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Cleavage of Anti-Apoptotic Bcl-2 Family Members after TCR Stimulation Contributes to the Decision between T Cell Activation and Apoptosis

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Engagement of the TCR induces activation-induced cell death (AICD) of T cells that have been previously stimulated. However, a portion of these T cells can survive and undergo further activation. The molecular mechanism that decides whether a T cell will live or die after TCR re-engagement is unclear. We found that cross-linking of TCR in preactivated primary mouse T cells led to the cleavage of anti-apoptotic Bcl-2 and Bcl-xL in dying cells. Cleavage-resistant Bcl-2 and Bcl-xL were more efficient than their wild-type counterparts in the inhibition of apoptosis in primary mouse T cells and in the H9 T cell line after TCR cross-linking. In contrast, the surviving T cells after TCR re-engagement displayed upregulation of Bcl-xL, and knockdown of Bcl-xL promoted AICD. This indicates that caspase-mediated cleavage of anti-apoptotic Bcl-2 or Bcl-xL facilitates AICD in T cells, whereas upregulation of Bcl-xL promotes T cell survival and allows further T cell activation. Our data suggest that cleavage of anti-apoptotic Bcl-2 and Bcl-xL contributes to the decision between T cell activation and apoptosis after TCR re-engagement. The Journal of Immunology, 2013, 190: 168–173.

The clearance of significantly expanded T cells after an immune response is critical for the maintenance of lymphocyte homeostasis and immune tolerance (1–4). At the peak of immune responses, activated T cells may undergo activation-induced cell death (AICD) that involves the induction of Fas-mediated apoptosis through autocrine secretion of Fas ligand (5, 6). Genetic disruption of the interaction between the death receptor Fas and Fas ligand results in the accumulation of lymphoid cells in systemic autoimmunity and lymphoproliferative disease (7–12). Mutations in Fas or Fas ligand have also been shown to cause defective AICD of T cells in the autoimmune lymphoproliferative syndrome in humans (2). AICD is important for limiting the scope of Ag-specific T cell activation and protecting against the development of autoimmunity (2).

Compound deficiencies of Fas and a proapoptotic Bcl-2 family member, Bim, have been shown to induce more severe lymphocyte accumulation and autoimmune manifestations in mice (13–15). This suggests that death receptor–mediated extrinsic apoptosis and mitochondrion-dependent intrinsic apoptosis pathways are both important for the regulation of lymphocyte homeostasis and immune tolerance. It has been shown that loss of Bim causes defective AICD in T cells (16). An activating mutation of NRAS can cause the development of autoimmune lymphoproliferative syndrome with downregulation of Bim and T cell expansion (17). Therefore, defects in the intrinsic apoptosis pathway may lead to decreased AICD in human autoimmune diseases.

Anti-apoptotic Bcl-2 family proteins, such as Bcl-2 and Bcl-xL, maintain the integrity of the mitochondrial outer membrane by inhibiting proapoptotic Bax and Bak (18, 19). During apoptosis, Bcl-2 and Bcl-xL can be cleaved by caspases and lose their anti-apoptotic functions (20–22). Bcl-2 and Bcl-xL with mutations of the cleavage sites are more efficient in inhibiting apoptosis than their wild-type counterparts, suggesting that caspase-induced cleavage indeed inactivates the anti-apoptotic functions of Bcl-2 and Bcl-xL (20–22). Notably, the loss of mitochondrial membrane potential (ΔΨm) during cell death is inhibited when caspase-9, or caspase-3 and caspase-7, are deficient (23–27). This suggests an important role for caspases in feedback disruption of mitochondria to promote apoptosis. We have found that dimerization of caspase-9 induces the activation of a downstream caspase cascade through caspase-3 (28) and triggers a feedback loop that promotes mitochondrial disruption by cleavage of anti-apoptotic Bcl-2 family proteins (22). However, whether caspase-mediated cleavage of anti-apoptotic molecules plays a physiological role in the execution of apoptosis in T cells is not known.

