Resting Human Memory B Cells Are Intrinsically Programmed for Enhanced Survival and Responsiveness to Diverse Stimuli Compared to Naive B Cells[1]

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Enhanced secondary Ab responses are a vital component of adaptive immunity, yet little is understood about the intrinsic and extrinsic regulators of naive and memory B cells that result in differences in their responses to Ag. Microarray analysis, together with surface and intracellular phenotyping, revealed that memory B cells have increased expression of members of the TNF receptor, SLAM (signaling lymphocytic activation molecule), B7, and Bcl2 families, as well as the TLR-related molecule CD180 (RP105). Accordingly, memory B cells exhibited enhanced survival, proliferation, and Ig secretion, and they entered division more rapidly than did naive B cells in response to both T cell-dependent and T cell-independent stimuli. Furthermore, both IgM and isotype-switched memory B cells, but not naive B cells, costimulated $CD4^+$ T cells in vitro through a mechanism dependent on their constitutive expression of CD80 and CD86. This study demonstrates that up-regulation of genes involved in activation, costimulation, and survival provides memory B cells with a unique ability to produce enhanced immune responses and contributes to the maintenance of the memory B cell pool. ***The Journal of Immunology,*** **2009, 182: 890–901.**

The pool of Ag-specific memory B cells generated during a primary immune response is a major contributor to the efficiency of secondary responses (1–4). It could be postulated that secondary Ab responses are qualitatively and quantitatively superior to primary responses simply because an increased number of Ag-specific, affinity-matured memory B cells exist that dominate the secondary response, thus facilitating a faster reaction time to foreign Ag. Alternatively, memory B cells may have properties imprinted within their cellular composition that are absent or present from naive B cells, and it is these properties that endow memory B cells with the ability to produce an accelerated and more robust response than naive B cells.

To understand the regulatory network underpinning the cellular dynamics of primary and secondary humoral responses, differences in gene expression, phenotype, and responses to stimuli between naive and memory B cells have begun to be elucidated. This has been performed using both murine (5–9) and human (10–19) systems. These studies have identified key differences between naive and memory B cells that allow memory cells to respond more efficiently than naive cells. Human memory B cells show superior proliferative capabilities and produce more Ig-secreting cells (ISCs)[4] than do naive B cells in response to diverse stimuli (10, 11, 14, 15, 17–19). As such, naive B cells require either more signals or a greater signal intensity in order to elicit a proliferative response that approximates that of memory B cells induced by fewer or weaker signals (11–15, 17, 18). This is likely due to intrinsic differences between naive and memory B cells. Indeed, gene expression analysis has revealed that resting memory B cells have down-regulated cell cycle regulators, including members of the Krüppel-like factor family, which endows them with the ability to enter division quickly, thus facilitating enhanced secondary responses (18, 20). However, there are many facets to such responses that remain to be elucidated. These include the generation, maintenance, and survival of B cell subsets, recruitment of T cell help, and terminal differentiation of B cells into plasma cells (PCs). Furthermore, recent discussion on the developmental history of IgM^+CD27^+ B cells as to whether they can be classified as "memory" B cells (21) compelled us to compare in depth the genotype and phenotype of this B cell population to the classically defined population of isotype-switched memory B cells. Therefore, global gene expression analysis and cellular assays were utilized to identify mechanisms that contribute to qualitative differences between primary and secondary humoral responses. All memory B cells were found to have high expression of activation and prosurvival molecules, consistent with their ability to respond quickly during an immune response, and for the memory B cell pool to persist for the lifetime of the host.

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[4] Abbreviations used in this paper: ISC, Ig-secreting cell; APRIL, a proliferation-inducing ligand; BAFF, B cell-activating factor of the TNF family; BCMA, B cell maturation Ag; CRACC, CD2-like receptor-activating cytotoxic cells; GC, germinal center; MNC, mononuclear cell; PB, peripheral blood; PC, plasma cell; PD, programmed death; SA, streptavidin; SLAM, signaling lymphocytic activation molecule; TACI, transmembrane activator and calcium modulator ligand interactor; TNFSF, TNF superfamily; TNFRSF, TNF receptor superfamily; ttfd, time to first division.

Materials and Methods

Abs and reagents

The following Abs were used in this study: PE-anti-CD27, PE-anti-Bcl-2, PE- and biotinylated-anti-IgM, -IgD, -IgA and -IgG mAbs, streptavidin (SA) conjugated to PerCp (SA-PerCp), FITC-anti-CD48 mAb, PE-anti-CD80, -CD84, -CD86 and TACI (transmembrane activator and calcium modulator ligand interactor) mAbs (BD Pharmingen); FITC-anti-CD20 and allophycocyanin-conjugated anti-CD38 mAb (BD Biosciences); PE-anti-CD38, SA conjugated to Tricolor (SA-TC; Caltag Laborabories); PE-anti-NTB-A, CD229 (Ly9), and CRACC (CD2-like receptor-activating cytotoxic cells) mAbs, biotinylated anti-BCMA (B cell maturation Ag) mAb, and purified anti-CD80 mAb (R&D Systems); PE-anti-BAFF (B cell-activating factor of the TNF family)-R, anti-TLR9, and anti-RP105 (CD180) mAb, biotinylated anti-signaling lymphocytic activation molecule (SLAM) mAb, biotinylated and allophycocyanin-anti-CD27 mAb, and purified anti-CD86 mAb (eBioscience). CFSE, SA-Alexa 594, and SA-Alexa 647 were from Molecular Probes; BAFF was from PeproTech; APRIL (a proliferation-inducing ligand) was from R&D Systems; DAPI (4′,6-diamidino-2-phenylindole) and demecolcine were from Sigma-Aldrich. Membranes of insect cells infected with baculovirus expressing recombinant human CD40L were prepared by Dr. Marylin Kehry (Boehringer Ingelheim) or Dr. Grant Shoebridge and Nathan Hare (Centenary Institute).

Isolation of human B cells

Human spleens were obtained from cadaveric organ donors and were received and processed as previously described (22) ~12–18 h following splenectomy (Australian Red Cross Blood Service, Sydney, Australia). Institutional human ethics review committees approved all studies. Total B cells were isolated and then fractionated into subsets of naive, IgM memory, and isotype-switched memory cells according to their differential expression of CD27 and Ig isotypes as described (14, 17). For molecular analyses, naive ($CD20^+CD27^-IgG^-/IgA^-/IgE^-$), IgM memory ($CD20^+CD27^+IgG^-/IgA^-/IgE^-$), and switched memory B cells ($CD20^+CD27^+IgG^+/IgA^+/IgE^+$) were simultaneously identified and isolated following labeling with mAb to CD20, CD27, and IgG/IgA/IgE. For functional experiments, subsets of memory B cells were purified by labeling with anti-CD20 and anti-CD27 mAb together with mAbs to IgM and IgD or IgG, IgA, and IgE to identify switched memory B cells and IgM^+, respectively (14, 17). This strategy of negative isolation of memory subsets avoided cross-linking the BCR. While this sorting strategy would potentially include transitional B cells ($CD20^+CD10^+CD27^-$) in the naive cell gate and germinal center (GC) B cells ($CD20^+CD27^+CD38^{high}$) in the memory cell gates, transitional and GC B cells are unlikely to represent a significant proportion of the resulting naive and memory cell populations because both transitional and GC B cells comprise <2% of human splenic B cells (23). Furthermore, expression of genes that are highly expressed by transitional (*CD5*, *CD10*) (23) and GC (*CD10*, *AICDA*, *Bcl-6*) (16) B cells were weakly (if at all) expressed by sort-purified naive, IgM memory, and switched memory B cell populations (not shown), confirming that the data generated by sorting and analyzing $CD20^+CD27^-IgG/IgA/IgE^-$ cells as naive B cells and $CD20^+CD27^+IgG/IgA/IgE^{+/-}$ cells as IgM memory and switched memory B cells is unlikely to have been influenced by the presence of any residual transitional or GC B cells that may have been purified with these cells. PCs were isolated by sorting $CD20^{low}CD38^{high}$ cells from splenic mononuclear cells (MNCs) (24). Cells were sorted on a FACStar Plus, FACSVantage, or FACSAria flow cytometer (BD Biosciences). Gating strategies excluded doublets. The postsort purity for each population was typically >98%.

