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T Cell Leukemia/Lymphoma 1 and Galectin-1 Regulate Survival/Cell Death Pathways in Human Naive and IgM+ Memory B Cells through Altering Balances in Bcl-2 Family Proteins

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BCR signaling plays a critical role in purging the self-reactive repertoire, or in rendering it anergic to establish self-tolerance in the periphery. Differences in self-reactivity between human naive and IgM+ memory B cells may reflect distinct mechanisms by which BCR signaling dictates their survival and death. Here we demonstrate that BCR stimulation protected naive B cells from apoptosis with induction of prosurvival Bcl-2 family proteins, Bcl-xL, and Mcl-1, whereas it rather accelerated apoptosis of IgM+ memory B cells by inducing proapoptotic BH3-only protein Bim. We found that BCR-mediated PI3K activation induced the expression of Mcl-1, whereas it inhibited Bim expression in B cells. Phosphorylation of Akt, a downstream molecule of PI3K, was more sustained in naive than IgM+ memory B cells. Abundant expression of T cell leukemia/lymphoma 1 (Tc11), an Akt coactivator, was found in naive B cells, and enforced expression of Tc11 induced a high level of Mcl-1 expression, resulting in prolonged B cell survival. In contrast, Galectin-1 (Gal-1) was abundantly expressed in IgM+ memory B cells, and inhibited Akt phosphorylation, leading to Bim up-regulation. Enforced expression of Gal-1 induced accelerated apoptosis in B cells. These results suggest that a unique set of molecules, Tc11 and Gal-1, defines distinct BCR signaling cascades, dictating survival and death of human naive and IgM+ memory B cells. The Journal of Immunology, 2009, 182: 1490–1499.

Primary human peripheral B cells are made up of heterogeneous subpopulations that include a high proportion of memory B cells compared with those in rodents. Due to the advantage conferred by the usefulness of CD27 as a memory marker in humans, peripheral B cells are divided into at least three distinct subsets: naive (IgM+CD27−), IgM+ memory (IgM+CD27+), and switched memory (IgG+ or IgA+CD27+) B cells (1). Of particular interest are IgM+ memory B cells in that they do not exist in mice and could develop through the novel germinal center-independent pathways and express somatically mutated IgM Abs (2). To date, IgM+ memory B cells have been proposed to be circulating splenic marginal zone (MZ) B cells and to play a critical role in the protection against encapsulated organisms (2, 3).

Although in vivo function of IgM+ memory B cells is becoming evident (4), the molecular mechanisms of activation of this subset remain poorly characterized.

Due to random rearrangements of the subunits of a functional BCR from genomic cassettes, a large proportion of developing human B cells in the bone marrow express self-reactive BCRs, but most of these potentially noxious BCRs are purged from the repertoire at several checkpoints in the bone marrow and the periphery (5). Nevertheless, up to 20% of mature naive B cells in normal peripheral blood still express low-affinity self-reactive BCRs (5). In sharp contrast, IgM+ memory B cells isolated from normal donors are devoid of such self-reactive BCRs (6). These findings suggest a distinct homeostatic control of human naive and IgM+ memory B cells.

BCR transmits the signals that are critical for both the elimination of self-reactive repertoire and the expansion of pathogen-specific repertoire. Upon BCR ligation by Ags, Lyn and Syk protein tyrosine kinases are initially activated. Syk propagates the signal by phosphorylating downstream signaling molecules. This results in activation of key signaling intermediates PI3K and phospholipase C (PLC)γ2. PI3K activates Akt kinase, which is critical for B cell survival (7). PLCγ2 activation leads to the release of intracellular Ca2+ and protein kinase C activation, which in turn cause activation of MAPK family kinases (ERK, JNK, and p38 MAPK) and transcription factors including NF-κB and NF-AT. These outputs subsequently connect with further-downstream molecules responsible for determining B cell fates such as survival, growth, and differentiation.

Abbreviations used in this paper: MZ, marginal zone; Gal-1, galectin-1; Tc11, T cell leukemia/lymphoma 1; PLC, phospholipase C; h, human; EGFP, enhanced green fluorescent protein; CLL, chronic lymphocytic leukemia; BAFF, B cell-activating factor of the TNF family; HDAC, histone deacetylase; TCR, T cell receptor; PI3K, phosphoinositide 3 kinase; MAPK, mitogen-activated protein kinase; TNF, tumor necrosis factor; Mcl-1, myeloid cell leukemia sequence 1; Bcl-2, B cell lymphoma 2; Bcl-xL, Bcl-2 homology domain-containing protein 4; TNF, tumor necrosis factor; BCR, B cell receptor.