Although engagement of the TCR on prestimulated T cells can trigger AICD, this usually does not lead to apoptosis of all T cells. A portion of the T cells can survive and become further activated. What decides whether a T cell lives or dies in response to TCR re-engagement is not clear. We detected cleavage of Bcl-2 or Bcl-xL and simultaneous upregulation of Bcl-xL in T cells after TCR re-engagement. Although cleavage of Bcl-2 and Bcl-xL was detected in apoptotic T cells, upregulation of Bcl-xL was found in surviving T cells. Our data suggest that cleavage of anti-apoptotic Bcl-2 or Bcl-xL can tip the balance between cell activation and cell death after T cells re-encounter the Ag.

Materials and Methods

T cell culture and flow cytometry

Splenocytes from wild-type or lpr mice on the C57BL/6 background were cultured in the presence of 5 μg/ml Con A in RPMI complete medium for 48 h, followed by culture with 100 U/ml IL-2 for 48 h. For small interfering RNA (siRNA) silencing, mouse splenocytes were stimulated with 5 μg/ml Con A for 48 h, followed with 100 U/ml IL-2 for 48 h, with the
presence of 1 μM Acell bcl-xL or nontargeting siRNA in Acell siRNA delivery media (Dharmacon) throughout the culture. For anti-CD3-induced cell death, T cells (2.5 × 10^5/ml) were cultured in 96-well flat-bottom plates with or without coating with 1 μg/ml anti-CD3 (2C11; BD Biosciences) for 24 h. The percentage of cell loss was quantitated by flow cytometry as described (22, 28). T cells were also stained with FITC–anti-CD4, cyochrome–anti-CD8, and PE-conjugated Abs to CD25, Fas, or ICAM (BD Biosciences), followed by flow cytometry. Intracellular staining for IFN-γ was performed as described (29). For Western blot analyses, T cells were cultured in 24-well plates with or without anti-CD3 coating (2 × 10^5 cell/well) for 8 or 24 h in the presence of 10 μM MG132 during the last 4 h of culture. The cells were lysed for Western blot analyses. In some experiments, T cells after 8-h culture on anti-CD3 plates were stained with FITC–annexin V, followed by sorting for annexin V+ dying cells or annexin V– live cells. The sorted cells were used for Western blot analyses.

Mouse T cells stimulated with Con A and IL-2 as above were cultured with or without anti-CD3 stimulation for 24 h. Live cells were isolated by Ficoll-gradient separation and washed to remove IL-2. The cells were then cultured in the absence or presence of 100 U/ml for 24 h. IL-2 withdrawal-induced cell death was determined. Cell lysates were also prepared for Western blot analyses of Bcl-2 and Bcl-xL.

Human H9 T cells were transfected with HA-tagged wild-type or cleavage-resistant Bcl-2 or Bcl-xL as described (22). The cells were cultured in 96-well plates coated with 10 μg/ml anti-CD3 (UCHT1; BioLegend) for 24 h, followed by Western blot or cell death assays.

**FIGURE 1.** Engagement of TCR on preactivated T cells leads to both cell activation and apoptosis. (A) T cells stimulated with Con A and IL-2 were cultured for 24 h in tissue culture plates with or without 1 μg/ml anti-CD3 coating. The cells were stained with 10 μg/ml PI and analyzed by flow cytometry. Forward scattering (FSC) versus side scattering (SSC) or FSC versus PI staining was plotted. (B) T cells without (control) or with anti-CD3 stimulation as in (A) were stained with various cell surface markers and analyzed by flow cytometry. Intracellular staining of IFN-γ was also performed. The expression of different surface markers and intracellular IFN-γ on live CD4+ or CD8+ T cells was plotted. (C) T cells stimulated with anti-CD3 were incubated with DEVD-FITC, followed by staining with PE–annexin V. (D) T cells stimulated with anti-CD3 and incubated with DEVD-FITC were stained with PE–anti-Fas or PE–anti-Fas ligand, followed by flow cytometry analyses. Cells positive or negative for DEVD staining were gated, and the expression of Fas or Fas ligand was plotted. (E) T cells as in (A) were cultured on plates coated with different concentrations of anti-CD3 for 24 h. Percentages of cell death were plotted. (F) T cells as in (A) were cultured with different concentrations of anti-CD3 for 6 h in tissue culture plates. The cells were incubated with DEVD-FITC and analyzed by flow cytometry.