Gene expression analysis

RNA was isolated from each B cell population (RNeasy total RNA isolation kit, Qiagen). Complementary RNA was synthesized and amplified using biotin-labeled ribonucleotides and T7 RNA polymerase (25, 26). Biotin-labeled cRNA was then hybridized to human genome U133A and B GeneChips (Affymetrix), which contain >44,000 probes sets representing ~39,000 transcripts. Resulting data were analyzed by Affymetrix Microarray Suite software (26–29). In-depth analyses and clustering of data were conducted using GeneSpring software (Agilent). After data transformation (to convert any negative value to 0.01), normalization was performed using a per-chip 50th percentile normalization and per-gene median normalization method. Genes that were consistently absent or below the noise level were excluded from analysis. To identify genes with statistically significant differences between B cell subsets, one-way ANOVA with a p value cutoff of 0.05 and the Benjamini and Hochberg false discovery rate as a multiple testing correction were performed. The Student-Newman-Keuls post hoc test was used to identify the specific groups in which significant differential expression occurred. Hierarchical clustering was performed on both genes and individual experiments, with Pearson correlation as a measure of similarity to group genes and samples with similar expression patterns. Data points were arranged in a hierarchy and were displayed in a phylogenetic tree of clusters of genes in a hierarchically ordered relationship. Branch lengths represent the degree of similarity between sets. Gene expression profiles that were similar across the experimental samples were clustered together. Heat maps were generated using Spotfire DecisionSite software (26–29). The signal number of a gene that generated an absent call was converted to 0 for clarity of display. Gene expression profiles of cell populations were performed in duplicate from different donors. Detailed descriptions of each microarray experiment are provided at www.ncbi.nlm.nih.gov/geo/ (accession no. GSE13411).

Immunofluorescence staining

MNCs were preincubated with mouse IgG (100 μg/ml) and then labeled with anti-CD20, anti-CD27, and a combination of Abs to IgG/IgA/IgE to resolve naive, IgM memory, and switched memory B cells. In some experiments, MNCs were stained with anti-CD38 and anti-CD20 mAb to detect PCs ($CD38^{+/+}CD20^{+/-}$). The cells were also labeled with a mAb to the protein of interest or control mAb and then fixed in 1% formaldehyde. To detect expression of intracellular proteins, cells were initially fixed in 2% formaldehyde for 20 min at room temperature and permeabilized in PBS-0.13% Tween 20 for a minimum of 16 h in the dark at room temperature. Afterward, the permeabilized cells were washed and stained as above. Samples were acquired on a FACSCalibur (BD Biosciences) and analyzed using FlowJo (Tree Star).

In vitro culture of human B cells to assess survival, proliferation, time to first division, and Ig secretion

Sort-purified B cell subsets ($20–50 \times 10^3$ cells/well) were cultured in B cell media (15) containing Normocin in 96-well round-bottom plates. To assess survival and/or proliferation, B cells were stimulated with combinations of CD40L alone, anti-CD180 (RP105) mAb (5 μg/ml, generously provided by Dr. Kensuke Miyake, Univeristy of Tokyo, Japan), the TLR9 ligand CpG2006 (Sigma-Aldrich/Proligo; 1 μg/ml) (13), BAFF (500 ng/ml) or APRIL (500 ng/ml). After 4 days, the cultures were pulsed with [^{3}H]thymidine, and its incorporation by proliferating B cells was determined 8–18 h later. Alternatively, a known number of CaliBRITE beads (BD Biosciences) were added to each well; the cells were then harvested and fixed in 1% formaldehyde. Data were collected on a FACSCalibur. The division history and absolute number of cells were determined by analyzing CFSE profiles and calculating the ratio of live cells to CaliBRITE beads. To determine the time to first division (ttfd), the mitotic inhibitor demecolcine (10 ng/ml), which allows cells to enter their initial S phase but blocks all subsequent rounds of division, was added to the cultures (14, 30). [^{3}H]thymidine incorporation (1 μCi/ml per well; ICN Biomedicals) was measured during either a 4-h pulse every 24 h, over a 6-day time period for the ttfd assays, or for the final 8–14 h of a 4-day culture period for proliferation assays performed in the absence of demecolcine. Cells were harvested and log-normal distributions fitted to the data for ttfd using Prism software (GraphPad Software) (14). To measure the effect of different stimuli on B cell differentiation, purified B cells were cultured with CpG 2006 alone or in the presence of CD40L, BAFF, and/or APRIL. After 10 days, culture supernatants were harvested and levels of secreted IgM, IgG, and IgA determined by Ig H chain-specific ELISA (15).

$CD4^+$ T cell costimulation assay

$CD4^+$ T cells were purified from PBMC obtained from healthy donors (Australian Red Cross Blood Service, Sydney, Australia) using the $CD4^+$ T cell-positive isolation kit (Dynal Biotech) following the manufacturer's instructions. These $CD4^+$ T cells (50×10^3/well) were cultured with increasing numbers of sort-purified and irradiated (4000 rad) splenic allogeneic naive, IgM^+ memory, or isotype class-switched memory B cells. After 5 days, T cell proliferation was measured by incorporation of [^{3}H]thymidine (1μCi/well) during the final 10 h of culture. The cells were harvested, scintillation counting was performed, and proliferation was measured. These cocultures were also performed in the presence of blocking mAb to CD80 and/or CD86 (10 μg/ml).

Results

Differences in the levels of expression of receptors and ligands may either enhance proliferation of B cells and stimulation of helper T cells or inhibit activation and in some cases induce apoptosis. Therefore, the expressions of members of various families

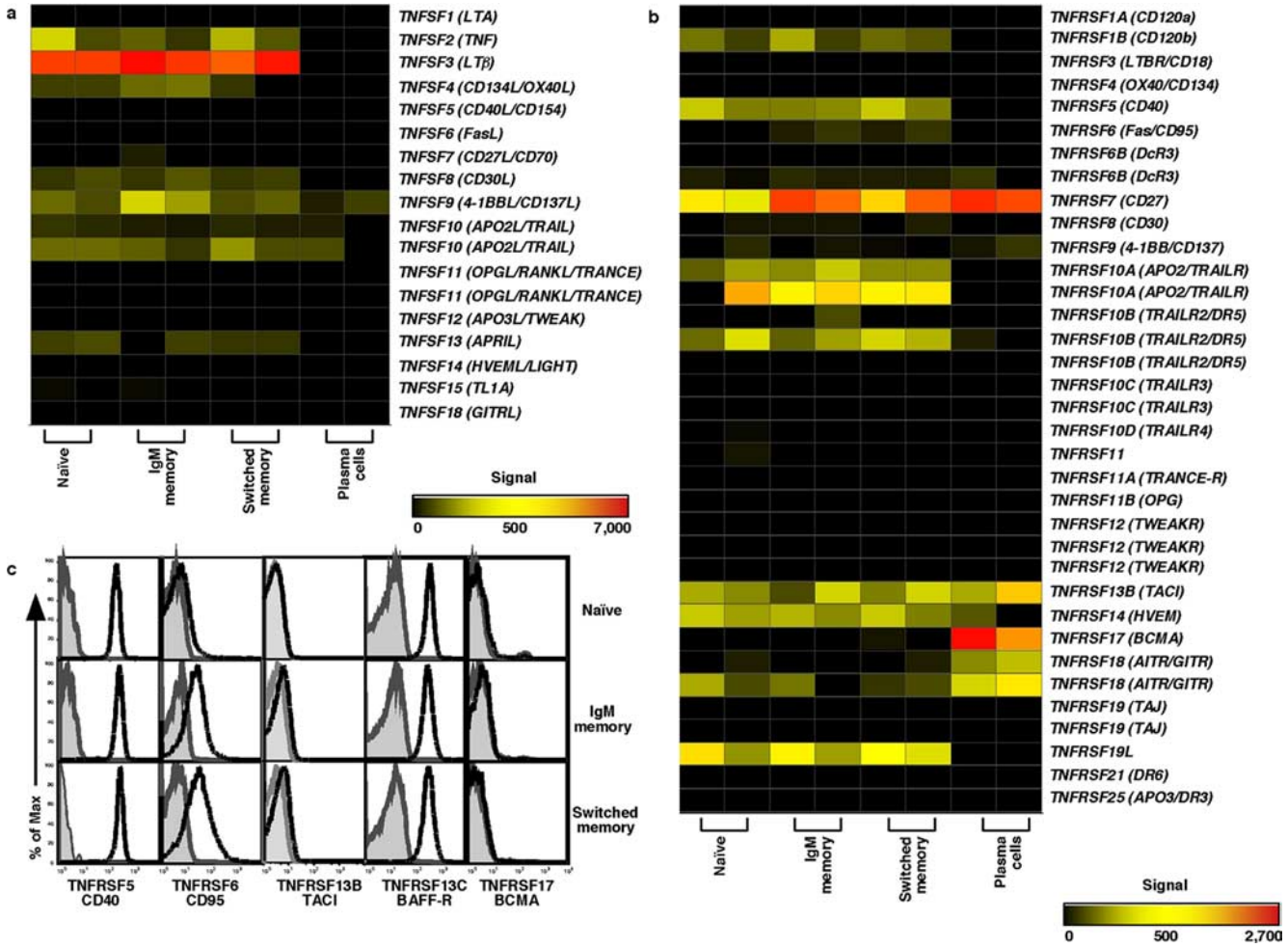

FIGURE 1. Expression of TNFSF and TNFRSF members by B cell subsets. *a* and *b*, Microarray expression profiles of (*a*) TNFSF and (*b*) TNFRSF members in naive B cells, IgM memory B cells, switched memory B cells, and PCs. The signal intensity was plotted from 0 (denoted as black squares), to 500 (yellow squares), to the high expression of either 7000 (*a*, TNFSF; red squares) or 2700 (TNFRSF; red squares). *c*, Human splenic MNCs were stained with mAb to CD20, CD27, and Ig isotypes to identify B cell subsets, together with mAbs to to TNFRSF5 (CD40), TNFRSF6 (CD95), TNFRSF13B (TACI), TNFRSF13C (BAFF-R), or TNFRSF17 (BCMA) (open histogram), or an isotype control (filled histogram).

