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Distinct but complementary roles of Fas ligand and Bim in homeostatic T cell apoptosis

Daniela Kassahn, Thomas Brunner* and Nadia Corazza*

Division of Immunopathology; Institute of Pathology; University of Bern; Switzerland

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Dear Editor

The adaptive immune system, as mediated by T cells and B cells, plays an essential role in host defense. Throughout adult life the total number of T cells remains fairly constant, suggesting that their expansion and retraction is tightly regulated. However, in response to infection or immunization antigen-specific T cells must expand and develop various effector functions enabling them to rapidly eliminate pathogens and thereby protect the host. After antigen removal the number of activated T cells must be reduced to initial levels, thereby maintaining T cell homeostasis. Rapid shout-down of the immune system prevents accumulation of potentially dangerous activated effector cells, leading to immune pathological disorders. Apoptosis is crucially involved in controlling T cell development in the thymus, homeostasis of peripheral T cells, and depletion of activated T cells after resolution of the immune response. In mammalian cells, including T cells, two major mechanisms of apoptosis induction have been described. They are summarized in the so-called intrinsic and extrinsic pathways. The intrinsic apoptosis pathway is initiated by signals that activate the mitochondrial apoptosis pathway, including growth factor withdrawal, and cytotoxic or DNA-damaging agents. This pathway is tightly regulated by the interplay between pro- and anti-apoptotic members of Bcl-2 family. Death-inducing signals activate so-called BH3-only (Bcl-2 homology domain 3-only) proteins of the pro-apoptotic subgroup of the Bcl-2 family (e.g., Bim), which may than initiate the mitochondrial apoptosis pathway through activation of other pro-apoptotic Bcl-2 homologs or neutralization of anti-apoptotic Bcl-2 members. The extrinsic pathway is activated by ligation of so-called death receptors of the Tumor Necrosis Factor (TNF) receptor superfamily. These include Fas (CD95), TNF receptor 1 and TRAIL receptor 1 and 2. Activation of these death receptors leads to the rapid activation of cellular proteases (caspases) causing the apoptotic demolition of the cell. Both death receptor signaling and the mitochondrial apoptotic pathway have been shown to be critical for lymphocyte homeostasis as documented by the dramatic phenotype of mice with either mutations in death receptor genes or genes that encode for members of the Bcl-2 family. Both, mice with mutations in the Fas receptor (*lpr*) or its ligand (*gld*), and mice deficient for the pro-apoptotic Bcl-2 member Bim accumulate autoreactive T cells and B cells, and develop autoimmune diseases. Similar observations are made in patients with autoimmune lymphoproliferative syndrome (ALPS), which is often caused by mutations in the Fas signaling pathway. The relative contribution of the two distinct pathways in the termination of T cells immune responses has been a matter of debate for a long time. Only recently three different independent publications demonstrated that both Fas/FasL and Bim cooperate in the shout-down of activated T cells. By crossing Bim-deficient mice with *lpr* mice they demonstrated that both pathways are essential for maintaining T cell homeostasis, preventing autoimmune diseases and downsizing the T cell responses after acute viral infection.[1-3]

Although it is now accepted that both mechanisms play an essential role in T cells apoptosis and homeostasis it is far from being understood how the extrinsic (by Fas) and the intrinsic (by Bim) apoptosis pathway cooperate in homeostatic T cell depletion. One possibility could be that Fas and Bim represent effector molecules of two autonomous apoptosis pathways controlling T cell death at different and independent checkpoints. The other possibility could be that Bim and Fas may not be involved in separate signaling pathways but may represent effector molecules of the same apoptosis signaling cascade.

