CD21 ligand, IL-4, and BAFF) exhibited 2-fold greater recovery of total cells (viable and apoptotic). Of these, 86% were daughter cells with 13, 22, 26, 13, and 12% of the progeny representing cells with 1, 2, 3, 4, and ≥5 divisions, respectively. As will be indicated later, the differences in the proportion of total recovered cells within each of the division subsets as compared with the distribution of viable cells (Fig. 2) is explained by a heightened apoptosis characteristic of cells undergoing division.

This new observation that B cell receptors for the innate immune system molecules, C3, IL-4, and BAFF, exhibit synergy in promoting the in vitro clonal expansion of recirculating B2 cells after suboptimal BCR engagement has important physiological implications. It suggests that, within an inflammatory milieu, even B cells of low affinity, e.g., those with autoreactive specificity, may be triggered to clonally replicate in a T cell-independent manner if they contact a moderately multivalent, C3dg-bound form of self Ag.

**Daughter cells generated upon BCR:CD21 coligation in the presence of IL-4 and BAFF are lymphoblasts with high levels of class II MHC and CD86 (B7.2) but without characteristics of the plasma cell lineage**

Progeny resulting from the synergistic response were characterized for surface molecule expression by two-color flow cytometric

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**FIGURE 1.** BCR:CD21 coligation, IL-4, and BAFF exhibit synergy in promoting S phase entry of resting human B2 cells. A. In six separate experiments (exp), purified B2 cells from tonsil (three experiments), spleen (one experiment), and peripheral blood (2 experiments) were stimulated with a limiting concentration (0.01 μg/ml) of moderately multivalent anti-IgM:dex conjugates, with differing Fab’ affinity for human membrane IgM (Cμ2), that did (●) and did not (○) coexpress anti-CD21. Cultures were supplemented with BAFF (50 ng/ml) ± IL-4 (5 ng/ml), or with medium alone. Cultures with no IgM specificity, i.e., IgG control:dex, are shown by free symbols. \[^{[3]}H\]Thymidine uptake was assessed at 45–65 h. Significance above that with IgG:Ctrl:dex for responses without CD21 coligation (+) and for responses with CD21 ligation (+). B. Characteristically heightened cluster formation at day 3 in B2 cell cultures activated by anti-IgM:anti-CD21:dex plus IL-4 and BAFF (×40).
analysis of CFSE-labeled cells after 6 days of culture with BCR:CD21 ligand, IL-4, and BAFF. The representative experiment in Fig. 3 shows that the daughter cells exhibit a surface Ag phenotype characteristic of optimal APC, but not plasmablasts or plasma cells. Thus, they expressed high levels of membrane class II MHC and CD86 (B7.2) and sustained expression of CD20, CD40, and IgM, molecules typically down-regulated by the plasma cell transcriptional regulator, Blimp (44). They did not express the plasma cell marker, syndecan (CD138). Fig. 3 also indicates that, like the divided population, most of the viable, undivided B cells in these stimulated cultures exhibit high levels of the activation-associated molecules, CD27 and CD86. The up-regulation in CD27 is characteristic of stimulation by BCR:CD21 and not seen at day 7 in cultures with IL-4 and BAFF alone (data not shown). Other past studies have shown that nearly all B2 cells up-regulate CD86 by days 2–3 of culture after stimulation by BCR:CD21 plus IL-4 (45).