of molecules known to have important roles in directing immune responses were investigated in human splenic B cells at different stages of immunopoiesis (i.e., naive, IgM memory, Ig isotype-switched memory, and PCs) using microarrays, flow cytometry, and functional assays. Overall, naive and memory B cell subsets had very similar expression patterns (supplemental Figs. 1 and 2),[5] confirming results from other studies (7, 9, 16). However, the gene expression profiles of PCs differed substantially from those of naive and memory B cells, with more than a thousand genes differentially expressed (supplemental Fig. 1). This is due to the presence of various genes involved in the production and secretion of Abs and the subsequent cellular physiology required to accommodate this function (31). The following *Results* sections detail differences in expression and function of families of molecules on naive and memory B cells that are involved in regulating lymphocyte activation, differentiation, and survival.

Increased expression of members of the TNFR superfamily (TNFSF) of molecules on memory B cells

The TNFSF of ligands and their receptors (TNFRSF) are intricately involved in regulating B cell survival, proliferation, and differentiation. They also allow B cells to mediate activation of hematopoietic and nonhematopoietic cells, such as $CD4^+$ T cells and follicular dendritic cells, respectively (32). From this family of ligands, *TNFSF2* (*TNF*) and *TNFSF3* (*lymphotoxin β*) were expressed highly in both naive and memory B cells; however, their expression was extinguished in PCs (Fig. 1*a*). *TNFSF4* (*OX40L*), *TNFSF8 (CD30L*), *TNFSF9* (*4-1BBL*), *TNFSF10* (*TRAIL*), and *TNFSF13* (*APRIL*) were also expressed by naive and memory B cells. There was a slight elevation of *OX40L* and *4-1BBL* in IgM memory B cells (~2-fold higher). Interestingly, PCs appeared to lack expression of most TNFSF members, at least at the transcriptional level (Fig. 1*a*).

There was a more distinct pattern of expression of members of the TNFRSF by human B cell subsets (Fig. 1*b*). Importantly, CD27 was more highly expressed in memory B cells relative to naive B cells, demonstrating the accuracy and reliability of the sorting strategy and the GeneChips. Expression of *CD95* and *TACI* was at least 2-fold higher on memory B cells than on naive B cells (Fig. 1, *b* and *c*), consistent with gene expression profiling of B cell subsets isolated from human tonsils (16). Furthermore, naive and memory B cells both expressed high levels of BAFF-R (Fig. 1*b*) (33) when assessed by flow cytometry, while expression of BAFF-R and TACI were down-regulated on PCs (see Ref. 34).

[5] The online version of this article contains supplemental material.

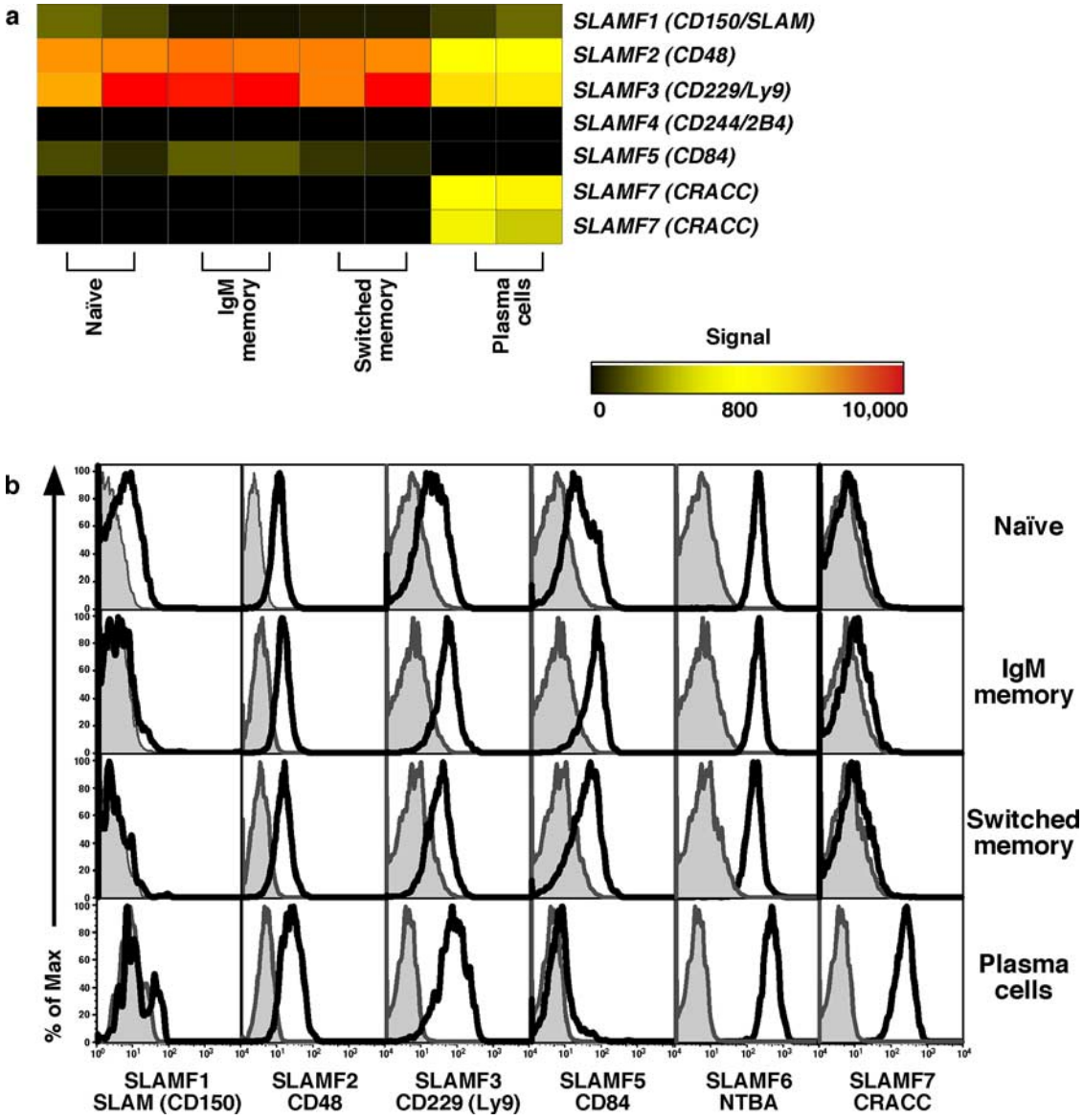

FIGURE 2. Expression of SLAM family members by B cell subsets. *a*, Microarray expression profiles of SLAM family members in naive B cells, IgM memory B cells, switched memory B cells, and PCs. The signal intensity was plotted from 0 (black squares), to 800 (yellow squares), to the high expression of 10,000 (red squares). *b*, Human splenic MNCs were stained with mAb to CD20, CD27, and Ig isotypes to identify naive, IgM memory, and switched memory B cells and with mAb to CD20 and CD38 to identify PCs, together with mAbs to either SLAM, CD48, CD229 (Ly9), NTBA, CD84, CRACC (open histogram), or an isotype control (filled histogram).

BCMA and *TNFRSF18* (*GITR*) were expressed in PCs but not in naive or memory B cells (Fig. 1*b*). Despite this, and consistent with previous findings (33, 34), splenic PCs did not express BCMA on their surface (Fig. 1*c*). While expression of *CD27* was increased in PCs (Fig. 1*b*), other TNFRSF members such as *CD40* and *CD95* were not, despite clear expression of these proteins on the surface of PCs (24).

