The NF-κB regulator Bcl-3 and the BH3-only proteins Bim and Puma control the death of activated T cells

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Apoptosis of activated T cells is critical for the termination of immune responses. Here we show that adjuvant-stimulated dendritic cells secrete cytokines that prime activated T cells for survival and analyze the roles of the NF-κB regulator Bcl-3 and the proapoptotic Bcl-2 family members Bim and Puma. Bcl-3 overexpression increased survival, and activated *bcl-3*$^{-/-}$ T cells died abnormally rapidly. Cytokines from adjuvant-stimulated dendritic cells induced Bcl-3, but survival through cytokine priming was Bcl-3-independent. Apoptosis inhibition by Bcl-3 involved blockade of Bim activation, because Bim was overactivated in Bcl-3-deficient cells, and Bcl-3 failed to increase survival of *bim*$^{-/-}$ T cells. However, adjuvants increased survival also in Bim-deficient T cells. This Bim-independent death pathway is at least in part regulated by Puma, as shown by analysis of puma$^{-/-}$ and noxa$^{-/-}$ T cells. IL-1, IL-7, and IL-15 primed T cells for survival even in the absence of Bim or Puma. Our data define interrelations and a Bim-independent pathway to activated T cell death.

adjuvant | apoptosis | Bcl-2-family | survival

In the adaptive immune system, apoptosis is critical for the contraction of the expanded T cell pool at the end of the immune response. T cells that recognize their cognate antigen are activated and expand to form reactive clones. Once the infectious agent is cleared, most of these activated T cells die by apoptosis, precluding immunopathology and autoimmunity (1, 2).

How this form of apoptosis is regulated at the molecular level has been the object of intense research. Antigen-activated T cells can be killed by a second signal through the T cell receptor (TCR), a process termed activation-induced cell death, regulated through the interaction of Fas/APO-1/CD95 and its ligand. The role of Fas in activated T cell death has been addressed in a number of studies (for a recent discussion, see ref. 2), and the most recent studies do not support a role for FasL/Fas in T cell immune response shutdown (3, 4).

The second pathway to apoptosis (the mitochondrial pathway) is regulated by the Bcl-2-family of proteins (5). This pathway starts with the activation of members of the BH3-only protein subgroup, which leads to the activation of the proapoptotic proteins Bax and/or Bak. Bax/Bak activation causes the release of cytochrome *c* from the mitochondria into the cytosol, where it induces the activation of caspases. The antiapoptotic Bcl-2 and closely related proteins such as Bcl-X_L inhibit this pathway by interfering with the BH3-only protein-mediated activation of Bax/Bak (6, 7).

The current model of activated T cell death proposes that the BH3-only protein Bim/Bod is activated in T cells at the end of the immune response, then leads to the activation of Bax and Bak. This model is based on the observation that the loss of Bim impairs the death of activated T cells *in vivo* and *in vitro* (3, 4, 8), and that transgenic expression of the Bim antagonist Bcl-2 can inhibit the death of activated T cells (9).

An unconnected observation is the finding that the NF-κB regulator Bcl-3 is involved in the inhibition of activated T cell death. The addition of adjuvants, such as LPS, to a TCR stimulus (superantigen) *in vivo* induced the up-regulation of Bcl-3 mRNA and prolonged the survival of activated T cells. Retroviral transduction with a Bcl-3 expression vector increased T cell survival in the absence of adjuvants (10). Bcl-3 therefore somehow delays apoptosis of activated T cells.

Thus, with Bim, Bcl-3 and adjuvant-dependent stimuli, three factors have been identified that affect the survival of activated T cells. Here, we aimed at clarifying the interrelation of these factors. Bcl-3 was found to inhibit the activation of Bim. Adjuvant-induced cytokines regulated Bcl-3 expression and, in addition, Bcl-3-independent survival pathways. Surprisingly, a Bim-independent death pathway for killing activated T cells was identified, and it appears to require the BH3-only protein Puma/Bbc3.