Although both BAFF and IL-4 are important early in activation, continued replication requires late signaling via BAFF, not IL-4 To unravel the mechanism(s) responsible for synergy in promoting B2 cell replication, it was important to evaluate the temporal requirements for each ancillary stimulus. Prior studies from this laboratory had shown that S phase entry of cells exposed to limiting BCR:CD21 ligand and IL-4 requires BCR engagement only during the first few hours of culture, but signals from IL-4 both early in the response and throughout G1 (42). In this study, we demonstrate that BAFF is also required at the initiation of the response for optimal day 2–3 DNA synthesis to BCR:CD21 ligand, IL-4, and
BAFF. This became evident by varying the time at which BAFF or TACI-Fc was added to cultures. TACI-Fc is a soluble form of one of three BAFF receptors that blocks BAFF binding to all its receptors (31). A delay of even 12 h in the addition of BAFF to cultures stimulated with BCR:CD21 ligand plus IL-4 impaired the response (Fig. 4A). Additionally, TACI-Fc was most effective at inhibiting the response of cultures stimulated with BCR:CD21 ligand, IL-4, and BAFF when added at culture initiation (Fig. 4B).

Importantly, BAFF was also essential later in the response for maximal yield of viable daughter cells. This was evidenced by the significant decline in viable cells representing three to six divisions following TACI-Fc addition at day 3 (Fig. 4C). Unlike BAFF, IL-4 had a lesser role after ≥3 days of culture, as indicated by negligible inhibition following a late pulse with neutralizing anti-IL-4 Ab (Fig. 4D). Thus, although IL-4 is quite important throughout the first cell cycle, in contrast to BAFF, its influence significantly wanes as the replication process continues.

Whereas BCR:CD21 engagement and IL-4 each promote viability early in culture, BAFF influences B cell viability at late, but not early, stages in the response.

To gain insight into the relative viability-promoting effects of each of the stimuli, i.e., BCR:CD21 coligation, IL-4 and BAFF, during the B2 cell activation process, cultures receiving different combinations of stimuli were assessed for the proportion of viable cells at 3–6 days after initiation. Unlike the notable early viability-promoting effects of IL-4 alone, or low dose BCR:CD21 engagement alone, BAFF minimally affected the viability of resting and recently activated human B2 cell cultures (Fig. 5A). This contrasts with the significant pro-viability effects of BAFF on resting murine B2 cells (reviewed in Ref. 31) but is in agreement with another recent study with human B2 cells (46). The negligible effect of BAFF on early culture viability suggests that its role early in the response may primarily reflect its enhanced expression of G1-related proteins, cyclin D2 and CDK4 (47). Beginning at day 4, however, cultures receiving stimuli from BCR:CD21 and IL-4 reproducibly exhibited greater viability when BAFF was also present (Fig. 5, A and B).

Because this late BAFF-associated increase in viability was temporally associated with the beginning of cell replication, it was not immediately clear whether the effect represented 1) selective effects of BAFF on the viability of dividing cells and/or 2) BAFF-enhanced replication which populated the cultures with new viable cells. To gain insight into these issues, it was important to distinguish the viability of nondividing and dividing cells and, additionally, to compare total cell recoveries (viable plus apoptotic) in cultures receiving, or not receiving, BAFF signals late in culture. The above was greatly facilitated by the fact that CFSE fluorescence is sustained in cells that become apoptotic (48), a finding that we reproduced in cells gated for apoptosis on the basis of light scatter (Fig. 6).

A validation of forward light scatter (FSC)-side light scatter gating for distinguishing viable from apoptotic cells, it
should be noted that this approach yielded results similar to FITC-annexin gating in past experiments with human B cells (40). Additionally, daughter cells designated as viable or apoptotic on the basis of scatter were negative and positive, respectively, for active caspase 3 (Fig. 6B).

Using the approach shown in Fig. 6 for delineating viable and apoptotic cells within each division subset, we examined the effect of curtailing BAFF signals from day 2(3) onward by addition of TACI-Fc. As indicated in Fig. 5D, late blockade of BAFF in cultures activated by intermediate affinity BCR:CD21 ligand, IL-4, and BAFF significantly impaired the viability of the replicating cells. The viability of the subpopulation that did not replicate was less affected by this abrogation of BAFF function. Although TACI-Fc addition at day 2–3 impaired daughter cell viability, there was no obvious effect on the replication process. This is shown in Fig. 5C by the comparable yields of total cells (viable plus apoptotic) within each division subset in cultures receiving TACI-Fc or medium (or in some experiments control IgG1 human myeloma protein). Taken together, the above results suggest that the major mechanism for increased day 5 culture viability in the presence of BAFF is a direct effect of BAFF at sustaining the viability of replicating lymphoblasts.