Cross-talk between the extrinsic and the intrinsic apoptosis pathway has been described in different systems. Currently the best characterized example is Fas-induced apoptosis in hepatocytes involving the BH3-only protein Bid and mitochondrial amplification of apoptosis signaling. Upon Fas ligation, Bid is cleaved by active caspase 8 and truncated Bid triggers the mitochondrial pathway of apoptosis by either neutralizing anti-apoptotic Bcl-2 members activating pro-apoptotic ones. Consequently, Bid-deficient hepatocytes are insensitive to Fas-induced apoptosis.[4,5] Our own recent results indicated, however, that the Fas signaling pathway in hepatocytes is even more complex and involves additional apoptosis effector molecules such as Bim. While wild type mice develop fulminant hepatitis after injection of an agonistic anti-Fas antibody, Bim-deficient mice are largely protected.[6] In light of our findings, demonstrating a critical interplay between Fas and Bim in hepatocyte apoptosis, and the dramatic accumulation of autoreactive lymphocytes in both Bim-deficient mice and Fas/FasL mutant animals, it is feasible to suggest that death receptors and Bim do not represent two completely independent apoptosis pathways mediating T cell homeostasis, but may act on the same apoptosis pathway. To explore this possibility we employed T cell blast from Bim-deficient and wild type mice, and compared their apoptosis sensitivity to recombinant FasL or to activation-induced cell death (AICD) by triggering the T cell receptor. T cell receptor-induced cell death of previously activated T cells is know to proceed via a FasL/Fas-dependent mechanism, and has been discussed as an in vitro model for deletion of chronically activated T cells in vivo.[7] T cell blasts were generated from wild type and Bim-deficient mice by activation of isolated spleen cells with the lectin concanavalin A and subsequent culture in IL-2 containing media for 5 days. Pre-activated T cells were then re-stimulated with immobilized anti-CD3 antibody, or triggered with recombinant FasL or, as positive control for Bim, with the glucocorticoid dexamethasone. Apoptosis was detected after 6 hours by Annexin V staining in the $CD4^+$ and $CD8^+$ T cell subsets. As illustrated in Figure 1A, the spontaneous apoptosis of both $CD8^+$ and $CD4^+$ T cell blasts from Bim-deficient mice was reduced compared to wild type mice. This observation support the role of Bim in growth factor withdrawal-induced apoptosis as already demonstrated in previous reports.[8,9] However, activated T cell subsets from both, Bim-deficient and wild type mice, were equally sensitive to recombinant FasL as well as to re-stimulation of the T cell receptor with anti-CD3 antibody. In contrast, substantially reduced apoptosis was observed in Bim-deficient T cells after treatment with dexamethasone, which induces apoptosis

*Correspondence to: Thomas Brunner; Division of Immunopathology; Institute of Pathology; University of Bern; Murtenstr. 31; Bern 3010 Switzerland; Tel.: +41.31.632.49.71/32.08; Fax: +41.31.381.87.64; Email: tbrunner@pathology.unibe.ch/ Nadia Corazza; Division of Immunopathology; Institute of Pathology; University of Bern; Murtenstr. 31; Bern 3010 Switzerland; Tel.: +41.31.632.49.71/32.08; Fax: +41.31.381.87.64; Email: nadia.corazza@pathology.unibe.ch

in a Bim-dependent manner via the intrinsic apoptosis pathway. Interestingly, AICD in both, wild type and Bim-deficient mice was blockable with anti-FasL antibody. As both FasL-induced apoptosis and FasL-dependent AICD were unaffected by the presence or absence of Bim these in vitro data support the conclusion that Bim does not contribute to the Fas signaling pathway.

In vitro experiments are often a poor substitute for in vivo processes. Given the strong similarities between the role of Fas and Bim in peripheral deletion in vivo we aimed at analyzing the relative contribution of Bim in the Fas signaling pathway also in vivo. To induce antigen-specific activation, expansion and deletion of T cells, we injected wild type, Bim-deficient and FasL mutant *gld* mice i.v. with 100 μg/mouse superantigen staphylococcal enterotoxin B (SEB). At different time points after injection the percentage of Vβ8$^+$ CD4$^+$ and CD8$^+$ T cells in lymph nodes and spleen was analyzed. Notably, superantigen-responsive Vβ8$^+$ T cells from wild type, Bim-deficient and *gld* mice showed a comparable expansion on day 4 (Fig. 1B). In contrast, while on day 11 after superantigen injection wild type mice demonstrated already a strong decrease in the levels of Vβ8$^+$ T cells, no deletion was observed in Bim-deficient mice and reduced deletion in *gld* mice, confirming an important role for Bim and FasL in antigen-induced peripheral deletion. Interestingly, the absence of FasL variably affected superantigen-induced depletion, depending on the T cell subset and the location. While FasL seemed always to be critical in the deletion of CD4$^+$ T cells in spleen and lymph node, no effects were seen for CD8$^+$ T cells in the spleen and only minor effects in the axial lymph node. This finding is in agreement with previous reports describing a role for TNFα in the antigen-induced deletion of CD8$^+$ T cells.[10]