Naive, memory, and plasma cells exhibit distinct expression patterns of the SLAM family of immunomodulators

The SLAM family of receptors includes CD150 (SLAM), CD84, CD229 (Ly9), CD244 (2B4), NTB-A, and CRACC/CS-1. All of these receptors recruit the cytoplasmic adaptor protein SAP and are involved in regulating leukocyte adhesion and activation (reviewed in Ref. 35). With the exception of CD244, which interacts with the related molecule CD48, all other SLAM family molecules are self ligands. Studies have focused on the role of the SLAM family in the activation of T and NK cells. However, since SAP-dependent signaling is required for the generation and maintenance of long-term humoral immunity (35), it is important to determine the expression of these molecules on human B cells.

All SLAM family members, except *CD244*, were expressed by at least one B cell subset (Fig. 2*a*). Both *CD48* and *CD229* were highly expressed in naive and memory B cells, but down-regulated in PCs (Fig. 2*a*). At the protein level, naive B cells, memory B cells, and PCs expressed comparable levels of CD48, while the expression pattern of CD229 resembled that observed at the transcript level, with expression being greatest on the memory B cell subsets (Fig. 2*b*). In contrast, CRACC was only expressed by PCs. Interestingly, SLAM and CD84 were differentially expressed among splenic B cell subsets, in that SLAM was highest on naive B cells and weakly expressed by PCs yet absent from memory B cells, while CD84 was higher on IgM memory B cells than on naive and switched memory B cells, and it was absent from PCs at

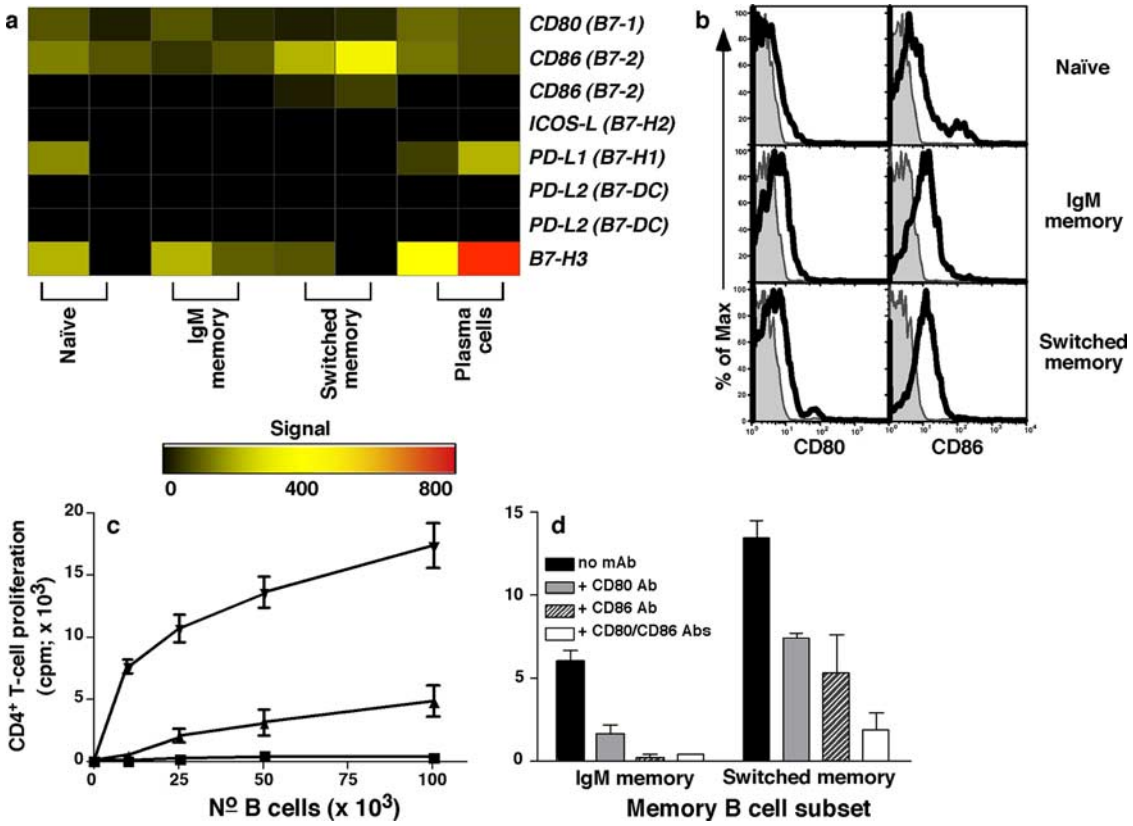

FIGURE 3. Memory, but not naive, B cells constitutively express CD80 and CD86 costimulatory molecules and potently stimulate allogeneic $CD4^+$ T cells. *a*, Microarray expression profiles of the B7 family members in naive B cells, IgM memory B cells, switched memory B cells, and PCs. The signal intensity was plotted from 0 (black squares), to 400 (yellow squares), to the high expression of 800 (red squares). *b*, Splenic MNCs were stained with anti-CD20 and anti-CD27 mAbs and a cocktail of Abs to IgG/IgA/IgE to identify B cell subsets. The cells were also stained with anti-CD80 or anti-CD86 (open histogram) mAbs or an isotype control (filled histogram) and analyzed on a FACSCalibur. *c*, Allogenic $CD4^+$ T cells (50×10^3) were isolated from PB by positive selection with CD4 Dyna beads and cultured alone, or with increasing numbers of sort-purified and irradiated splenic naive (■), IgM memory (▲), and switched memory B cells (▼). After 5 days, the cultures were pulsed with [^{3}H]thymidine (1μCi/well) for the final 10 h. *d*, PB $CD4^+$ T cells and memory B cell subsets were isolated and cultured as for *c* in the absence or presence of blocking mAb to CD80 (10 μg/ml), CD86 (10 μg/ml), or both. Proliferation was assessed by measuring incorporation of [^{3}H]thymidine during the final 10 h of culture.

both the transcript (Fig. 2*a*) and protein (Fig. 2*b*) levels. Although a probe for *NTBA* (*SLAMF6*) was not present on the microarray chips used in this study, similar levels of this receptor were detected on all subsets of splenic B cells when assessed by flow cytometry (Fig. 2*b*). Therefore, the differential expression of SLAM family members on B cell subsets may provide them with different abilities to communicate and receive help from other immune cells that also express these self ligands.

IgM and switched memory, but not naive, B cells constitutively express B7 molecules, which underlies their ability to activate $CD4^+$ T cells

The B7 and CD28 families have important roles in the collaboration between APCs and T cells. B7 family members expressed by B cell subsets bind their corresponding CD28 family members on T cells and NK cells. Since B7/CD28 family members have both stimulatory and inhibitory effects on lymphocytes (36), the expression pattern of these families during B cell differentiation was investigated. *CD80* and *CD86* were expressed by mature B cells (Fig. 3*a*). Although there was little difference in expression of *CD80* transcripts between subsets (Fig. 3*a*), switched memory B cells had a higher level of CD80 on their surface than did IgM memory B cells, while expression on naive B cells was only slightly above background (Fig. 3*b*). This confirms previous reports that examined naive and total memory B cells in human and murine spleens and human tonsils (8, 16, 24, 37, 38). CD86 was more highly expressed on memory B cell subsets compared with naive B cells (Fig. 3, *a* and *b*). *ICOS-L* transcript was not expressed in any of the subsets (Fig. 3*a*), although ICOS-L has been reported to be constitutively expressed on naive B cells, but down-regulated on memory B cells (36). Although programmed death (*PD*)-*L2* was not expressed in any of the human B cell subsets examined, *PD-L1* was detected in PCs, while *B7-H3* was present in all B cell subsets, with expression being greatest in PCs (Fig. 3*a*). While a role for B7-H3 in B cells has not yet been reported, these data suggest a possible function of B7-H3 and PD-L1 in PCs. Murine PCs and malignant human PCs have been found to express CD28 (39, 40). However, neither CD28 mRNA (not shown) or protein was detectable on primary PCs, nor naive or memory B cells isolated from human spleens (Ref. 24 and J. I. Ellyard and S. G. Tangye, unpublished observations), consistent with the phenotype of normal PCs present in human tonsil and bone marrow (40).