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3 Abbreviations used in this paper: MZ, marginal zone; Gal-1, galectin-1; Tc11, T cell leukemia/lymphoma 1; PLC, phospholipase C; h, human; EGFP, enhanced green fluorescent protein.

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GFP: CLL, chronic lymphocytic leukemia; BAFF, B cell-activating factor of the TNF family.

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The Bcl-2-regulated pathway plays a critical role in BCR-induced survival and death (8, 9). The Bcl-2 family proteins fall into three subgroups: the first subgroup including Bcl-2, Bcl-xL, and Mcl-1 inhibits some apoptotic pathways; the second subgroup including Bax and Bak directly induces apoptosis by promoting cytochrome c release from the mitochondria; the third subgroup, called BH3-only proteins, consists of at least eight mammalian proapoptotic proteins and is activated in a stimulus-specific, as well as a cell type-specific, manner. Among Bcl-2 family proteins, a BH3-only protein Bim is particularly important in controlling lymphocyte apoptosis. Bim deficiency causes a substantial expansion of autoreactive B cells leading to autoimmune diseases (10). B cells lacking Bim are refractory to BCR-induced apoptosis (10). Bim preferentially binds anti-apoptotic Mcl-1 (11, 12). Conditional knockout of Mcl-1 causes premature death of immature and mature B cells (12), suggesting a pivotal role of Mcl-1 in B cell survival. Based on these findings, tipping the balance between Mcl-1 and Bim expression may be a critical determinant for B cell survival and death. To date, little is known about how BCR signaling dictates the survival and death of human B cell subsets via the Bcl-2-regulated pathway.

In this study, we demonstrate that BCR stimulation rescued naive B cells from apoptosis with Bcl-xL and Mcl-1 induction, whereas it rather accelerated apoptosis of IgM⁺ memory B cells with Bim induction. Sustained Akt activation in naive but not IgM⁺ memory B cells appears to be critical for reciprocal expression pattern of these Bcl-2 family proteins. Moreover, we demonstrate that T cell leukemia/lymphoma 1 (Tcl1) and galectin-1 (Gal-1), abundantly expressed in naive and IgM⁺ memory B cells, respectively. To play a crucial role in regulating Akt activation, thereby affecting their survival and death via the Bcl-2-regulated pathway.

Materials and Methods

Reagents
PE-Cy5-conjugated mouse anti-human (h) CD3, -hCD4, -hCD8, -hCD11b, -hCD14, -hCD56, and -human glycophorin A mAbs; FITC-conjugated mouse anti-hCD19, -hCD69, -hCD86, -hCD95 mAbs; and PE-conjugated mouse anti-hCD27 mAb were purchased from BD Immunocytochemistry. FITC-conjugated goat anti-hlgM, -h-lgD, -h-lgGl, -h-lgA, rabbit anti-hGal-l sera and recombinant hGal-1 were obtained from MBL. Goat anti-hlgM and IgG1/2/3 IgG (Fab’), fragments were purchased from Jackson Immunoresearch Laboratories. Rabbit anti-human phospho-ZAP70/Syk, anti-human phospho-PLCγ2 (Y1217), anti-human phospho-JNK, anti-human phospho-ERK, anti-human phospho-Akt, anti-human Bim, anti-human Tcl1 sera, and rabbit anti-human phospho-p85/p70 S6K, anti-human phospho-NF-κB p65, and anti-human Bcl-xL mAbs were from Cell Signaling Technology. Mouse anti-β-actin mAb and rabbit anti-human Mcl-1 sera were from Sigma-Aldrich. A P3K inhibitor (Ly294002) was purchased from Calbiochem (EMD Biosciences).

Isolation and culture of B cell subsets

Human PBMCs were separated from buffy coats kindly provided by Fukuoka Red Cross Blood Center (Chikushino, Japan). The buffy coats originate from kind whole blood donations of RBC transfusion by healthy origins. Isolated B cells were from kind whole blood donations of RBC transfusion by healthy origins. The buffy coats were from kind whole blood donations of RBC transfusion by healthy origins. Isolated B cell subsets exhibited >95% viability by flow cytometry (Fig. 1B). Cells were cultured at 1 × 10⁶ cells/ml in a flat-bottom 96-well microtiter plate in complete RPMI 1640 supplemented with 10% FCS. Preliminary experiments showed that trace levels of phosphorylation of BCR signaling molecules are observed in B cell subsets immediately after purification probably due to mechanical stress. The cells were thus rested for a couple of hours and used for further analysis throughout the study. Consistent with a previous study (2), IgM⁺ memory B cells exhibited a slightly higher level of IgM than did naive B cells (Fig. 1C). Absence of surface expression of CD95, CD69, and CD69, representative activation markers, in both subsets before stimulation, suggests that these cells are rested (Fig. 1C).