Thus, these results suggest that both Bim and to a lesser extent FasL/Fas participate in antigen-induced depletion of T cell in vivo. How can we reconcile our results with conflicting reports in the literature? The role of FasL/Fas interactions in T cell retraction after SEB administration has been questioned by several investigators.[9-11] While some reports suggested that Fas ligand is required in the depletion of activated T cells after SEB administration, others showed that superantigen-activated T cells die even in the absence of Fas/FasL but fail to deplete in the absence of Bim. Given the new view that both FasL and Bim contribute to peripheral deletion the reason for this discrepancy is most likely attributed to the administration procedure of SEB, i.e., amounts of superantigen, and single vs. repeated doses. Thus, in case where animal are injected with high or repeated doses of SEB, mimicking high antigen loads as are observed during chronic infection with pathogens, Fas/FasL seems to play a more dominant role in the activation-induced retraction of antigen-specific T cells. On the other hand, single low doses of antigen were found to induce FasL-independent but Bim-dependent deletion. These observations are in accordance with the notion that during shutdown of an acute immune response once the pathogen is eradicated the elimination of T cells does not occur by repeated activation of the T cell receptor, which would induce activation of the Fas/FasL pathway, but involves lack of survival signals and Bim. In our own experiments mice were treated with a single high dose of SEB, and we observed that both Bim and FasL/Fas participate in T cell depletion. We thus wondered whether during this FasL and Bim-dependent retraction phase in vivo Bim may be part of the Fas signaling pathway and thereby amplify Fas-induced apoptosis via the mitochondrial pathway. Thus, on day 4 after SEB injection (at the maximal expansion phase of activated Vβ8$^+$ T cells) (Fig. 1B) we isolated lymphocytes from the lymph nodes and spleen of wild type and Bim-deficient animals, and re-stimulated them with anti-CD3 antibody, or treated them with recombinant FasL or dexamethasone (Fig. 1C). An obvious first observation was that Vβ8$^+$