Results

A Soluble Dendritic Cell (DC)-Derived Factor Mediates Adjuvant-Induced T Cell Survival. Superantigens such as staphylococcal enterotoxin B (SEB) activate T cells expressing TCRα/β receptors containing certain Vβ segments by direct TCR crosslinking. In mice, this activation is visible as clonal expansion, followed by deletion (11). This is, however, probably not an ideal model for a T cell response to a pathogen, because in an infection, T cell costimulatory signals are provided by activated DC, both soluble and membrane-bound.

A model system more closely resembling an infection is therefore the coinjection of SEB and, as an adjuvant, a DC stimulus such as LPS. As reported (10, 12), injection of LPS prolonged survival of SEB-stimulated T cells *in vivo* and *ex vivo* (Fig. 7, which is published as supporting information on the PNAS web site). We noted a similar prosurvival effect *in vitro*. When total spleen cells were stimulated with the mitogen ConA, washed on day 3, and tested for survival 20 h later, the presence of LPS during the initial stimulation phase increased survival (Fig. 1*A*). This effect could be mimicked by stimulating purified T cells with ConA, antibodies to CD3, or antibodies to CD3 and CD28 in the presence of supernatant derived from LPS-stimulated DC (Figs. 1*B* and 7). Similar effects on T cell survival (*in vivo* and *in vitro*) were seen when LPS was replaced with CpG-DNA, although the magnitude of the effect was somewhat smaller (data not shown). Thus, the presence of DC-derived

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Abbreviations: SEB, staphylococcal enterotoxin B; DC, dendritic cell; TCR, T cell receptor; BMDDC, bone-marrow-derived DC.

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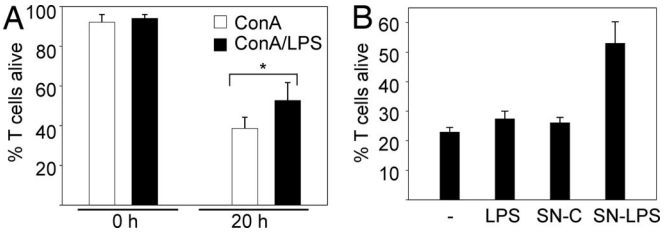

Fig. 1. DC-derived factors promote survival of activated T cells. (*A*) Spleen cells from C57BL/6 mice were stimulated *in vitro* for 3 days with ConA with or without LPS. On day 3, T cells were purified and transferred to 96-well plates. T cell survival was measured directly after washing and after 20 h of incubation in simple medium (no growth factors or mitogens). Values are mean ± SD from four independent experiments (*, $P < 0.001$). (*B*) T cells were purified from spleen by negative sorting and stimulated for 3 days with ConA, with or without the addition of LPS (1 μg/ml), supernatant from unstimulated BMDDC (SN C, 50% vol/vol) or supernatant from LPS-stimulated BMDDC (SN LPS, 50% vol/vol). On day 3, cells were washed and transferred into flat bottom 96-well plates, incubated for 20 h and cell death was measured by flow cytometry. Results are mean/SD of three independent experiments.

factors during TCR-stimulation conditions activated T cells for cell autonomous survival.

The DC supernatant did not induce expression of Bcl-2 or Bcl-X_L in activated T cells as assessed by Western blotting (not shown). Likewise, a reduction in the levels of Bax/Bak was not seen by Western blotting (not shown).

Bcl-3 Is Up-Regulated in the Presence of Adjuvant but Is Not the Sole Mediator of the Prosurvival Effect. Bcl-3 is structurally defined as a member of the IκB group of inhibitors of NF-κB transcription factors but is unusual in that it can be directly involved in the formation of transcriptional complexes at promoters (13). When T cells were activated by superantigen *in vivo*, the coadministration of LPS caused an up-regulation of bcl-3 mRNA (10). We observed an up-regulation of Bcl-3 protein in T cells stimulated *in vitro* in the presence of LPS or supernatant from LPS-stimulated DC (Fig. 2*A*). To test the relevance of this up-regulation, we analyzed T cell immune responses in mice lacking Bcl-3. Interestingly, LPS costimulation enhanced survival of activated T cells from *bcl-3*$^{-/-}$ T cells both in culture (Fig. 2 *B* and *C*) and within the whole animal (Fig. 8, which is published as supporting information on the PNAS web site). Factors present in conditioned medium of adjuvant-activated DC thus induce Bcl-3 expression, but Bcl-3 is dispensable within T cells for the prosurvival effects of adjuvant. This indicates that additional Bcl-3-independent adjuvant-induced signaling pathways promote survival of activated T cells.