Expression constructs and transient transfection of human B subsets

 Constructs encoding human Tcl1- or Gal-1-enhanced GFP (EGFP) fusion proteins (pEGFP-Tcl1 or -Gal-1) were generated by inserting sequence encoding the full-length protein into the pEGFP-N3 vector (Clontech). Transient transfections of B cell subsets with pEGFP-Tcl1 or pEGFP-Gal-1 were conducted using the Nucleofector protocol from AMAXA Biosystems. Cells (1 × 10⁶) were suspended in 100 µl of Nucleofector solution with 5 µg of plasmid DNA and then electroporated using program U-15. Cells were transferred to 2.5 ml of medium containing 15% FCS and harvested 24 h after transfection. The transfection efficiency ranges between 20 and 30% for each experiment.

Annexin V staining

After culture, cells (1–2 × 10⁶) were washed twice with PBS and then suspended in 85 µl of binding buffer (MBL). The suspension was supplemented with 10 µl of annexin V-FTTC or -PE (MBL) and 5 µg of propidium iodide or 1 µg of 7-aminocoumarin D was incubated at room temperature for 15 min in the dark. Subsequently, 600 µl of binding buffer were added, and the percentage of early apoptotic cells was measured using flow cytometry.

Mitochondria membrane potential

Assessment of mitochondria membrane potential was performed using Mitotracker Red CMXRs (Invitrogen). Cells were incubated in 50 nM Mitotracker Red at 37°C for 1 h in the dark. Flow cytometric analysis (50,000 events/sample) was performed on FACSCalibur (BD Biosciences). Cell debris was electronically gated out based on the forward scatter. Data were further analyzed using FlowJo software.

Measurement of intracellular free calcium

Cells were washed with RPMI 1640 containing 10% FCS and adjusted at 1 × 10⁶ cells/ml. After incubation at 37°C for 15 min, 1 µg/ml fluo-4-acetoxymethyl ester (Dojindo) was added, and the cells were incubated for a further 30–45 min with resuspension every 15 min. The cells were centrifuged and resuspended in RPMI 1640 at 2 × 10⁶ cells/ml. The cells were stimulated with anti-IgM (20 µg/ml), and the fluorescence intensity of intracellular free fluo 4 was monitored and analyzed using flow cytometry.

Western blot analysis

Unstimulated or stimulated cells (1 × 10⁶) were lysed as described (13). Lysates were then denatured in an equal volume of 2% SDS sample buffer, resolved by a 10% SDS-PAGE gel and electrotransferred to nitrocellulose membranes in non-SDS-containing transfer buffer (25 mM Tris, 0.2 M glycine, 20% methanol, pH 8.5). Western blotting was performed with anti-phospho-Syk (1/2,000), anti-phospho-PLCγ2 (1/2,000), anti-phospho-p85/p70 S6 kinase (1/2,000), anti-phospho-jNK (1/2,000), anti-phospho-ERK (1/2,000), anti-phospho-Akt (1/2,000), anti-phospho-p65 NF-κB (1/1,000), anti-phospho-Bcl-xL (1/2,000), anti-phospho-Mcl-1 (1/2,000), anti-phospho-Bcl-xL (1/2,000), anti-phospho-Mcl-1 (1/2,000), anti-phospho-Gal-1 (1/2,000), and anti-phospho-Akt (1/2,000) followed by horseradish peroxidase-conjugated IgG (Jackson ImmunoResearch Laboratories). Blots were developed with ECL plus kit (Amersham Biosciences). The chemiluminescence intensity was monitored with a laser3000 (FujiFilm) instrument. We quantitated band intensity of the proteins using ImageGauge software (FujiFilm) and normalized their expression in reference to β-actin levels. Using these normalized data, relative expression is subsequently calculated as fold changes in protein expression compared with the controls.

Quantitative real-time PCR

Total RNA was extracted from sorted human B cell subsets using Isogen reagent (Nippon Gene) and treated with DNase I (Invitrogen) to remove contaminating genomic DNA. First-strand cDNA was synthesized using a
QuantiTect reverse transcription kit (Qiagen). Quantitative real-time PCR was conducted in the ABI Prism 7700 Sequence Detector (Applied Biosystems). Reactions were performed in triplicate wells in 96-well plates. TaqMan target mixes for Bim, Bcl-xL, Mcl-1, Tcl1, and Gal-1 were purchased from Applied Biosystems. 18S rRNA was separately amplified in the same plate to be used as an internal control for variances in the amount of cDNA in PCR. Collected data were analyzed with Sequence Detector software (Applied Biosystems). Data were expressed as a fold change in gene expression relative to those from unstimulated naive B cells.