Although BAFF delayed the activation-related apoptosis of these lymphoblasts, the protection was transient and a substantial proportion did eventually succumb to apoptosis. This was evident by phase microscopy at day 5–6 of culture. Shrunken cells with an apoptotic morphology were noted as an avalanche released from the large clusters (data not shown). The timing for these microscopic indications of apoptosis paralleled the temporal appearance of apoptotic CFSE-labeled daughter cells observed by flow cytometry (data not shown). Interestingly, the kinetics is also concordant with the collapse of in vivo germinal centers formed in response to T cell-independent Ags (49).

Within BCR:CD21-triggered cultures lacking IL-4, BAFF fails to augment daughter cell viability

Because cultures stimulated with BCR:CD21 ligand and only Baff often exhibited slightly lesser levels of day 3 DNA synthesis than...
cultures stimulated by BCR alone plus BAFF (Fig. 1A), we compared BAFF effects on daughter cell recovery and viability in IL-4-deficient cultures stimulated by high affinity BCR vs BCR:CD21 ligand. These studies showed that, even in the absence of IL-4, BAFF significantly augments the replication of B2 cells, regardless of whether BCR-triggered cells received ancillary signals via CD21/CD19 (Fig. 7A). Importantly, however, whereas the progeny of cells triggered by BCR alone received positive signals for viability from BAFF, the progeny generated following BCR:CD21 engagement did not (Fig. 7B). Corresponding to this lesser viability was a slightly diminished yield of cells representing ≥2 divisions in cultures receiving ancillary signals via CD21/CD19 (Fig. 7A; closed triangles vs closed circles). This difference from cultures triggered by BCR alone was statistically significant \( (p < 0.05) \) and may reflect greater apoptosis during the prior division. The latter interpretation is supported by the fact that the slight reduction in yield of progeny lagged by one division behind the reduction in viability.

**FIGURE 6.** Assessment of CFSE fluorescence in scatter-gated apoptotic cells within day 6 cultures shows that many replicating B2 cells succumb to activation-associated apoptosis. A, Cells from day 6 cultures (high affinity BCR:CD21:dex used as BCR ligand) were gated into viable (high FSC) or apoptotic (low FSC) subsets, and each subset was assessed for replication by CFSE division slicing (histograms). The division status of each peak is indicated. We interpret the peak designated as 0* as representing undivided cells that underwent apoptosis soon after placement in culture and thus failed to process short-lived CFSE-labeled proteins during the first 24 h after labeling (41). α, Anti; Ctrl, control; Via, viable; Apo, apoptotic; SSC, side scatter. Values to the right indicate the percent of the total events collected within each division subgroup. B, Expression of active caspase 3 in CFSE-labeled cells stimulated for 6 days with anti-IgM:anti-CD21:dex plus IL-4 and BAFF; cells were stained with PE-mAb specific for active caspase 3 and gated by light scatter into viable and apoptotic subsets. undiv, undivided.

**BCR:CD21 coligation augments B cell expression of BR3 and TACI receptors for BAFF, and this is down-modulated by IL-4**

Available evidence suggests that the three known BAFF receptors, i.e., BR3 (BAFF-R), TACI, and BCMA, differentially regulate B cell responses. Although signaling via BR3 and BCMA appears to be uniformly positive, TACI ligation can have both stimulatory and inhibitory effects on B cell responses (31, 50, 51). It was, therefore, important to determine whether stimulus-specific changes in these receptors might explain the varying effects of BAFF on daughter cell viability (Figs. 5 and 7).