Memory B cells express higher basal levels of CD80 and CD86 (Fig. 3, *a* and *b*) than do naive B cells, and they up-regulate these molecules more rapidly than do naive B cells following in vitro stimulation (38). While increased expression of B7 molecules on human tonsillar memory B cells (defined as IgD^-CD38^-) allows them to strongly stimulate allogeneic $CD4^+$ T cells (38), it is unknown whether IgM and switched memory B cells have an equivalent ability to stimulate $CD4^+$ T cells. In fact, previous studies

that compared the abilities of B cell subsets to activate allogeneic $CD4^+$ T cells unintentionally excluded IgM^+ memory B cells from this analysis because they would not have been isolated along with the IgD^-CD38^- cells (38). Therefore, the ability of splenic B cell subsets to induce proliferation of $CD4^+$ T cells was determined. Purified allogeneic peripheral blod (PB) $CD4^+$ T cells were cultured either alone or with increasing numbers of irradiated naive, IgM memory, or switched memory B cells. T cell proliferation was measured by determining incorporation of [^{3}H]thymidine during the final 10 h of a 5-day culture. Allogeneic $CD4^+$ T cells proliferated in response to as few as 10×10^3 switched memory B cells, while almost 3-fold more IgM memory B cells were required to achieve the same effect (Fig. 3*c*). Furthermore, even in the presence of the highest number of IgM memory B cells, the response of $CD4^+$ T cells was still less than that invoked by fewer numbers of switched memory B cells (Fig. 3*c*). In contrast to both populations of memory B cells, naive B cells failed to elicit a proliferative response by cocultured $CD4^+$ T cells irrespective of the number of B cells tested (Fig. 3*c*). To determine whether memory B cells costimulated $CD4^+$ T cells through CD80, CD86, or both, IgM memory and switched memory B cells were cultured with allogeneic $CD4^+$ T cells alone or in the presence of neutralizing mAb. Addition of blocking mAb to CD80 reduced T cell proliferation induced by IgM memory and switched memory B cells by 3-fold and ~2-fold, respectively (Fig. 3*d*). Blocking CD86 had a similar effect as neutralizing CD80 on proliferation of T cells that were cultured with switched memory B cells (Fig. 3*d*). When both blocking Abs were added to the cultures, $CD4^+$ T cells failed to respond to stimulation by IgM memory B cells, while proliferation induced by switched memory B cells was reduced a further 2-fold compared with either Ab alone (Fig. 3*d*). Therefore, memory B cells use both CD80 and CD86 to induce T cell proliferation.

Differential expression of TLRs and the TLR-related receptor CD180 on naive and memory B cells

The human TLR family contains 10 members that recognize conserved microbial or viral Ag as a component of the innate immune system (41). An equally important role for TLRs in adaptive immunity and B cell responses has emerged (41). From the microarray profiles, it was found that *TLR9* and *TLR10* were expressed highly in B cells, whereas *TLR1*, *TLR6*, and *TLR7* were only weakly expressed (Fig. 4*a*). These results support findings from studies of total peripheral blood or tonsillar B cells (42–45). *TLR9* and *TLR10* were expressed at slightly higher levels in memory B cells than in naive B cells (Fig. 4*a*), while expression of both of these TLRs was down-regulated in PCs relative to memory B cells (Fig. 4*a*). We also examined expression of CD180 (RP105), a member of the leucine-rich repeat family of molecules with homology to TLR4 (41). Although CD180 was highly expressed by naive and memory B cells, it was 2–5-fold higher on memory cells compared with naive cells (Fig. 4, *b* and *c*). On the other hand, *CD180* was absent from PCs (Fig. 4*b*).

The consequence of constitutively high expression of CD180 on naive and memory B cell subsets was investigated by utilizing a ttfd assay (14, 17, 18). When B cells were cultured with CD40L together with anti-CD180 mAb, the proportion of cells entering division, as revealed by the height of the fitted curve, was increased 1.5- to 2.5-fold for all B cell subsets, compared with CD40L alone (Fig. 4, *d–f*). Costimulating human naive B cells through CD180 accelerated their entry into cell division, as the ttfd compared with that in response to CD40L alone was reduced by ~25% (from 80 to 60 h; Fig. 4*d*). In contrast, the effect of anti-CD180 mAb on the ttfd of CD40L-stimulated memory B cells was less pronounced, reducing the ttfd for IgM memory B cells by ~10%, and having little if any effect on switched memory B cells (Fig. 4, *e* and *f*). In agreement with these findings, when B cell subsets were cultured with CD40L and increasing doses of anti-CD180 mAb, memory B cell subsets proliferated to a greater extent than did naive B cells (Fig. 4*g*), yet naive B cells exhibited the greatest fold increase in proliferation compared with CD40L alone (Fig. 4*h*). It is possible that the inability of anti-CD180 mAb to have as great an effect on the memory B cell response compared with naive B cells reflects the intrinsically robust response of memory B cells. Similar to the results obtained following stimulation of human B cells through CD40L alone or in combination with anti-Ig (17, 18), the response of naive B cells to CD40L plus anti-CD180 mAb resembled that of memory B cells stimulated with CD40L alone.

Memory B cells strongly up-regulate TACI expression in response to TLR9 signaling, which correlates with enhanced proliferation, survival, and Ig secretion in response to TACI ligands

Microarray and flow cytometric analysis revealed greater expression of TACI on memory B cells than on naive B cells (see Fig. 1, *b* and *c*) (46). It has recently been reported that activation of human naive B cells with the TLR9 ligand CpG induces expression of TACI to a level comparable to that on resting memory B cells (47). However, the functional significance of CpG-induced expression of TACI on naive B cells and whether memory B cells respond similarly to CpG stimulation are unknown. To examine this, naive, IgM memory, and switched memory B cells were cultured in vitro in the absence or presence of CpG. Compared with the basal level of expression, TACI was increased on both naive and memory B cells when cultured in the absence of any exogenous stimuli (Fig. 5*a*). However, exposure to CpG led to a further 10- to 20-fold increase in expression, with both IgM memory and switched memory populations exhibiting 5- to 10-fold higher levels of TACI than naive B cells (Fig. 5*a*). CpG also induced low level expression of BCMA on memory, but not naive, B cells (Fig. 5*b*), while expression of BAFF-R was unaffected (data not shown). In contrast to CpG, stimulation with CD40L or anti-Ig, alone or together, had only a small, if any, effect on TACI expression, and using these reagents together with CpG did not further augment the expression level achieved by CpG alone (data not shown). By demonstrating that CpG up-regulates expression of TACI on both naive and memory B cell populations, these findings reveal that this is a general effect on all B cell subsets and is not restricted to naive B cells, as originally detailed by Darce et al. (47).

Memory B cells can undergo proliferation in response to CpG alone, whereas naive B cells seemingly only enter cell division following concomitant engagement of TLR9 and the BcR (12, 13). Since memory B cells exhibit higher basal expression of TLR9 (Fig. 4, *a* and *c*) (12, 13) and TACI (Fig. 1, *b* and *c*) (46, 47) than do naive B cells, and they have greater responses to CpG stimulation with respect to proliferation (12, 13) and TACI up-regulation (Fig. 5*a*), we examined the consequences of engaging BAFF receptors on CpG-stimulated human B cell subsets. While stimulation of naive B cells with CpG alone induced a low amount of proliferation, >50% of both IgM memory and switched memory B cells underwent at least one cell division, as revealed by dilution of CFSE (Fig. 5*c*). Furthermore, CpG improved the recovery of viable naive and memory B cells by ~5-fold and ~25-fold, respectively, compared with cultures of unstimulated B cells (Fig. 5*d*). Addition of BAFF, but not APRIL, increased CpG-induced proliferation of all human B cell subsets, as shown both by the accumulation of cells that had undergone increased rounds of cell division (Fig. 5*c*) and by the recovery of 2- to 3-fold more viable