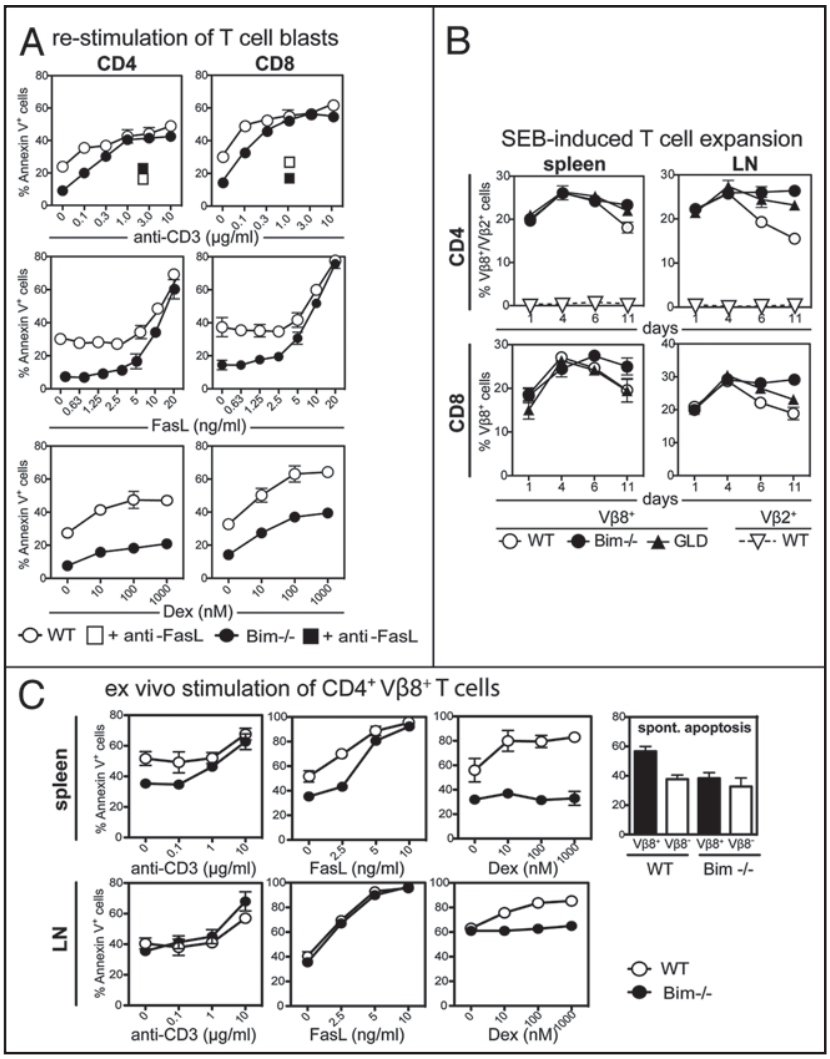

Figure 1. FasL/Fas and Bim represent two independent T cell apoptosis pathways. (A) T cell blasts were generated from wild type and Bim-deficient mice by activation of isolated spleen cells with the lectin concanavalin A for 48 hours and subsequent culture in media containing 50 U/ml IL-2 for 5 days. Pre-activated T cells were then re-stimulated with different concentration of immobilized anti-CD3 antibody, or triggered with recombinant FasL or with dexamethasone (Dex). Apoptosis was detected after 6 hours by Annexin V staining in the CD4$^+$ and CD8$^+$ T cell subsets. AICD in both, wild type and Bim-deficient mice was blockable with 3 μg/ml anti-FasL antibody. (B) Superantigen-induced T cell expansion. Wild type, Bim-deficient and FasL mutant *gld* mice were injected i.v. with 100 μg/mouse staphylococcal enterotoxin B (SEB). At different time points (4, 6, 11 days) after injection the percentage of Vβ8$^+$ CD4$^+$ and CD8$^+$ T cells in lymph nodes (LN) and spleen was analyzed by flow cytometry. As negative control Vβ2$^+$ CD4$^+$ and CD8$^+$ T cells were monitored in parallel (non-SEB-responsive). (C) Ex vivo stimulation of Vβ8$^+$ CD4$^+$ T cells. Lymphocytes were isolated 4 days after in vivo SEB injection from the lymph nodes (LN) and spleen of wild type and Bim-deficient animals, and re-stimulated with different concentrations of plate-bound anti-CD3 antibody, or incubated with recombinant FasL or dexamethasone (Dex). Apoptosis was detected after 6 h by Annexin V staining in the Vβ8$^+$ CD4$^+$ and Vβ8$^+$ CD8$^+$ T cell subsets. Spontaneous apoptosis of Vβ8$^+$ CD4$^+$ T cell and Vβ8$^-$ CD4$^+$ T cells was assessed in splenocytes isolated from both wild type and Bim-deficient mice.

CD4+ T cells isolated from spleen of wild type mice were clearly more sensitive to spontaneous ex vivo apoptosis than Vβ8- T cells (Fig. 1C, upper right). This observation is in agreement with the idea that the pre-activated T cells are more prone to apoptosis than the resting T cell pool. In contrast, Bim-deficient Vβ8+ and Vβ8- T cells showed equally low spontaneous apoptosis. While absence of Bim clearly favored increased survival of splenic T cells from spontaneous apoptosis, no differences were seen between wild type and Bim-deficient T cells isolated from the axial lymph node, suggesting that Bim-induced apoptosis may be more relevant for T cells in the spleen. An important role for Bim was also confirmed in dexamethasone-induced cell death, in splenic and lymph node CD4+ Vβ8+ T cells. However, comparable sensitivity to apoptosis was found in wild type and Bim-deficient Vβ8+ CD4+ T cells when stimulated ex vivo with either plate-bound anti-CD3 or recombinant soluble FasL. Again, we observed that AICD of ex vivo T cells was blockable by neutralizing anti-FasL antibody confirming the important role of FasL/Fas interaction in AICD (Fig. 1C). Very similar results were obtained with Vβ8+ CD8+ T cells (data not shown).

These finding lead to the conclusion that Bim does not participate in FasL/Fas apoptosis signaling in T cells neither in vitro nor in vivo. Although both, Bim and FasL clearly participate in T cell homeostasis and peripheral deletion, they represent effector molecules of two distinct apoptosis pathways. These results contrast our previous findings in murine hepatocytes where an important role of Bim in the Fas signaling pathway was established,[6] and suggest a cell type-specific use of the mitochondrial amplification of death receptor apoptosis pathways. While our present study demonstrates an important but independent role for Bim and FasL in T cell apoptosis, their respective role at different stages and check-points during an immune response remains to be established.

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