**Western blotting**

Cells were lysed in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1× protease inhibitor mixture from Roche, 2 μM NAD-fmk) and used for Western blotting as described (22, 28). The following primary Abs were used for Western blotting: mouse mAbs to Bcl-2 (BD Biosciences, San Jose, CA) and anti-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit polyclonal Ab to Bcl-xL (Cell Signaling Technology, Danvers, MA). The blots were then incubated with HRP-conjugated secondary Abs (Southern Biotech, Birmingham, AL), followed by development with the SuperSignal West Dura substrate (Thermo Scientific, Waltham, MA).

**Transduction of T cells**

cDNA for wild-type or cleavage-resistant Bcl-2 or Bcl-xL was fused to HA and cloned into a lentiviral plasmid, pTRIP, under the control of a CMV-promoter and followed by IRES-GFP (30). Recombinant pseudotyped lentiviral vector was generated by cotransfecting pTRIP vectors with pCMV-Δ R8.9 encoding gag and pol, and pMD.G encoding env into 293T cells as described (31). The viral supernatants from 24 to 72 h after transfection were centrifuged at 140,000 × g for 2 h, and viral pellets were resuspended in RPMI complete medium and titrated using NIH3T3 cells as described (30). Mouse T cells after stimulation with 5 μg/ml Con A were exposed to five multiplicities of infection of the lentiviral vectors and 8 μg/ml polybrene, followed by centrifugation at 1000 × g for 45 min at room temperature. After culture in the presence of 100 U/ml IL-2 at 37˚C for
12 h, T cells were washed and cultured in fresh medium containing 100 U/ml IL-2 for another 36 h. T cells were then added to tissue culture plates with or without anti-CD3 coating and cultured for 24 h. The loss of GFP+ transduced T cells was determined by flow cytometry.

Results

Cross-linking of TCR on prestimulated T cells leads to both T cell apoptosis and T cell activation

It is well established that stimulation of T cells sensitizes T cells to apoptosis (32, 33). Although re-engagement of TCR on pre-activated T cells led to significant induction of cell death (Fig. 1A), a portion of these T cells survived as shown by the exclusion of the staining with propidium iodide (PI) after re-engagement of the TCR (Fig. 1A). These surviving T cells displayed increased cell size by forward scattering (Fig. 1A). They also retained surface expression of Fas, but showed increased CD25 and ICAM-1 after anti-CD3 stimulation (Fig. 1B). In addition, anti-CD3 also induced the expression of IFN-\(\gamma\) in the surviving CD4+ and CD8+ T cells (Fig. 1B). These data suggest that restimulation of T cells via the TCR not only triggers apoptosis in some T cells but also induces activation in the surviving T cells with increased expression of cytokines, cytokine receptors, and adhesion molecules that may allow better responses to cytokines and interactions with other cell types. However, what intracellular signaling events might decide whether a T cell undergoes apoptosis or survives and becomes further activated after TCR re-engagement is not clear.

We next compared the surviving and dying cells after TCR cross-linking. As expected, the dying cells positive for annexin V staining also displayed caspase activation by cleavage of DEVD-FITC (Fig. 1C). T cells with or without caspase activation expressed similar levels of Fas or Fas ligand after TCR cross-linking (Fig. 1D), suggesting that cell death or cell activation is not determined by differential expression of these apoptosis molecules on the cell surface. We also observed that increased concentrations of anti-CD3 led to more cell death (Fig. 1E) and caspase activation (Fig. 1F). This suggests that stronger intensity of TCR cross-linking favors cell death over cell activation, whereas weaker initial TCR stimulation may lead to T cell activation without triggering cell death.