Intracellular flow cytometry

After two washings with PBS containing 1% FCS, $5 \times 10^3$ cells were placed in a 96-well microtiter plate. Cells were resuspended with 50 μl of medium plus 50 μl of fixation buffer (BD Biosciences) and incubated for 10 min at 37°C. After washing again with PBS containing 1% FCS, cells were resuspended with 50 μl of saponin permeabilization buffer (BD Biosciences) and spun down. The cell pellet was incubated with primary Abs (anti-human Mcl-1 or Bim) in saponin buffer at room temperature for

**FIGURE 1.** Isolation of purified human peripheral B cell subsets. A, Phenotypic analysis of B cell subsets in human peripheral blood. Donor B cells were purified by staining with Abs to CD3, CD4, CD8, CD11b, CD14, CD56, and glycophorin A (GPA) and were then evaluated by flow cytometry. B cell subsets were identified according to surface IgG/IgA and CD27 expression: IgG$^+$CD27$^+$ B cells (naive), IgG$^+$CD27$^-$ B cells (IgM$^+$ memory), and IgG$^+$CD27$^+$ B cells (switched memory). Data are presented as density plots. B, Highly purified B cell subsets were separated after cell sorting. C, Surface marker expression in human B cell subsets. Purified B cell subsets before (Unstim) and after stimulation (Stim) with 20 μg/ml F(ab')$_2$ goat anti-IgM (36 h) were analyzed separately for IgM, IgD, CD95, CD86, and CD69 surface expression. Bold line, naive B cells; gray area, IgM$^+$ memory B cells; thin line, isotype control line. These results are representative of peripheral blood samples from more than 10 different donors.
Results

Early BCR signaling is exaggerated in IgM+ memory but not naive B cells

BCR signaling is critical for B cell fate decisions such as B cell survival, growth, and differentiation (14). We first tested whether the profile of early BCR signaling is different between naive and IgM+ memory B cells. Phosphorylation of Syk, one of the earliest events in BCR signaling, was more pronounced in IgM+ memory B cells (Fig. 2A). Two enzymes, PI3K and PLCγ2, function as critical mediators downstream of Syk activation in B cells (15). Phosphorylation of p85/p70 S6K, a downstream molecule of PI3K, and PLCγ2, was more pronounced in IgM+ memory B cells (Fig. 2A). Activated PLCγ2 converts phosphatidylinositol 4,5-bisphosphate into IP3 and diacyl glycerol, the former of which is critical for calcium flux in B cells (14). Consistent with PLCγ2 phosphorylation, BCR-induced calcium flux was higher in IgM+ memory B cells (Fig. 2B). Calcium flux and diacyl glycerol led to activation of NF-κB and MAPKs such as JNK and ERK. Phosphorylation of JNK and ERK was more pronounced in IgM+ memory B cells, whereas p65 NF-κB phosphorylation was comparable in both subsets (Fig. 2A). Taken together, during the early phase of BCR activation, downstream signaling is pronounced especially in IgM+ memory B cells as compared with naive B cells.

BCR stimulation rescues naive but not IgM+ memory B cells from apoptosis

Following anti-IgM stimulation alone, naive and IgM+ memory B cells did not either divide or release Igs in the culture (data not shown), suggesting that BCR signaling alone is not sufficient to induce the growth and differentiation of human B cell subsets. We then tested whether the BCR signaling affects the survival and death of naive and IgM+ memory B cells. In the absence of stimuli, a considerable fraction of purified naive and IgM+ memory B cells underwent apoptotic cell death within 2 days in vitro (Fig. 3A). Spontaneous cell death was more pronounced in naive B cells than in IgM+ memory B cells. BCR stimulation, however, significantly rescued naive B cells from apoptosis, whereas IgM+ memory B cells were not rescued (Fig. 3, A and B). Thus, BCR signaling can protect naive, but not IgM+ memory B cells from apoptotic cell death.

Mitochondrial perturbations including cytochrome c release and inner membrane depolarization correlate with BCR-induced apoptosis (16). We thus tested whether BCR-induced depolarization of the mitochondrial inner membrane could be altered in naive and IgM+ memory B cells. High levels of mitochondrial membrane potential were observed in both subsets immediately after sorting, indicating their highly viable state (Fig. 3C, a and d). A 2-day culture of these subsets without stimuli caused a remarkable decrease in mitochondrial membrane potential (Fig. 3C, b and e). BCR stimulation for 2 days, however, partially abrogated the loss of mitochondrial membrane potential in naive, but not IgM+ memory B cells (Fig. 3C, c and f). Thus, BCR signaling rescues the B cell apoptosis pathway upstream of mitochondrial damage in naive, but not IgM+ memory B cells.