Membrane expression of BR3, TACI, and BCMA was evaluated with specific biotinylated mAbs after 2 days of culture with differing stimuli (Fig. 8A). Signaling via BCR induced an up-regulation in both BR3 and TACI, paralleled by an increase in the binding of biotin BAFF. Importantly, the BCR-triggered increase in TACI and, to a lesser extent, BR3 was augmented by coligation with CD21/CD19. BCMA was minimally if at all apparent under any stimulation condition.
The BCR-triggered up-regulation of BR3, but not BCMA, is in agreement with a study evaluating mRNA levels of these receptors in 18- to 24-h anti-IgM-stimulated mouse B cells (52). However, although the earlier study did not find TACI mRNA augmented following BCR cross-linking (52), we consistently observed BCR-triggered up-regulation of membrane TACI protein in human B2 cells with two distinct mAbs directed to TACI. (This was always most evident with BCR:CD21 coligation.) Parenthetically, what appears to be a lesser expression of BR3 vs TACI, based on relative CyChrome-streptavidin binding to the biotinylated mAbs (Fig. 8A), was not observed with a second biotinylated lot of the same anti-BR3 mAb. Rather the binding intensity of anti-BR3 was \(-1\) log greater than that of anti-TACI (data not shown). This likely reflects differences in the relative biotinylation of reagents and indicates that one cannot here fully equate differences in fluorescence intensity of the distinct probes with differences in receptor density.

Quite importantly, in the presence of IL-4, BCR:CD21-triggered cells manifest substantially less up-regulation of TACI, and BR3 as well (Fig. 8B). Given the reports that TACI can, under certain circumstances, be inhibitory (50, 51), this capacity of IL-4 to dampen its expression may be quite relevant. Indeed, it might help to explain the opposing conclusions regarding the function of BAFF in BCR:CD21-triggered cultures with IL-4 vs without IL-4 (Fig. 5 vs Fig. 7).

BAFF viability-promoting function correlates with sustained Mcl-1 levels within replicating B cells

In an effort to obtain a molecular explanation for the augmented daughter cell viability in BCR:CD21-triggered cultures containing both IL-4 and BAFF, CFSE-labeled cells were assessed for intracellular expression of three anti-apoptotic proteins, Bcl-2, Bcl-xL, and Mcl-1, following 6 days of culture with BCR:CD21 ligand \(\pm\) IL-4 and/or BAFF. This involved intracellular staining of fixed and permeabilized CFSE-labeled cells followed by two-color flow cytometry. As shown by the representative experiment in Fig. 9A, viable cells in BCR:CD21-stimulated cultures without added cytokines were largely undivided, strongly positive for Bcl-2, and weakly positive for Bcl-x and Mcl-1 (MFI of 218, 25, and 18, respectively). Irrespective of the cytokine present, Bcl-2 levels within the viable cells fell with increasing division (Fig. 9, A and B). Although less apparent, Bcl-x levels declined with increasing division. In contrast, Mcl-1 levels tended to be sustained, or even rise, within viable progeny in cultures containing either IL-4 alone or IL-4 and BAFF (Fig. 9, A and B). Thus, Mcl-1 expression is the anti-apoptotic molecule that best correlates with sustained viability of dividing cells.

The BCR-triggered up-regulation of BR3, but not BCMA, is in agreement with a study evaluating mRNA levels of these receptors in 18- to 24-h anti-IgM-stimulated mouse B cells (52). However, although the earlier study did not find TACI mRNA augmented following BCR cross-linking (52), we consistently observed BCR-triggered up-regulation of membrane TACI protein in human B2 cells with two distinct mAbs directed to TACI. (This was always most evident with BCR:CD21 coligation.) Parenthetically, what appears to be a lesser expression of BR3 vs TACI, based on relative CyChrome-streptavidin binding to the biotinylated mAbs (Fig. 8A), was not observed with a second biotinylated lot of the same anti-BR3 mAb. Rather the binding intensity of anti-BR3 was \(-1\) log greater than that of anti-TACI (data not shown). This likely reflects differences in the relative biotinylation of reagents and indicates that one cannot here fully equate differences in fluorescence intensity of the distinct probes with differences in receptor density.