Cleavage of Bcl-2 and Bcl-xL during AICD in H9 cells

We have previously observed cleavage of Bcl-2 and Bcl-xL in H9 T cell lines after chemically induced dimerization of a transfected caspase-9 (22). Cleavage-resistant Bcl-2 and Bcl-xL also provide resistance to etoposide-induced cell death in H9 cells (22). However, whether cleavage of Bcl-2 and Bcl-xL plays a role in the T cells (Fig. 1B). These data suggest that restimulation of T cells via the TCR not only triggers apoptosis in some T cells but also induces activation in the surviving T cells with increased expression of cytokines, cytokine receptors, and adhesion molecules that may allow better responses to cytokines and interactions with other cell types. However, what intracellular signaling events might decide whether a T cell undergoes apoptosis or survives and becomes further activated after TCR re-engagement is not clear.

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Cleavage of Bcl-2 and Bcl-xL after TCR cross-linking in primary T cells

We have previously observed cleavage of Bcl-2 and Bcl-xL in H9 T cell lines after chemically induced dimerization of a transfected caspase-9 (22). Cleavage-resistant Bcl-2 and Bcl-xL also provide resistance to etoposide-induced cell death in H9 cells (22). However, whether cleavage of Bcl-2 and Bcl-xL plays a role in the T cells (Fig. 1B). These data suggest that restimulation of T cells via the TCR not only triggers apoptosis in some T cells but also induces activation in the surviving T cells with increased expression of cytokines, cytokine receptors, and adhesion molecules that may allow better responses to cytokines and interactions with other cell types. However, what intracellular signaling events might decide whether a T cell undergoes apoptosis or survives and becomes further activated after TCR re-engagement is not clear.

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primary T cell apoptosis is unknown. It is well established that Fas ligand–induced apoptosis through Fas mediates AICD in T cells in an autocrine fashion. As expected, TCR cross-linking induced apoptosis in wild-type mouse T cells stimulated with Con A and IL-2, whereas Fas-deficient lpr T cells were resistant to such AICD (Fig. 2A). Bcl-2 and Bcl-xL were also processed in wild-type T cells after 8 h of stimulation with anti-CD3 (Fig. 2B). However, the processing of Bcl-2 and Bcl-xL was lacking in Fas-deficient T cells (Fig. 2B), indicating a critical role for Fas-mediated caspase activation in the cleavage of Bcl-2 and Bcl-xL. However, these experiments do not distinguish whether the processing of Bcl-2 and Bcl-xL contributes to cell death or is merely a consequence of cell death.

**Inhibition of AICD in H9 T cells after TCR cross-linking by Bcl-2<sup>ΔDA</sup> or Bcl-xL<sup>ΔDA</sup>**

We detected cleavage of Bcl-2 and Bcl-xL in H9 T cells after TCR cross-linking (Fig. 3A). To investigate whether cleavage of Bcl-2 and Bcl-xL contributed to the induction of cell death in H9 cells, we expressed wild-type and cleavage-resistant Bcl-2 and Bcl-xL (22) with aspartate to alanine substitution at the caspase cleavage sites (Bcl-2<sup>ΔDA</sup> or Bcl-xL<sup>ΔDA</sup>) in H9 cells (Fig. 3B). As expected, TCR cross-linking led to the cleavage of wild-type but not cleavage-resistant Bcl-2 or Bcl-xL (Fig. 3C). TCR cross-linking also efficiently induced AICD in H9 cells (Fig. 3D). Bcl-2<sup>ΔDA</sup> or Bcl-xL<sup>ΔDA</sup> but not wild-type Bcl-2 or Bcl-xL<sup>Δ</sup>, significantly inhibited AICD in H9 cells (Fig. 3D). This is consistent with the possibility that cleavage of Bcl-2 and Bcl-xL facilitates the induction of AICD in H9 T cells.