BCR stimulation induces anti-apoptotic Mcl-1 in naive B cells, whereas it induces proapoptotic Bim in IgM+ memory B cells at the protein level

Bcl-2 family proteins are the primary regulators of mitochondrial membrane integrity and play a vital role in the control of apoptosis (9). We tested whether BCR signaling affects gene expression of Bim, Bcl-xL, and Mcl-1 in naive and IgM+ memory B cells (Fig. 4A). Bcl-xL mRNA expression was induced after BCR stimulation in both subsets, and such induction was more pronounced in naive B cells. In contrast, the expression level of Bim mRNA was slightly higher in IgM+ memory B cells irrespective of BCR stimulation. Mcl-1 mRNA expression was not significantly changed in both subsets. We next tested whether BCR signaling affects protein...
expression of Bim, Bcl-xL, and Mcl-1 in naive and IgM+ memory B cells (Fig. 4B). Three isoforms (Bim-EL, Bim-L, and Bim-S) are expressed in various cell types, including lymphocytes (17). In the absence of stimuli, Bim-EL was weakly expressed in both subsets, but in IgM+ memory B cells BCR stimulation induced all of three Bim isoforms at the level higher than those in naive B cells. On the other hand, the expression level of anti-apoptotic proteins Bcl-xL and Mcl-1 was higher in naive B cells after BCR stimulation. Given that the difference in expression levels of surface IgM between two subsets (Fig. 1C) might cause these phenomena, we tested Mcl-1 expression in naive and IgM+ memory B cells using titrated doses of anti-IgM Ab. Higher levels of Mcl-1 in naive B cells were observed at all doses of anti-IgM tested (Fig. 4C). Collectively, these results suggest that after BCR stimulation, anti-apoptotic Bcl-xL and Mcl-1 are predominantly expressed in naive B cells, whereas the proapoptotic protein Bim was more abundantly expressed in IgM+ memory B cells. The discrepancy of mRNA and protein levels strongly suggests the existence of post-transcriptional regulation of Bim and Mcl-1 expression in both subsets.
PI3K activation plays a role in reciprocal regulation of Mcl-1 and Bim protein in B cell subsets

A previous report suggests a critical role of the PI3K pathway in Mcl-1 expression (18). We tested an effect of a selective PI3K inhibitor Ly294002 on BCR-induced Mcl-1 expression in naive B cells (Fig. 5A). Treatment of cells with Ly294002 strongly inhibited Mcl-1 induction in naive B cells, suggesting a critical role of PI3K activity in Mcl-1 expression in naive B cells. In contrast, Ly294002 treatment induced the expression of Bim-EL protein after BCR stimulation (Fig. 5A). These results suggest reciprocal Mcl-1 and Bim expression is dictated by the PI3K pathway. We thus monitored the phosphorylation of Akt, a downstream molecule of PI3K, during BCR stimulation. Akt phosphorylation was sustained for longer periods in naive B cells than IgM+ memory B cells (Fig. 5, B and C). A previous study showed that PI3K activation is critical for BCR-mediated induction of CD86 and CD69 surface expression in murine B cells (19). As shown in Fig. 1C, expression levels of CD86 and CD69 are higher in naive than IgM+ memory B cells at a late time point. Collectively, these results suggest that reciprocal expression of Mcl-1 and Bim protein in both subsets could be explained by their distinct regulation of the PI3K pathway.

Tcl1 and Gal-1 are the critical mediators for B cell to express Mcl-1 and Bim proteins, respectively

To identify the molecule responsible for sustained activation of the PI3K pathway, we conducted gene expression profiling of B cell subsets before and after BCR stimulation. A subset of genes displayed >2-fold differences between naive and IgM+ memory B cells (data not shown). Among these genes, we focused on Tcl1, a potent Akt kinase coactivator (20, 21). We tested Tcl1 mRNA expression in both subsets (Fig. 6A). In the absence of stimuli, a higher level of Tcl1 mRNA was observed in naive B cells than in IgM+ memory B cells. BCR stimulation resulted in more than 10-fold mRNA induction of Tcl1 in naive B cells. We next evaluated the level of Tcl1 protein in both subsets (Fig. 6B). Tcl1 protein was detected only in naive B cells irrespective of stimulation. Reduction in the expression of Tcl1 protein implies the existence of a posttranscriptional inhibitory mechanism of Tcl1 expression. To determine whether Tcl1 expression can induce Mcl-1 expression to promote B cell survival, Tcl1 transgene was overexpressed in naive B cells. Enforced Tcl1 expression induced a high level of Mcl-1 expression in IgM+ memory B cells and protected their apoptotic cell death (Fig. 6, C and D). Thus, Tcl1 expression in naive, but not IgM+ memory B cells plays a critical role in Mcl-1 expression that in turn promotes their survival.