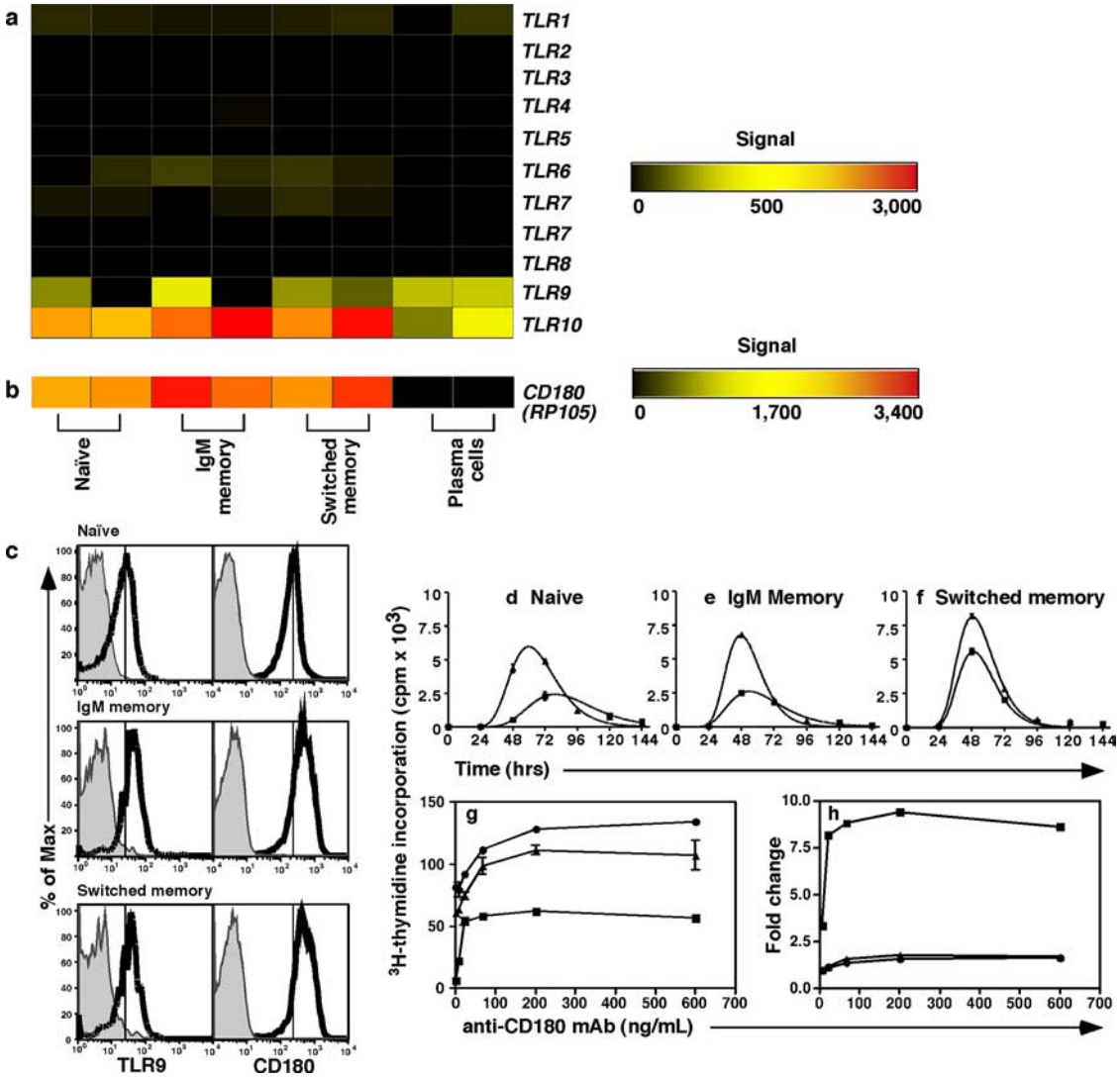

FIGURE 4. Expression of TLRs and CD180 by human B cell subsets. *a* and *b*, Microarray expression profiles of TLRs and CD180 in naive B cells, IgM memory B cells, switched memory B cells, and PCs. The signal intensity was plotted from (*a*) 0 (black squares), to 500 (yellow squares), to the high expression of 3,000 (red squares), or (*b*) 1700 (yellow squares) to 3400 (red squares). *c*, Human splenic MNCs were stained with mAb to CD20, CD27, and Ig isotypes to identify B cell subsets, together with an isotype control (shaded histogram). The cells were also stained with anti-CD180 mAb (open histogram) or fixed and permeabilized and then stained with anti-TLR9 (filled histogram) mAb. *d–f*, Sort-purified (*d*) naive, (*e*) IgM memory, and (*f*) switched memory B cell subsets were cultured with CD40L alone (■) or in combination with anti-CD180 mAb (5 μg/ml; ▲) for 6 days. Every 24 h, proliferation was assessed by the incorporation of [^{3}H]thymidine by the cell subsets during a 4-h pulse. The mitotic inhibitor demecolcine (10 ng/ml) was added at the onset of cultures to measure entry of the cells into their first S phase, and therefore their ttfd. The center of the curve is taken as the mean ttfd, and the height of the curve is proportional to the number of cells entering division. *g* and *h*, Sort-purified naive (■), IgM memory (▲), and switched memory B cell (●) subsets were cultured with CD40L alone or in combination with increasing concentrations of anti-CD180 mAb for 4 days. On the last day of culture, the cells were pulsed with [^{3}H]thymidine for 14 h. The data in *g* represent the mean thymidine incorporation (±SEM) of triplicate samples; *h* represents the fold increase in B cell proliferation mediated by the indicated concentration of anti-CD180 mAb relative to that induced by CD40L alone.

naive and memory B cells from cultures stimulated with CpG plus BAFF, compared with CpG alone, or CpG plus APRIL (Fig. 5*d*). We also examined Ig secretion under these stimulatory conditions. CpG alone induced secretion of detectable amounts of Ig by naive, IgM memory, and switched memory B cells, and this was significantly augmented by costimulation through CD40 (Fig. 5*e*). Both BAFF and APRIL increased Ig secretion induced by CpG alone or CpG/CD40L; however, the effect of BAFF exceeded that of APRIL by 2- to 3-fold (Fig. 5*e*), which parallels the superior effect of BAFF on survival of CpG-stimulated B cells (Fig. 5*d*). Thus, BAFF is capable of strongly increasing proliferation induced by CpG. Furthermore, BAFF, and to a lesser extent APRIL, can also augment Ig secretion by CpG-stimulated B cell subsets. These effects are likely to be mediated, at least in part, through CpG-induced up-regulation of TACI expression.

Elevated expression of the anti-apoptotic molecules Bcl-2, A1, and Mcl-1 by memory B cells correlates with improved survival in vitro

Cell survival is regulated by the interplay between pro- and anti-apoptotic molecules, such as those belonging to the Bcl-2 family (48). Although both anti-apoptotic and pro-apoptotic proteins were expressed in B cell subsets, only the anti-apoptotic proteins exhibited different expression patterns in naive and memory B cells (Fig. 6, *a* and *b*). *Bcl-2* message and protein were increased in both memory B cell subsets compared with naive B cells (Fig. 6, *a* and