**Inhibition of AICD in primary T cells after TCR cross-linking by Bcl-2<sup>ΔDA</sup> or Bcl-xL<sup>ΔDA</sup>**

To determine the effects of Bcl-2 and Bcl-xL on AICD in primary T cells, we generated retroviral vectors expressing either wild-type or cleavage-resistant Bcl-2 or Bcl-xL followed by a GFP reporter gene (Fig. 4A). We observed that Bcl-2<sup>ΔDA</sup> and Bcl-xL<sup>ΔDA</sup> were more efficient than their wild-type counterparts in the inhibition of AICD in T cells (Fig. 4B). This supports the conclusion that cleavage of Bcl-2 and Bcl-xL facilitates the induction of AICD in primary T cells.

*FIGURE 5.* Cleavage of Bcl-2 and Bcl-xL is correlated with cell death, whereas upregulation of these molecules is correlated with T cell activation. (A) Mouse T cells stimulated with Con A and IL-2 were cultured with or without anti-CD3 for 8 or 24 h. The cells were lysed for Western blot analyses. (B) Mouse T cells stimulated with Con A and IL-2 were cultured on anti-CD3–coated plates for 8 h. The cells were stained with FITC–annexin V. Cells positive or negative for annexin V staining were sorted (left panel). The cells were lysed and used for Western blot analyses (right panel). The intensities of the protein bands were determined using ImageJ. The ratios of intensities of full-length Bcl-2 versus tubulin: 0.895 (no anti-CD3), 0.966 (anti-CD3 live), and 0.650 (anti-CD3 dying); ratios for full-length Bcl-xL versus tubulin: 1.354 (control, no anti-CD3), 4.386 (control, anti-CD3), 0.208 (Bcl-xL siRNA, no anti-CD3), and 0.733 (Bcl-xL siRNA, anti-CD3). Percentages of cell death after anti-CD3 stimulation for 24 h were also quantitated (lower panel). **p < 0.01.

*FIGURE 6.* T cells surviving anti-CD3 stimulation are susceptible to IL-2 withdrawal–induced cell death. (A) Mouse T cells stimulated with Con A and IL-2 were cultured with or without anti-CD3 stimulation. Live cells were isolated and cultured in the absence or presence of IL-2 for 24 h. Percentage of cell loss induced by IL-2 withdrawal was quantitated in T cells with or without anti-CD3 pretreatment. (B) T cells with or without anti-CD3 pretreatment were cultured in the presence or absence of IL-2 as in (A) and used for Western blot analyses.
loration of Bcl-xL in the sorted live cells after anti-CD3 stimulation (Fig. 5B). To determine whether upregulation of Bcl-xL contributes to T cell survival after TCR cross-linking, we silenced bcl-xL in T cells using the Acell siRNA delivery method (Dharmacon) with ~80% knockdown of Bcl-xL protein expression (Fig. 5C, upper panel). We found that suppression of Bcl-xL led to significant increases of AICD in mouse primary T cells (Fig. 5C, lower panel). This suggests that the upregulation of Bcl-xL indeed leads to better T cell survival after TCR cross-linking.

Surviving T cells after TCR re-engagement are susceptible to IL-2 withdrawal–induced cell death

AICD is expected to take place at the peak of an immune response to delete Ag-specific T cells. This is likely to be important for controlling the magnitude of Ag-specific immune responses. After the clearance of Ags, T cells that have escaped AICD may undergo cell death due to lack of cytokines or other stimulation. We therefore isolated live T cells after TCR re-engagement. Indeed, T cells that had survived anti-CD3 restimulation underwent rapid cell death after IL-2 withdrawal (Fig. 6A). Moreover, these T cells downregulated Bcl-xL without the detectable cleavage of Bcl-2 or Bcl-xL (Fig. 6B). Therefore, T cells that survived repeated Ag stimulation are nevertheless sensitive to cytokine withdrawal–induced cell death, which may involve downregulation of Bcl-xL but not cleavage of anti-apoptotic Bcl-2 family members.