We also sought to identify a molecule involved in Bim expression and apoptosis in IgM+ memory B cells. In a list of genes identified in microarray analysis, higher levels of a glycoprotein Gal-1 in IgM+ memory B cells were noted (data not shown). In the absence of stimuli, Gal-1 mRNA was more expressed in IgM+ memory B cells and BCR stimulation of this subset caused drastic mRNA induction of this gene (Fig. 7A). Consistent with its mRNA expression, Gal-1 protein was abundantly expressed in IgM+ memory B cells (Fig. 7B). To test whether Gal-1 expression can induce Bim expression in B cells and enhance their apoptosis, Gal-1 transgene was overexpressed in naive B cells. Enforced Gal-1 expression resulted in higher levels of Bim expression in naive B cells, which is associated with the increment of apoptotic cells by >2-fold (Fig. 7, C and D). These results suggest that Gal-1 plays a vital role in promoting Bim expression and inducing apoptosis in IgM+ memory B cells. Interestingly, Gal-1 also functions

![FIGURE 4. BCR-induced expression of Bcl-2 family proteins in B cell subsets. Naive and IgM+ memory B cells were incubated for the indicated time periods in the absence or presence of 20 mg/ml F(ab')2 goat anti (α)-IgM. A, Quantitation of Bim, Bcl-xL, and Mcl-1 mRNA by real-time PCR in B cell subsets. Data are normalized to the expression of 18S rRNA. Results are representative of three independent experiments. *p < 0.01. B, Cell lysates were separated on a 12.5% SDS-PAGE gel, and analyzed by Western blotting with anti-Bim and -Mcl-1 sera and anti-Bcl-xL and -β-actin mAb. The results shown are representative of five independent experiments. RE, Relative expression. C, Effect of a titrated dose of anti-IgM on Mcl-1 induction in B cell subsets. Results are representative of three independent experiments. RE, relative expression.](http://www.jimmunol.org/doi/abs/10.4049/jimmunol.1495076)
as a soluble cytokine (22). Because activation of Akt and JNK is critical for regulating Bim expression (23, 24), we tested the effect of recombinant Gal-1 on BCR-induced phosphorylation of Akt and JNK in B cells. As shown in Fig. 7E, Gal-1 remarkably inhibited Akt phosphorylation, whereas it slightly enhanced JNK phosphorylation in B cells upon BCR stimulation. Taken together, Gal-1 regulates Bim expression through its effects on activation of Akt and JNK in B cells.

**FIGURE 5.** Regulation of Mcl-1 and Bim protein expression by the PI3K pathway. A, Naive B cells were pretreated with or without Ly294002 (Ly; 10 μM) for 30 min and stimulated for 36 h in the absence or presence of 20 μg/ml F(ab')2 goat anti-(α)-hIgM. Cell lysates were separated on a 12.5% SDS-PAGE gel, and analyzed by western blotting with anti-Bim, -Mcl-1 sera, and anti-β-actin mAb. The results shown are representative of three independent experiments. B, Naive and IgM+ memory B cells were pretreated with or without Ly294002 (10 μM) for 30 min and stimulated for the indicated time periods in the absence or presence of 20 μg/ml F(ab')2 goat anti-human IgM. Cell lysates were separated on a 10% SDS-PAGE gel and analyzed by Western blotting with anti-phospho-Akt sera and anti-β-actin mAb. Results are representative of three independent experiments. C, Densitometric analyses of Akt phosphorylation in B cell subsets. The resulting values were expressed as the percentage in reference to that of BCR-stimulated naive B cells at 0.5 h. Values are the mean ± SD of three independent experiments. *, p < 0.05; **, p < 0.01.