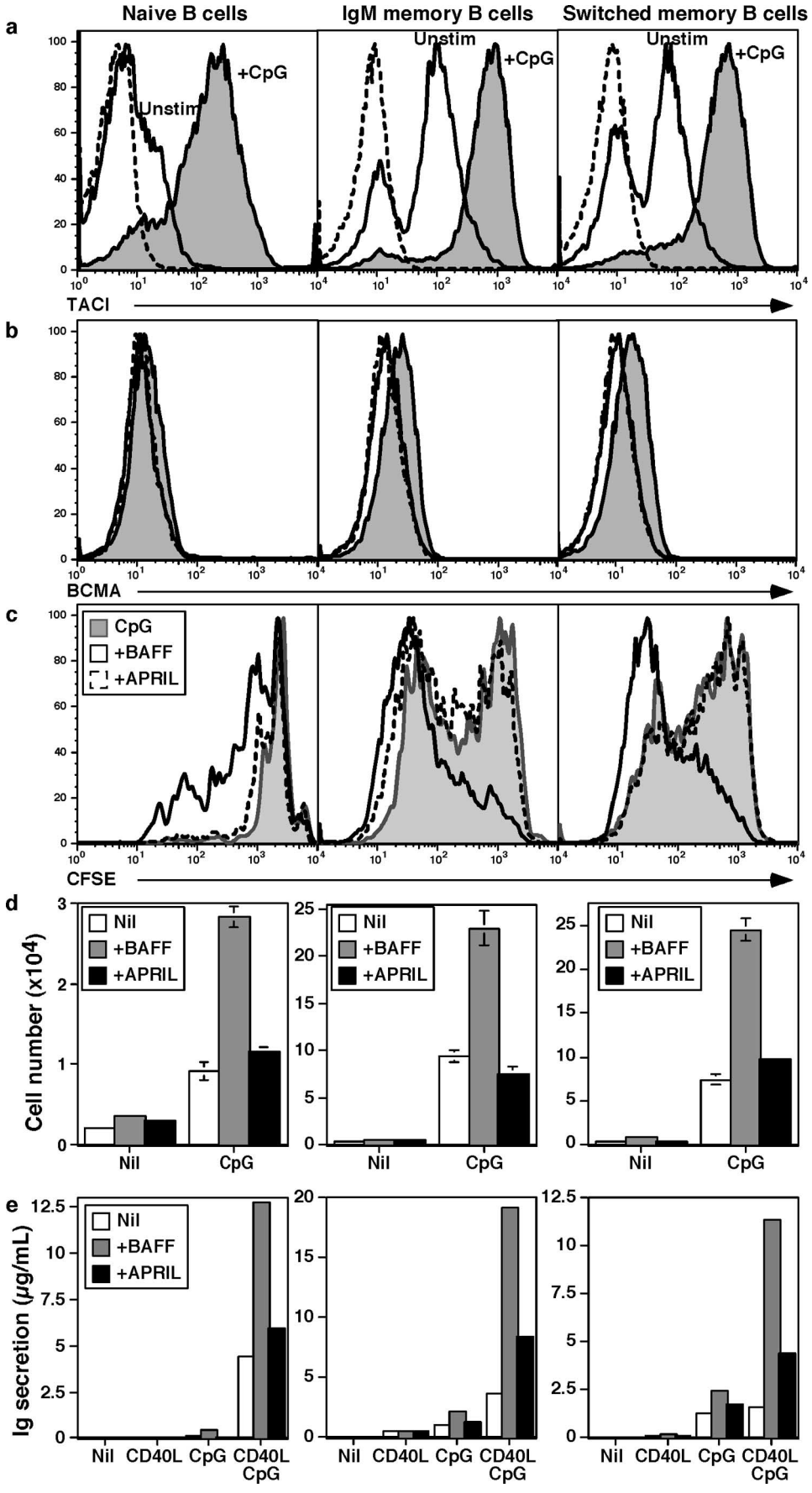

FIGURE 5. TLR9-mediated stimulation of memory B cells strongly up-regulates expression of TACI, improving their response to BAFF. *a* and *b*, Naive, IgM memory, and switched memory B cell subsets were cultured in the absence (open, solid black line histogram) or presence of CpG (filled histogram) for 48 h. After this time, expression of (*a*) TACI and (*b*) BCMA was determined. The dashed line histograms represent fluorescence of cells incubated with isotype control mAb. *c* and *d*, CFSE-labeled naive, IgM memory, and switched memory B cell subsets were cultured with CpG alone (filled gray histograms) or together with BAFF (open, solid black line histogram) or APRIL (open, dashed line histogram). After 4 days, CFSE profiles, indicative of division history (*c*), as well as the number of viable cells (*d*) were determined. These results are the means ± SEM of triplicate cultures and are representative of five independent experiments using cells from different donor spleens. *e*, Naive, IgM memory, and switched memory B cell subsets were cultured in medium alone or with CD40L, CpG, or CD40L/CpG alone or in the presence of BAFF or APRIL. Total Ig secretion (IgM + IgG + IgA) was determined after 10 days. The values represent the mean of triplicate cultures; similar results were obtained in additional independent experiments.

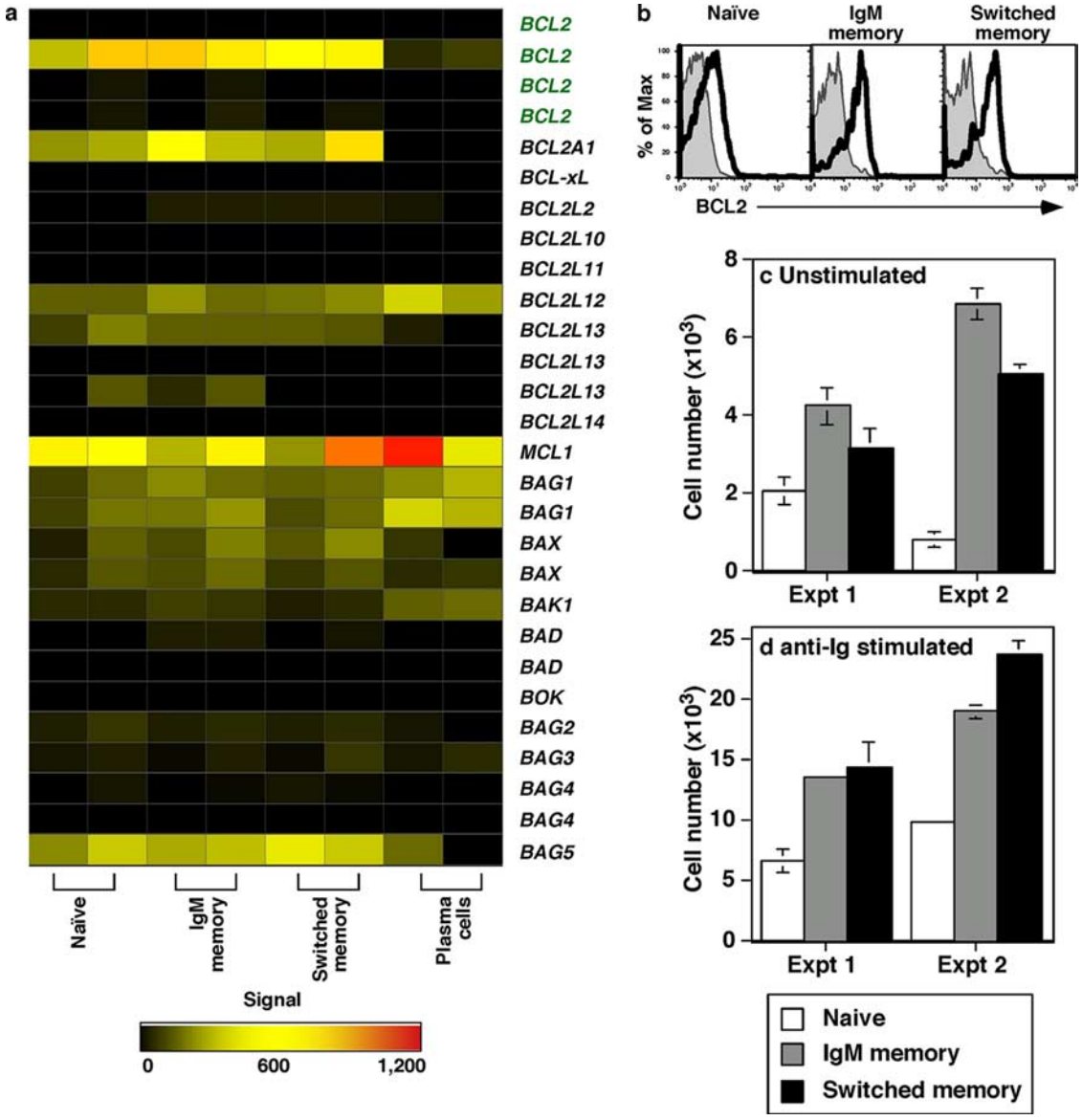

FIGURE 6. Increased expression of Bcl-2 family members by memory B cells correlates with improved survival in vitro. *a*, Microarray expression profiles of the indicated anti- and pro-apoptotic molecules in naive B cells, IgM memory B cells, switched memory B cells, and PCs. The signal intensity was plotted from 0 (black squares), to 600 (yellow squares), to the high expression of 1200 (red squares). *b*, Human splenic MNCs were stained with mAb to CD20, CD27, and Ig isotypes to identify B cell subsets. The cells were then fixed in 2% formaldehyde and permeabilized overnight in PBS-0.13% Tween 20, after which they were stained with anti-Bcl-2 (open histogram) or an isotype control (filled histogram) mAb, and analyzed on a FACSCalibur. *c* and *d*, Sort-purified naive B cells (open columns), IgM memory B cells (gray columns), and Ig isotype-switched memory B cells (black columns) were cultured in vitro (20×10^3/200 μl) in the absence (*c*) or presence of $F(ab')_2$ fragments of anti-human Ig (*d*). The numbers of surviving cells were enumerated after 4 days of culture. The values in *c* and *d* represent the mean $\pm$ SD of triplicate cultures and correspond to experiments performed using B cell subsets isolated from two different donor spleens.

b). Bcl-2 homolog *A1*, which interacts with Bcl-2 and Bcl-x_L to promote cell survival (48), was also increased in memory B cells (Fig. 6*a*). Lastly, *Mcl-1*, which is known to have an important role in survival (48), was increased in switched memory B cells and PCs (Fig. 6*a*).

We examined whether the differential expression of pro-survival genes in human B cell subsets altered their survival. Human splenic naive, IgM memory, and isotype-switched memory B cells were cultured in vitro in the absence or presence of $F(ab')_2$ fragments of anti-human Ig. It was found that while ~60–90% of unstimulated cells underwent apoptosis within 4 days of culture, there was consistently more (2- to 5-fold) viable memory B cells recovered than there were of naive B cells (Fig. 6*c*). Engagement of the BCR improved survival of both naive and memory B cells by ~3- to 5-fold (compare Fig. 6, *c* and *d*). Despite this, memory B cells continued to exhibit enhanced survival over naive B cells (Fig. 6*d*). Thus, it is likely that increased expression of Bcl-2, A1, and Mcl-1 in memory B cells contributes to their improved survival in vitro compared with naive B cells.