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**FIGURE 8.** BCR:CD21 coligation augments expression of BR3 and TACI on human B2 cells, whereas IL-4 dampens this up-regulation. A, Spleen B2 cells were cultured for 2 days with the indicated mAb:dex conjugates and membrane expression of BCMA, BR3, and TACI was assessed by immunofluorescent staining and flow cytometry. Total BAFF receptors reflects binding of biotinylated BAFF. The percent increase in BR3, TACI, or total receptors above that in cells cultured with control (Ctrl):dex is indicated. B, Pooled comparisons of the levels of BR3, TACI, and total BAFF receptors in spleen and tonsil B2 cell cultures stimulated as above \(\pm\) IL-4 (n = 5 experiments (exp)). *, Expression in cultures with IL-4 was significantly diminished. α-, Anti-.
Immunoblotting analyses of total culture lysates obtained on days 3 and 5 (6) (Fig. 10) concur with the above findings. Thus, whereas IL-4 substantially increased expression of Bcl-xL and Mcl-1 in day 3 stimulated cultures (particularly with BAFF present), by day 5–6 of culture, the expression of Bcl-xL and that of Bcl-2 had significantly declined. Mcl-1 levels, in contrast, were more sustained. Comparable changes in Bcl-2 and Mcl-1 expression have been observed within human B lymphoid tissue; Bcl-2 levels were lower, and Mcl-1 levels were higher, within the germinal center, as compared with the follicular mantle by immunohistological staining (53).

Although Mcl-1 expression within viable in vitro replicating cells on day 6 was relatively high, regardless of whether the B cells were cultured with only IL-4 or both IL-4 and BAFF (Fig. 9B), this was not observed when Mcl-1 expression of all progeny was considered (viable plus apoptotic) (Fig. 9D). In the latter case, it became apparent that optimal Mcl-1 expression in the divided population required the presence of BAFF. This reflects the accelerated demise of progeny generated in the absence of BAFF (Fig. 9C) and the fact that Mcl-1 levels drop significantly with apoptosis (Fig. 9D). Corresponding to this greater overall Mcl-1 expression in daughter cells from cultures with both IL-4 and BAFF, as compared with IL-4 alone (Fig. 9D), is a slight, albeit statistically significant, increase in Mcl-1 within lysates of cultures with both IL-4 and BAFF (Fig. 10B). Taken together, these results strongly suggest that BAFF signals contribute to the sustained viability of dividing lymphoblasts, at least in part, through sustaining Mcl-1 expression.

**Discussion**

The present study presents novel insights into a mechanism for innate immune system-driven clonal expansion of recirculating human B2 cells. Using surrogate C3dg-bound and C3dg-free Ags, the study shows that both the repertoire of innate immune system cytokines available upon limiting BCR engagement with Ag and the capacity of the Ag to present C3 ligands for CD21 strongly influence the potential for clonal expansion. B2 cells can integrate signals from BCR and the costimulatory receptors for C3dg, IL-4, and BAFF to 1) optimize cell cycle transitions, 2) regulate BAFF receptor density, and 3) sustain levels of antiapoptotic proteins that curtail mitochondria-dependent apoptosis. Such interdependence between costimuli may have evolved to permit significant TI clonal amplification of recirculating B2 cells only when multiple arms of the innate immune system are concomitantly activated, e.g., as can occur upon microbial invasion. Because self-Ags lack the pathogen-associated molecular patterns for eliciting cytokine production from innate cells, even if they can on occasion be quite effective activators of complement (e.g., apoptotic cells or IgG complexes), clonal expansion of autoreactive B2 cells is unlikely
under most circumstances. The outcome may be quite different, however, if autoreactive B2 cells encounter C3dg-bearing self Ags in a milieu rich with IL-4 and BAFF generated by effective inducers of innate immunity. Under these circumstances, atypical clonal expansion of autoreactive B2 cells, even those of relatively low affinity, may ensue. The latter is supported by the present finding that BCR:CD21 coligation, IL-4, and BAFF reproducibly promote multiple B cell divisions in a fraction of B2 cells within cultures containing a low concentration of low affinity BCR ligand.

