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Cutting Edge: Bim Is Required for Superantigen-Mediated B Cell Death

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To impair B cell clonal regulation, the microbial virulence factor, protein A of Staphylococcus aureus, can interact with evolutionarily conserved BCR-binding sites to induce a form of Fas-independent activation-associated B cell death that results in selective immune tolerance. We now show that this in vivo death pathway is associated with induction of increased transcript and protein levels of Bim, a BH3-only proapoptotic Bcl-2 family protein, which is inhibited by excess B cell-activating factor. An absolute requirement for Bim was documented, since Bim-deficient B cells were protected from in vivo superantigen-induced death and instead underwent persistent massive supraclonal expansion without functional impairment. These studies characterize a BCR-dependent negative clonal selection pathway that has been co-opted by a common bacterial pathogen to induce selective defects in host immune defenses. The Journal of Immunology, 2007, 178: 2636–2640.

During their coevolution with mammalian immune systems, microbial pathogens have developed diverse strategies that block essential lymphocyte clonal regulatory mechanisms. The virulence factor, protein A of Staphylococcus aureus (SpA), can impair host B cell-mediated defenses through specific binding interactions with a V_H framework site conserved in many BCR (1), and this superantigen (SAg)-mediated depletion of large supraclonal B cell sets is a T cell-independent process (2–4 and reviewed in Ref. 5). SpA production during S. aureus infection can result in lasting V_H-targeted changes in the expressed repertoire (6). Despite the great molar excess of circulating Ig with the same Fab-binding specificity, even microgram doses of this SAg can still induce quantitative deletion of BCR-targeted B cells (7).

In recent studies, SpA was shown to induce a form of activation-induced cell death (AICD) (3, 4), which was unimpared in mice with deficiencies in Fas and Fas ligand (3) and also TNFR1 death receptor (C. S. Goodyear and G. J. Silverman, unpublished observations). Although caspase inhibition slowed but did not block death, the efficiency of supraclonal deletion was attenuated by Bcl-2 overexpression (3). However, Bcl-2 can potentially suppress both intrinsic mitochondrial death pathways and apoptotic pathways mediated by membrane-associated death receptors. Moreover, apoptosis pathways may be influenced by many of the related members of the Bcl-2 family, which can differ greatly in their expression of domains that convey either prosurvival or prodeath effector functions.

In the current studies, we have investigated the potential roles of the Bcl-2 family regulatory proteins, with special interest in the superantigenic proteins that express only BH3 domains. We show that the special BCR-dependent pathway of AICD induced by SpA is linked to a potent capability for in vivo induction of the BH3-only member Bim, at both transcript and protein levels. Our studies demonstrate an absolute requirement for Bim and suggest that Bim plays nonredundant roles in postexpansion B cell clonal regulation.

Materials and Methods

T15 Ig knock-in (B6.129P2-Igh<sup>tm1Cgn</sup>) and Bim<sup>−/−</sup> (B6.129-Bcl2l11tm1.1Ast/J) (8) mice (gift from A. Strasser (The Walter and Eliza Hall Institute of Medical Research, Australia), provided by P. Oliver and P. Marrack (National Jewish Medical and Research Center, Denver, CO)) were crossed under specific pathogen-free conditions to generate T15<sup>+</sup>Bim<sup>−/−</sup> mice (B6.129-Bcl2<sup>−/−</sup>Igh<sup>−/−</sup> <sup>tm1.1Ast/J</sup>) (9), with treatments that included rSpA as described (2–4). Transcripts from negatively selective splenic B cells (CellMem Technologies) were measured by TaqMan (2). Immunohosting of splenocytes extracts used Abs to Bim protein (Santa Cruz Biotechnology), normalized for Hsc70 (Stressgen). Flow methods were as reported previously (2–4, 7), with intracellular staining for Bim (Santa Cruz Biotechnology), Bcl-x<sub>L</sub>, and Bcl-2 (BD Pharmingen) or phosphorotyrosines (4G10 mAb; Upstate Biotechnology). For digital confocal studies, images of splenocytes were collected as described (3, 4). For three-dimensional images, Z-stacks were processed by the San Diego Supercomputer Center. Tumorigenesis studies were as described elsewhere (3). Unpaired, two-tailed t tests were performed with InStat (GraphPad).