Discussion

Primary and secondary T cell-dependent humoral immune responses are driven by cognate interactions between Ag-specific naive and memory B cells, respectively, with $CD4^+$ T cells. Stimulation of B cell subsets through different ligand/receptor pairs, as well as subsequent cytokine secretion by activated T cells, directs the fate of responding B cells, whether it be proliferation, differentiation to ISCs, Ig isotype switching, survival, or death. A B cell

population exhibiting increased expression of activating receptors or survival molecules will have an advantage, with regard to the magnitude of a response, over a population with reduced or no expression of these proteins. Memory B cells can also persist for long periods of time in the absence of both cell division and immunizing Ag (6, 49, 50), indicating that they have enhanced survival mechanisms compared with naive B cells, although these mechanisms remain largely unknown (51). To date, there has not been a thorough investigation of the expression of families of genes in naive, IgM memory, and switched memory B cells, as well as in PCs from human spleen, an important site of immune activation. Furthermore, there has been much debate recently about the origin, function, and biology of IgM^+CD27^+ memory B cells (21). Therefore, this study provides a detailed analysis of the expression of molecules that may be involved in regulating the unique behavior of these B cell subsets in vivo.

The gene expression profile of PCs was very distinct to that of naive and memory cells. This is likely due to changes in physiology that take place upon terminal differentiation and their function as ISCs. A number of genes were specifically expressed in splenic PCs, compared with naive and memory B cells, such as *CRACC*, *B7-H3*, *CCR2*, and *CCR10* (Figs. 2 and 3; data not shown). These genes may be utilized to further understand the characteristics of long-term resident PCs in the spleen, as well as provide candidate targets for immunomodulation in autoimmune or malignant conditions. Indeed, humanized mAbs to CRACC have recently been tested in in vivo models as potential therapeutics for the treatment of the PC malignancy multiple myeloma (52, 53). In contrast, only a small number of differences were observed between naive and memory B cells. Thus, it appears that naive and memory B cell responses are controlled by relatively small changes in expression levels of molecules that enhance or inhibit an immune response. The similarities in gene expression are indicative of the fact that naive and memory B cells have a fundamentally similar function in vivo, that is, to respond to foreign Ag by differentiating into PCs; however, the memory B cell response is heightened as a result of Ag-induced modifications in cellular physiology (18).

Members of the TNF/TNFR (32), SLAM (35), and B7/CD28 (36) families provide or receive signals that instruct B and T lymphocyte responses. The gene array profiles revealed significant differences in expression of numerous molecules involved in T/B cell interactions. For example, *4–1BBL*, CD27, CD80, CD86, CD84, CD229 (Figs. 1–3) and CD1c (data not shown) (54) were all expressed more highly on memory cells than on naive B cells. This finding would suggest memory B cells have an enhanced ability to stimulate T cells. Accordingly, both IgM and switched memory splenic B cells could induce cocultured allogeneic $CD4^+$ T cell proliferation, while naive B cells did not (Fig. 3*c*). Notably, the effect of IgM memory B cells was ~40% of that of switched memory B cells. Memory B cell-induced $CD4^+$ T cell proliferation was mediated through both CD80 and CD86 (Fig. 3*d*), although blocking CD86 alone on IgM memory B cells was sufficient to abrogate T cell proliferation.

Previous studies have suggested that T cell-independent mechanisms, such as polyclonal stimulation through TLRs, play important roles in regulating B cell responses and maintaining humoral memory (12, 13). For instance, RT-PCR analysis revealed that human memory, but not naive, B cells uniquely express TLRs (12). This led to the proposal that constitutive expression of TLRs provides memory B cells with an advantage over naive B cells in that they can respond to innate stimuli alone without the need for costimulation either in the form of BCR engagement or T cell help (12, 13). We also detected elevated expression of TLR9 and *TLR10* in memory B cells (Fig. 4, *a* and *c*). However, naive B cells also expressed detectable amounts of these TLRs. Taken together with findings by others, who also detected TLR expression by subsets of human B cells (44, 55), it would appear that the original conclusion that naive B cells lack expression of TLRs may not be correct; this likely reflects insensitivities in the assay used earlier (12). Indeed, the finding that CpG improves survival and induces proliferation of both human naive and memory B cells (Fig. 5) (12, 18, 56) is consistent with basal expression of TLR9 by these cells. A striking consequence of engaging TLR9 on both naive and memory B cells was the substantial increase in TACI expression. The physiological significance of this is currently unknown; however, it is likely to increase responsiveness of human B cells to the stimulatory effects of BAFF (which would deliver activating signals through not only BAFF-R but also TACI (and BCMA)) and APRIL (which would bind only TACI (and BCMA)) (57). Since APRIL did not improve CpG-induced B cell proliferation or survival (Fig. 5), it could be inferred that TACI may not contribute to the enhanced responses of human B cells to BAFF. However, APRIL could modestly increase Ig secretion by human activated B cells (Fig. 5). This, together with the greater affinity of the BAFF/TACI vs APRIL/TACI interaction (58), attests to the possibility that TACI may be involved in BAFF-mediated augmentation of CpG-induced responses of human B cells. The concept that BAFF would utilize distinct receptors (i.e., BAFF-R, TACI) to exert divergent biologically outcomes (proliferation/survival vs Ig secretion) is consistent with a recent study in gene-targeted mice where it was found that BAFF improved B cell survival largely through BAFF-R, while induction of Ig secretion predominantly occurred in response to signals delivered downstream of BAFF-R and TACI (59). We attempted to definitively demonstrate that TACI mediates some of the effects of BAFF by using putative blocking mAb to prevent binding of BAFF to either BAFF-R or TACI, or agonsitic anti-BAFF-R and anti-TACI mAbs, to specifically engage these receptors. Unfortunately, neither of these approaches was successful (not shown). One approach that may reveal a role for TACI in the responses of CpG-stimulated human B cells to BAFF may be to examine the behavior of CpG-stimulated naive B cells from patients with common variable immunodeficiency that is associated with loss-of-function mutations in *TACI* (57). This is currently under investigation. Notably, memory B cells exhibit enhanced survival mechanisms in vitro (Fig. 6) and in vivo (50), relative to naive B cells, and they respond better than naive B cells to stimuli that activate the NF-κB pathway (e.g., CD40L, anti-Ig (17, 18)). Thus, increased basal expression of Bcl-2, *A1*, and *Mcl-1* in memory B cells (Fig. 6) (16, 24, 38) may facilitate enhanced survival during activation, independent of TACI up-regulation.

Similar to TLR9 and TLR10, the TLR homolog CD180 was expressed on all B cell subsets, and its expression was higher on memory than on naive cells (Fig. 4, *b* and *c*). Signaling through CD180 in combination with CD40L decreased the time taken to enter division for naive and memory B cells compared with CD40L alone, and it had a more potent effect on naive B cells than on memory B cells (Fig. 4, *d* and *e*). However, memory B cells still entered division earlier, and exhibited a greater response, than did naive B cells. It could be postulated that potent B cell stimulators have a greater effect on naive B cells than on memory B cells because the threshold for activation of memory cells is much less than that required for corresponding naive cells; consequently, memory B cells attain their maximal response more readily following activation with fewer or reduced stimuli than do naive B cells (18). Thus, when additional stimuli are provided to activate B cells, the number of responding memory B cells is increased yet there is little effect on the time required to enter the cell cycle.

In conclusion, our results underscore the important roles played by members of the TNFR, B7, TLR, SLAM, and Bcl-2 families in facilitating the maintenance of the memory B cell pool, as well as the rapid elicitation of memory B cell proliferation and differentiation into ISCs during a secondary humoral response to eliminate Ag substantially more quickly than the primary response. Furthermore, analysis of the transcriptome and function of IgM memory and switched memory B cells indicated that expression of molecules important in directing B cell responses is comparable between these subsets, adding to the evidence that IgM^+CD27^+ B cells are involved in rapid memory responses. Overall, the gene and protein expression profiles and functional assays presented herein have provided insight into the mechanisms by which memory B cells interact with and recruit the help of other immune cells, as well as maintain humoral immunity for extended periods of time. Targeting these pathways may represent strategies to improve or attenuate humoral immune responses in cases of immunodeficiency or autoimmunity.

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Disclosures

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