Because the sources of the above costimulatory molecules, i.e., mast cells, basophils, eosinophils, neutrophils, macrophages, and dendritic cells, are found throughout the body, and not simply in organized lymphoid tissue, there are undoubtedly many peripheral sites where recirculating B2 cells could encounter C3dg-bound self Ag in the presence of both IL-4 and BAFF. The cytokine shown to have a critical role early in the response, i.e., IL-4, is rapidly produced by many innate cells, in addition to its more delayed production by Th2 (25–29). Indeed, mast cells initiate IL-4 mRNA transcription at substantially lower levels of receptor occupancy than required for degranulation (54), and can release preformed IL-4 protein within seconds after stimulation (28). Thus, early inflammatory responses could easily provide the IL-4 necessary for initiating the cycling of B cells with specificity for any C3dg-bound Ag present. The concurrent and sustained production of BAFF from macrophages, dendritic cells, and/or neutrophils (31–33, 55) recruited to the inflammatory site should significantly heighten the likelihood of a proliferative response and prolong the viability of the actively dividing progeny.

Importantly, whereas the viable replicating cells generated in this innate immune system-driven response showed little evidence of plasma cell differentiation (either on the basis of surface Ag expression (Fig. 3) or IgM secretion in culture supernatants; our unpublished results), they did express high levels of class II MHC and CD86, two molecules critical for APC function. Although APC activity of daughter cells was not directly examined, it is quite likely that they exhibit this function. Past studies showed that B cell blasts with elevated class II MHC and CD86 after 2 days of culture with BCR:CD21 ligand plus IL-4 have significantly heightened capacity to activate naïve alloreactive T cells (45). In the context of the present findings, it is of particular interest that the B lymphocytes that accumulate in the pancreatic islets of type I diabetes-susceptible NOD mice before insulitis and that appear to be responsible for T cell stimulation and T cell-mediated pathology express high levels of CD86 (56). Furthermore, the C3dg ligand, CD21, has been implicated in the development of this disease (57).

The above route of expanding Ag-specific B2 cells may lead to situations in which autoreactive B cells predominate as the relevant APC population. A local increase in the frequency of rare autoreactive B cells in peripheral sites, the heightened class II MHC and CD86 expression on these cells, and the substantially greater ability of specific B cell APC to capture limiting amounts of Ag for processing and presentation to T cells (58), may all contribute to effective B cell APC function, under conditions where dendritic cells are less active.

Insights into how a resting B2 cell converts stimuli from limiting BCR:CD21 coengagement, IL-4 and BAFF into a robust proliferative response becomes evident by integrating this study’s new observations with previously established functions for each stimulus. Taken together, the information indicates that optimal entry into the cell cycle requires excitation by C3dg-bound Ag in the presence of both IL-4 and BAFF. Although the BCR signal need not be prolonged under these conditions (42), IL-4- and BAFF-induced signals are necessary throughout the first cell cycle. Thereafter, the influence of IL-4 wanes, while that of BAFF is sustained (schematic in Fig. 11). During this orchestrated response, quantitative, qualitative, and temporal changes in cell surface and intracellular molecules take place which regulate both B cell cycle progression and viability at onset of the response and throughout the replication process.