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4 Abbreviations used in this paper: SpA, protein A of Staphylococcus aureus; AICD, activation-induced cell death; BAFF, B cell activation-induced factor of the TNF family; LN, lymph node; ΔΨ<sub>mem</sub>, mitochondrial membrane potential; SAg, superantigen.
Results and Discussion

In time course studies of T15i+/+ knock-in mice in which all B cells are SpA susceptible (2–4), we quantified transcript levels of Bcl-2 family member transcripts in splenic B cells (Fig. 1) and found that SpA treatment increased only the prodeath BH3-only Bcl-2 family member Bim (BimS isoform), which peaked at 8 h after SpA treatment to 3.5-fold the level after control treatment (Fig. 1A). By contrast, prosurvival and other BH3-only Bcl-2 family members decreased by 8 h, but later normalized likely due to the loss of affected B cells. RT-PCR studies, which evaluated a greater range of Bim transcript isoforms, confirmed that SpA induced the greatest relative increases of the BimS isoform, with more modest increases in the BimL and BimEL isoforms (data not shown).

Although Bim protein was not detected in B cell extracts from control-treated mice, levels were increased of BimL and BimEL protein isoforms and some BimS by 8 and 12 h after in vivo SpA treatment (Fig. 1B). This time course suggested a temporal linkage for marked induced increases in Bim transcript and protein levels in B cells undergoing SpA-induced death. As previously reported (3), flow cytometric analyses, which showed that SpA encounters induced expression of B cell activation markers (e.g., CD86) detectable by 12 h after exposure, also confirmed induced increases in intracellular levels of Bim as early as 4 h (data not shown), with the highest levels documented at 12 and 24 h after SpA treatment (Fig. 1C). By contrast, SpA-affected B cells did not display reproducible changes in Bcl-xL, Bcl-2, and Mcl-1 levels (data not shown). This in vivo induction of Bim was specific for SpA interactions, because neither anti-IgM nor LPS challenge increased the levels of Bim (data not shown).

Notably, we found similar increases in the Bim levels in splenic CD9+ B220+ cells (predominantly marginal zone B cells) and in CD9− B220+ (primarily follicular (i.e., B2) cells; data not shown). To determine whether or not this increase in Bim levels could be demonstrated in nontransgenic mice, we treated C57BL/6 with fluorochrome-labeled SpA or control protein. At 12 h, we were able to demonstrate that SpA-binding CD9-positive B220+ and CD9-negative B220+ B cells had higher levels of Bim (data not shown).

Deconvolution digital microscopy studies showed that in control-treated mice the BCR were widely distributed along the B cell membrane (Fig. 2, A and C), whereas by 8 h after SpA treatment the BCR became colocalized into tight clusters of bead-like cappings with some intracellular BCR clusters (Fig. 2D). In fact, SpA-induced BCR cocomplexes were more tightly clustered than could be induced by in vitro or in vivo anti-IgM or anti-IgM treatment in control studies (data not shown). In control-treated mice, Bim in B cells was detected as small areas of punctate staining (Fig. 2, B and C) akin to reported patterns of Bim staining that colocalized with mitochondria in resting B cells (9). By contrast, by 8 h posttreatment, the B cells with BCR-SpA co-caps also displayed profound increases in Bim protein (Fig. 2, E and F).

By costaining for the nuclear envelope, Bim in SpA-affected B cells was shown to accumulate solely within the B cell cytoplasm (i.e., outside the nuclear membrane) (Fig. 2G). B cells at later apoptotic stages had localized nuclear condensation with invaginating folds of the nuclear envelope, and Bim accumulated in the enlarged local cytoplasmic areas that were adjacent to, but still outside of, the nuclei of the apoptotic B cells (data not shown). Bim did not primarily segregate with actin in the cytoskeleton in these lymphoid cells (data not shown). These findings contrast with a report of posttranscriptional regulation...
of Bim, which was sequestered within the dynein motor complex in healthy cells, whereas induction of apoptotic death resulted in cytoskeletal changes and translocation of Bim complexes to inactivate prosurvival Bcl-2 members and caused apoptotic death (10).

Although the targeted deletion of B cells by SpA is heralded by a progressive dissipation of mitochondrial membrane potential (Δψm), the efficiency of deletion is greatly attenuated in B cells that overexpress Bcl-2 (3, 4). To assess the relevance of the modulation of other Bcl-2 family members, Bcl-2, or Bcl-xL at the protein level (data not shown). These findings suggest that the BAFF prosurvival factor can block the SAg-mediated in vivo death of primary B cells.