**FIGURE 6.** Tcl1 is critical for Mcl-1 expression and survival of B cell subsets. Naive and IgM+ memory B cells were incubated for the indicated time intervals in the absence or presence of 20 μg/ml F(ab')2 goat anti-(α)-hIgM. A, Quantitation of Tcl1 mRNA by real-time PCR in naive and IgM+ memory B cells. Data are normalized to the expression of 18S rRNA. Results are representative of five independent experiments. *, p < 0.01 (with reference to unstimulated naive B cells). B, Cell lysates were separated on a 12.5% SDS-PAGE gel, and analyzed by Western blotting with anti-Tcl1 sera, and anti-β-actin mAb. Results are representative of four independent experiments. IgM+ memory B cells were transfected with either pEGFP-empty or -Tcl1 for 18 h and then stimulated with 20 μg/ml F(ab')2 goat anti-hIgM for 24 h. C, After culture, intracellular Mcl-1 expression of GFP-positive cells was analyzed by flow cytometry. Insets, Expression of Tcl1 transgene. Results are representative of three independent experiments. D, After culture, GFP-positive cells were stained with PE-labeled annexin V and analyzed using flow cytometry. Percentages of annexin-positive cells are shown. Results are representative histogram of three independent experiments. Cont, Control.
Our study shows that BCR stimulation rescued naive B cells from apoptosis with Mcl-1 induction, whereas it rather accelerated apoptosis of IgM memory B cells with Bim induction. Sustained Akt activation in naive but not IgM memory B cells appears to be critical for reciprocal expression pattern of these Bcl-2 family proteins. Tcl1 and Gal-1, abundantly expressed in naive and IgM memory B cells, respectively, play a crucial role in regulating Akt activation, thereby affecting their survival and death via the Bcl-2-regulated pathway.

BCR signals regulated Mcl-1 expression primarily at the protein level (Fig. 4B), presumably because Akt up-regulates Mcl-1 post-transcriptionally via regulating activation of glycogen synthase kinase-3 (25). Sustained Akt activation in naive B cells (Fig. 5, B and C) may thus be indispensable for continuous replenishment of Mcl-1 protein due to extraordinary short half-life of Mcl-1 (26). In contrast to Mcl-1, Bim transcription is negatively regulated by Akt through via regulating activation of the forkhead transcription factor FOXO3a (27). A small but significant increase in Bim mRNA in IgM memory B cells (Fig. 4A) in response to BCR stimulation might be induced by immediate inactivation of Akt (Fig. 5, B and C) in this subset. Thus, Akt signals might play a critical role in controlling Mcl-1 and Bim expression reciprocally in these B cell subsets.

In contrast to BCR-induced death, spontaneous cell death is more pronounced in naive than in IgM memory B cells (Fig. 3A). In addition, 2-day culture of naive B cells without stimuli caused a further decrease in mitochondrial membrane potential (Fig. 3C), suggesting that spontaneous cell death is regulated at the mitochondrial level presumably by Bcl-2 family proteins. We, however, found that in the absence of stimuli, expression levels of Bim and Mcl-1 in naive and IgM memory B cells are comparable (data not shown). Therefore, Bim-Mcl-1 balances are not the main determinant of spontaneous cell death in two subsets. Collectively, Bim-Mcl-1 balances can regulate activation-induced death of B cell subsets, whereas other Bcl-2 family proteins might be more critical for the longevity of B cell subsets in the periphery.

We show here that Tcl1 and Gal-1 are differentially expressed in human naive and IgM memory B cells. Tcl1 interacts with Akt and functions as a potent Akt coactivator (20, 21). In Tcl1-deficient mice, the number of splenic follicular, germinal center, and MZ B cells is reduced (28). Our data suggest that Tcl1 positively