The BCR signal provides the major eliciting stimulus; it enhances B cell viability, regulates the cell cycle, and influences receptivity to IL-4 and BAFF. C3dg-dependent recruitment of the CD21/CD19 costimulatory complex amplifies the above functions to differing degrees. Consistent with the low BCR engagement threshold for sustaining resting B cell viability (45, 59), recruitment of CD21/CD19 only slightly improves B cell viability (42, 45), likely via elevating Bcl-2 (40, 60, 61). In contrast, as suggested from the notable effect of BCR:CD21 coligation on B cell S phase entry (22, 45), the latter likely influences the expression of at least some cell cycle proteins. Finally, engagement of CD21/CD19 can reduce B cell expression of molecules that negatively regulate the BCR signaling process (62), raising still another contribution of innate immune system-derived C3.

IL-4, the second innate immune system player in the synergistic response, is well known to have pleotropic effects on B cell activation. These include down-regulation of molecules that inhibit BCR signaling (63), modulation of cell cycle proteins (64–66), and up-regulation of antiapoptotic molecules, Bcl-xL and Mcl-1 (67). It is likely that the dependence on IL-4 during the first 2 days of activation in part reflects the effectiveness of this cytokine at
boosting early expression of Bcl-xL and McI-1 (67) (Fig. 10), as well as IL-4–triggered increases in cyclin D2, cyclin E, and associated cdk kinases, and decreased p27Kip1 (64–66). Importantly, the BCR signaling pathway is less effective at inducing many of the latter cell cycle–promoting events (64, 65, 68). Furthermore, although BAFF up-regulates both cyclin D2 and cdk4, it does not affect cyclin E or p21Kip1 (47). Thus, synergy between IL-4, BAFF, and BCR:CD21 engagement in promoting early cell cycle progression likely reflects both cooperation and complementation: maximal augmentation of those cell cycle proteins that are limiting (but commonly activated via each receptor pathway); and compensation for those ineffectively activated via any one pathway.

Despite the importance of IL-4 early in activation, the positive effects of this cytokine decline after day 3. This phenomenon is consistent with past studies showing that activated human B cells are poorly responsive to IL-4 (69, 70) and may reflect lower IL-4Rα expression (71) and/or negative regulatory effects of IL-4 (72, 73) during the clonal expansion phase.

As IL-4 loses importance, BAFF assumes the primary role for sustaining the viability of progeny in this TI response. This was evidenced by: 1) lesser apoptosis of daughter cells in BCR:CD21 plus IL-4–stimulated cultures that also contain BAFF; 2) increased frequency of apoptotic daughter cells upon ablation of BAFF function late in culture; 3) more sustained expression of the antiapoptotic molecule, McI-1, within daughter cells in cultures containing both cytokines. Although other recent studies showed that BCMA–induced McI-1 is responsible for BAFF–mediated survival of plasmablasts and plasma cells (46, 74), a similar, but nonidentical, mechanism is involved here. Firstly, the daughter cells affected by BAFF did not have surface Ag characteristics of cells of the plasma cell lineage (Fig. 3), nor did the actively replicating cultures show evidence of a decline in PAX-5, a B cell lineage transcription factor down-regulated upon plasma myelocyte differentiation (44) (P. Mongini, unpublished immunoblotting results). Additionally, at day 5, only a small subpopulation of viable daughter cells express BCMA (P. Mongini, unpublished results). Thus, BAFF appears to positively influence the viability of replicating human lymphoblasts before their transition into the plasma cell differentiation pathway via a mechanism that involves McI-1, but likely not BCMA.

Somewhat unexpectedly, BAFF failed to promote the viability of daughter cells under certain culture conditions. When IL-4 was absent and B cells received a stronger signal via high affinity BCR:CD21 coengagement, the presence of BAFF was not associated with increased viability (Fig. 7B). This may reflect two factors: 1) BCR:CD21 coligation promotes the expression of TACI, a Janus-like BCR receptor with inhibitory, as well as stimulatory, functions (50, 51); 2) IL-4 dampens this induced expression of TACI (Fig. 8B). It is possible that the outcome of TACI signaling is strongly influenced by the density of TACI and that, without IL-4, the TACI–mediated inhibitory pathway predominates.

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Disclosures

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