To directly test whether Bim is essential, T15i+/- mice were backcrossed to the Bim-deficient state. Confirming earlier reports (12), naive adult Bim-deficient T15i+/- mice had significant increases in mature splenic B cells, with greater individual variations among these mice, compared with Bim-sufficient T15i+/- mice (Fig. 4, A and C, and data not shown). At 6 days after SpA treatment, we found a mean 59% depletion of splenic B cells in Bim-sufficient mice, while there was a mean 63% increase in splenic B cells in Bim-deficient compared with control-treated mice (Fig. 4, A and C). Notably, compared with control groups, SpA also induced modest increases of splenic B cells in Bim+/- mice (mean, 17.8 × 10^6 ± 5.4 to 20.6 × 10^6 ± 6.8, n = 5), which represented an intermediate phenotype between Bim wild-type and Bim homozygotic-deficient mice. Even at 14 days after treatment, the same distinct patterns were found, with a mean 64% depletion of splenic B cells in Bim-sufficient mice, while there was a persistent mean 69% increase in splenic B cells in Bim-deficient mice compared with control-treated mice (data not shown), whereas the level of splenic B cells in SpA-treated Bim-sufficient mice had begun to normalize to the levels in control-treated mice (data not shown).

During in vivo responses to LPS, a different type of B cell mitogen, by 72-h splenic B cell expansions, was initially induced in both Bim-deficient and Bim-sufficient mice (T15i+/-...
Bim\(^{+/+}\) control, 12.8 × 10\(^6\) vs LPS, 23.4 × 10\(^6\) compared with T15\(^{+/+}\)/Bim\(^{-/-}\) control, 19.7 × 10\(^6\) vs LPS, 26.1 × 10\(^6\)). However, by 14 days, these LPS-induced B cell expansions had completely normalized in both types of mice (Fig. 4, B and C). Hence, even though Bim is required for SAg-induced BCR-mediated AICD, Bim is not required for the collapse of LPS-induced, presumably TLR4-mediated, activation-associated B cell expansion.

To assess the level of proliferation induced by SpA and track the newly generated B cells, we used BrdU-labeling studies to show that despite concurrent modest-induced increases in newly generated lymphocytes among the residual B cells, SpA treatment of Bim-sufficient mice still induced massive overall net B cell deletion (Fig. 4D) (3, 7). Significantly, while at 14 days after control treatment, Bim-deficient mice had a mean of 3.0 × 10\(^6\) ± 0.7 BrdU\(^+\) splenic B cells, after SpA treatment there was a mean of 10.4 × 10\(^6\) ± 4.3 BrdU\(^+\) splenic B cells (p = 0.007; Fig. 4D), representing a 240% increase compared to control-treated mice. Furthermore, surveys of systemic lymph nodes (LN) and mesenteric LN in Bim-deficient mice demonstrated comparable levels of SpA-induced B cell expansion and proliferation (LN: control, 17% ± 0.1; SpA, 37.4% ± 1.8; mesenteric LN: control, 21.1% ± 1.1; SpA, 34.7% ± 2.3, and data not shown). Hence, SpA induced a generalized B cell expansion in Bim-deficient mice, with persistence at diverse peripheral sites even 2 wk after exposure, indicating that these changes cannot be solely attributed to altered trafficking.

To rule out the possibility that these differences in Bim-deficient mice were due to B cell extrinsic influences (i.e., non-B cell effects), splenic T15\(^{+/+}\) B cells were CFSE-labeled and adoptively transferred into congenic mice with B cells marked by a distinct CD45 isoform. When examined at 6 days after control treatment of either of the two types of mice, only limited background levels of proliferation were detected (Fig. 4E). At this time point, SpA treatment induced an overall mean 48% depletion of the Bim-sufficient B cells, even though most Bim-sufficient B cells had undergone one or more rounds of proliferation (Fig. 4E) as previously reported (3). Confirming our results from BrdU-labeling studies, SpA induced a mean 285% increase of CFSE-labeled Bim-deficient B cells (Fig. 4E), which is akin to the increased survival of Bim-deficient anti-hen egg lysozyme-Ig transgene-expressing B cells after encounter with soluble hen egg lysozyme-Ag (13). Hence, these adoptive transfer studies in Bim-sufficient hosts clearly documented that Bim acts as a B cell intrinsic influence during SAg-induced death. Moreover, despite the greater survival of Bim-deficient B cells, the Bim-deficient and Bim-sufficient B cells that remained after SpA challenge had undergone similar distributions of rounds of proliferation (data not shown). Hence, Bim-deficient B cells did not display major abnormalities in their entry or rate of proliferative cycling.