**FIGURE 7.** Gal-1 is critical for Bim expression and apoptosis of B cell subsets. Naive and IgM memory B cells were incubated for the indicated time intervals in the absence or presence of 20 μg/ml F(ab′)2 goat anti-(α) IgM. A, Quantitation of Gal-1 mRNA by real-time PCR in naive and IgM memory B cells. Data are normalized to the expression of 18S rRNA. The results shown are representative of four independent experiments; *, p < 0.01 (with reference to unstimulated naive B cells). B, Cell lysates were separated on a 12.5% SDS-PAGE gel, and analyzed by Western blotting with anti-Gal-1 sera, and anti-β-actin mAb. Results are representative of three independent experiments. Naive B cells were transfected with either pEGFP-empty or -Gal-1 for 18 h and then stimulated with 20 μg/ml F(ab′)2 goat anti-human IgM for 24 h. C, After culture, intracellular Bim expression of GFP-positive cells was analyzed by flow cytometry. The insets depict the expression of Gal-1 transgene. Results are representative of three independent experiments. Cont, Control. D, After culture, GFP-positive cells were stained with PE-labeled annexin V and analyzed using flow cytometry. Percentages of annexin-positive cells are shown. Results are representative histograms of three independent experiments. E, Human B cells (CD19+) were pretreated with or without recombinant Gal-1 (10 μg/ml) for 12 h and stimulated for 5 min in the absence or presence of 20 μg/ml F(ab′)2 goat anti-human IgG/IgA/IgM. Cell lysates were separated on a 10% SDS-PAGE gel, and analyzed by Western blotting with anti-phospho-Akt, -JNK sera, and anti-β-actin mAb. The results shown are representative of three independent experiments. RE, Relative expression.
regulates Akt activation, resulting in Mcl-1 expression in B cells (Fig. 6). To date, three Tcl1 isoforms have been identified in mice and humans: Tcl1, TCL1B, and MTCP1. Our analysis showed that Tcl1 and MTCP1 but not TCL1B mRNA are expressed in human naive and IgM memory B cells, whereas the expression level of Tcl1 mRNA is different between the subsets (data not shown). These data suggest that the difference in Tcl1 expression between the subsets (Fig. 6) does not reflect the expression patterns of Tcl1 isoforms in each subset. In contrast to Tcl1, Gal-1 induced Bim protein and enhances apoptosis in B cells (Fig. 7, C and D). Furthermore, Gal-1 significantly inhibited BCR-dependent activation of Akt, leading to the up-regulation of proapoptotic Bim (Fig. 7, C and E). Gal-1 slightly enhanced BCR-induced JNK phosphorylation (Fig. 7E). Because JNK activation positively regulates Bim-induced apoptosis (24, 29), Gal-1 may induce Bim expression in IgM memory B cells also by positively regulating JNK activation.

Gal-1 may play a critical role in the maintenance of B cell tolerance. In fact, anergic B cells express higher levels of Gal-1 than wild-type cells do (30). Gal-1 induces tolerogenic dendritic cells and promotes the expansion of regulatory T cells in vivo (31). In addition, a high level of Gal-1 is required for naturally occurring CD4+CD25+ T cells to maintain their optimal Treg function (32). These data raise an interesting possibility that human IgM memory B cells play a critical role in the regulation of DC and Treg functions through Gal-1 production. In contrast, abnormal expression of Tcl1 could link to the pathogenesis of B cell malignancies. Tcl1-transgenic mice reveal an expansion of the CD5+IgM+ population that is reminiscent of human B cell chronic lymphocytic leukemia (CLL) (33), and high Tcl1 expression in human B cell CLL correlates with an aggressive CLL phenotype showing unmutated Ig variable region genes and ZAP70 positivity (34). These data collectively suggest that fine-tuning of the balance between Gal-1 and Tcl1 expression is critical for the homeostasis of human B cell subsets.

Random generation of BCRs results in the emergence of a large number of self-reactive B cells, together with pathogen-specific B cells. BCR-induced cell death and anergy are thus crucial for purging or silencing self-reactive B cells. However, there are significant differences in self-reactivity between human B cell subsets: in healthy individuals; up to 20% of naive B cells express self-reactive BCRs, whereas IgM memory B cells are devoid of such self-reactive BCRs (5, 6). Bim plays a critical role in BCR-induced cell death and anergy based on the fact that Bim deficiency causes a substantial expansion of autoreactive B cells leading to autoimmune diseases (10, 35). Thus, Bim expression in IgM memory B cells may serve a novel safeguard mechanism that allows efficient elimination or inactivation of the self-reactive repertoire. Our data suggest that the balance between Mcl-1 and Bim is critical in determining B cell survival and death. It has been shown that constitutive expression of B cell-activating factor of the TNF family (BAFF), a survival-promoting cytokine for murine B cells, can break B cell tolerance through expanding self-reactive B cell populations in MZ (36, 37). BAFF exerts its effects on murine B cell survival through down-regulating Bim and up-regulating Mcl-1 (38, 39). We found that BCR-induced death in human IgM memory B cells is abrogated in the presence of BAFF (data not shown). Because patients with systemic lupus erythematosus and Sjögren’s syndrome have elevated levels of serum BAFF (37), it is important to test whether self-reactive IgM memory B cells are expanded in these autoimmune diseases.

In summary, BCR signaling dictates survival and death in human naive and IgM memory B cells, respectively. These phenotypes are driven by reciprocal expression of Bcl-2 family proteins such as Mcl-1 and Bim in these B cell subsets. Tcl1 and Gal-1 are expressed in naive and IgM+ memory B cell subsets, respectively. Tcl1 and Gal-1 might play critical roles in the expression of Mcl-1 and Bim, at least through regulating Akt activation. Therefore, a unique set of molecules such as Tcl1 and Gal-1 defines distinct BCR signaling cascades, dictating fate of human naive and IgM memory B cells.

Disclosures
The authors have no financial conflict of interest.

References