Discussion

Our results suggest that caspase-mediated cleavage of Bcl-2 and Bcl-xL can tip the balance between life and death in T cells reactivated through their TCR. Cross-linking of the TCR led to both cleavage and upregulation of Bcl-2 and Bcl-xL in activated primary T cells. Western blot analyses of sorted live and apoptotic T cells after TCR cross-linking showed the cleavage of Bcl-2 and Bcl-xL in apoptotic cells. Upregulation of Bcl-xL, but not cleavage of Bcl-2 or Bcl-xL, was found in live cells. Retroviral transduction of T cells showed that caspase-resistant Bcl-2 and Bcl-xL were efficient in inhibiting AICD in T cells. Our data suggest that re-engagement of the TCR leads to a certain level of initial caspase activation, resulting in cleavage of Bcl-2 and Bcl-xL and amplification of mitochondrial disruption to promote cell death.

Fas plays an important role in activation-induced cell death in T cells (5). Recent studies suggest mitochondrial-dependent cell death regulated by Bim is also involved in inducing AICD in T cells (16). As expected, Fas-deficient T cells were resistant to AICD. Cleavage of Bcl-2 and Bcl-xL was not detectable in Fas-deficient T cells. We have previously observed that caspase-3 is the major effector caspase for the processing of Bcl-2 and Bcl-xL (22). The activation of mitochondrial-dependent apoptosis by cleavage of Bcl-2 and Bcl-xL is not merely the consequence of apoptosis, but rather is likely to contribute to AICD in T cells. Consistent with this possibility, cleavage-resistant Bcl-2 and Bcl-xL both showed better protection of the H9 T cell line or primary T cells against AICD. This supports our conclusion that cleavage of Bcl-2 and Bcl-xL helps to tip the balance between life and death for T cells re-encountering Ags. We found that more cells displayed caspase activation and underwent cell death with increased concentrations of anti-CD3 (Fig. 1C, 1D). This suggests that stimulation over a certain threshold will favor the induction of cell death. We propose that a certain level of initial caspase activation leads to cleavage of Bcl-2 and Bcl-xL, which leads to amplification of mitochondrial disruption, more caspase activation, and cell death.

AICD is likely to take place at the peak of immune responses when T cells are activated and Ags are still abundant. AICD is therefore potentially important for limiting the scope of Ag-specific immune responses. This may help prevent uncontrolled T cell expansion and the induction of inflammatory and autoimmune responses. In addition to triggering cell death in activated T cells, TCR engagement also upregulated Bcl-xL in T cells that survived. siRNA silencing experiments suggest that upregulation of Bcl-xL is critical for suppressing mitochondrion-dependent apoptosis to protect T cells from AICD (Fig. 5C). This may allow some T cells to survive longer in the presence of Ag stimulation.

After the clearance of Ags, lack of active immune responses likely causes the decrease in T cell stimulation and cytokine production. T cells that have survived the restimulation from TCR may subsequently undergo cell death due to lack of cytokines. Different from AICD, IL-2 withdrawal did not trigger the cleavage of anti-apoptotic Bcl-2 family members, but rather caused the downregulation of Bcl-xL. The contribution of cleavage of Bcl-2 or Bcl-xL to AICD suggests that different cell death pathways interact to make the decision between life and death for T cells during immune responses. This may help to fine-tune the duration and magnitude of immune responses to various bacterial or viral infections that have different Ags and infectious cycles.

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Disclosures

The authors have no financial conflicts of interest.

References