To directly assess the functional status of B cells that persist following in vivo SpA treatment, we evaluated splenic B cells 6 days after challenge and found that posttreatment B cells vigorously respond to in vitro anti-IgM treatment for induction of intracellular phosphatidylinositol expression and for the induction of calcium flux, documenting a lack of induced functional defects (data not shown). In fact, B cells from SpA-treated Bim-deficient mice displayed enhanced responses to stimulation with the B cell mitogens, LPS, or anti-IgM (i.e., IgG F(ab\(^{\prime}\))\(_2\)), or CD40 ligand, compared to those from control-treated mice (data not shown). Hence, B cells surviving from SpA exposure are not commonly anergic (12) and, under the conditions evaluated, Bim-deficient B cells instead displayed enhanced responsiveness to restimulation, which is consistent with evidence from other systems that Bim may contribute to non-deletional negative selection mechanisms (i.e., anergy) (12, 14).

In the current studies, we characterize a novel pathway by which a microbial toxin induces AICD with the selective induction of high intracellular transcript and cytoplasmic protein levels of Bim. An absolute requirement for Bim was demonstrated, since the mature B cells in Bim-deficient mice instead underwent massive proliferative expansions. Our studies provide further evidence of the inverse regulatory roles for Bim and the prosurvival Bcl-2 family members, which is consistent with analyses showing that increased Bim levels may inactivate Bcl-x\(_L\) and/or induce the executioner Bcl-2 family members, Bak and Bax, responsible for apoptosome-mediated death (15). We were also surprised to find that the mature B cell populations expanded by BCR encountered in Bim-deficient mice, unlike LPS-expanded populations, persisted long after SAg encounter, with only limited normalization even after 5 wk. Our investigations therefore suggest that there are no other compensatory mechanisms that efficiently act to normalize these supralclonal expansions since the influence of Bim on postexpansion clonal regulation appears largely nonredundant with other deletional pathways (e.g., Fas and other death receptors).

Our findings contrast with earlier reports in which the Fas death receptor system was reported to be essential for the elimination of self-reactive B lymphocytes and the maintenance of self-tolerance (16). More recently, Bim has also been implicated in TCR-triggered death (17), and the death induced by a staphylococcal T cell SAg, although in this setting Bim levels did not change following exposure (18). Although less is known about B cells, studies of BCR-mediated death of human B cell lymphoma lines have implicated Bim, although here Bim transcript levels were also unaffected since apoptosis was instead dependent on posttranscriptional regulatory events. In fact, most studies of primary B cells have focused on immune development (11–13, 19). By contrast, our studies have examined the roles of Bcl-2 family members after in vivo challenge with a natural BCR-targeted toxin of mice with established peripheral B cell compartments.

Our findings therefore support the hypothesis that stress stimuli, including the exhaustion of growth survival factors, can induce a mitochondrial death pathway dependent on shifts in the balance of prosurvival Bcl-2 members and the induction of prodeath BH3-only members (8, 10, 13). We speculate that a B cell SAg more commonly delivers a very strong BCR signal that is associated with greater cellular requirements for cytokine or cognate help that are critical for survival and/or cell cycle progression, as well as because a SAg also enhances cellular competition among the great numbers of B cells affected by this BCR-targeted ligand (3). This model is consistent with our demonstration that supraphysiological levels of BAFF can inhibit both the BCR-mediated up-regulation of Bim and subsequent apoptotic death akin to other systems (11, 12, 14). This model is also supported by our recent findings that relatively larger SAg doses induce limited proliferation with more rapid and larger scale death, presumably due to the more rapid exhaustion of available prosurvival signals (7).
Our studies therefore indicate that posttranslational mechanisms are not alone responsible for the role(s) of Bim in BCR-mediated death, and future studies should characterize transcriptional regulation during AICD. Taken together with other recent studies (3, 4, 7), we have provided evidence that the death pathway of BCR-mediated B cell death induced by the bacterial toxin SpA is initiated by the formation of highly avid BCR cocomplexes that are especially well suited to the induction of Bim in mature peripheral B cells (Fig. 2). We believe that our studies of this natural B cell toxin have revealed a pathway for BCR-mediated negative selection that is likely common to B cell SAGs, contributing to infection-associated immune modulation, and which also may be potentially accessed by engineered B cell toleragens (discussed in Ref. 5).

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Disclosures
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References

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