Bcl-3 Has a Prosurvival Effect. Although adjuvant-conditioned medium enhanced T cell survival in the absence of Bcl-3, activated T cells from mice lacking Bcl-3 died more rapidly in culture than WT T cells. This was not seen in resting T cells but was evident in T cells activated with ConA *in vitro* for 1, 2, or 3 days (Fig. 3*A*). There was no further enhanced susceptibility of *bcl-3*$^{-/-}$-activated T cells to a range of apoptotic stimuli tested (staurosporine, etoposide, UV irradiation, ionomycin, taxol, or dexamethasone; data not shown). Bcl-3 therefore appears to specifically regulate the death of mitogen- or antigen-activated T cells.

Because loss of Bcl-3 accelerated the death of activated T cells, we tested whether forced expression of Bcl-3 could prolong their survival. As reported previously for SEB-activated T cells *in vivo* (10), transduction of T cells *in vitro* with a retroviral Bcl-3 expression construct prolonged survival of activated T cells (Fig. 3*B*). Bcl-3 thus clearly has a function in the regulation of T cell apoptosis, and the increase in Bcl-3 protein levels may contribute

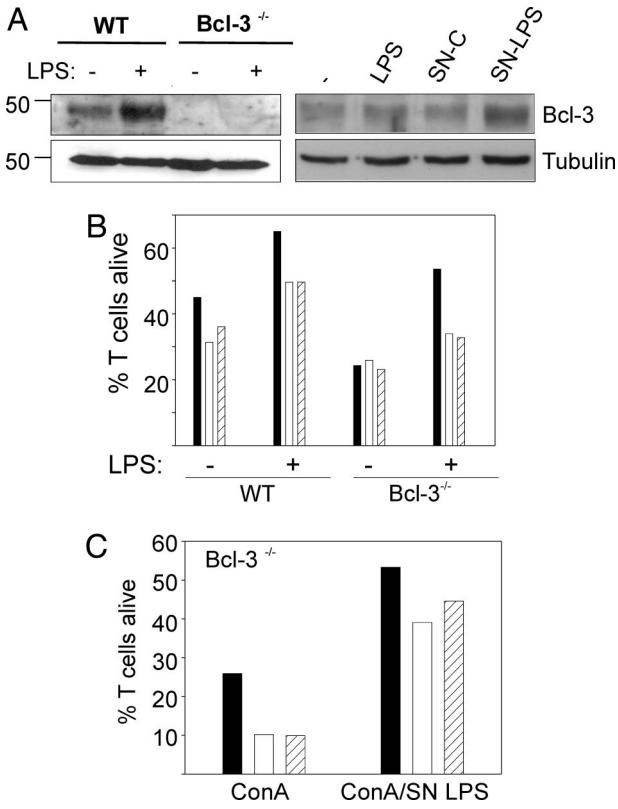

Fig. 2. Adjuvants cause up-regulation of Bcl-3 protein but can still improve T cell survival in its absence. (*A*) (*Left*) Spleen cells from WT or *bcl-3*$^{-/-}$ mice were stimulated *in vitro* for 3 days with or without addition of LPS. T cells were then purified and subjected to Western blot analysis. Similar results were seen in three separate experiments. Spleen cells from *bcl-3*$^{-/-}$ mice were used as a specificity control and detection of β-actin served as a loading control. (*Right*) Purified splenic T cells were stimulated *in vitro* for 3 days in six-well cell culture plates with ConA, ConA plus LPS, or ConA plus supernatant from unstimulated (SN C) or LPS-stimulated BMDDC (SN LPS). Cells were washed and analyzed by Western blotting. (*B*) Spleen cells from WT or *bcl-3*$^{-/-}$ mice were stimulated *in vitro* for 3 days with ConA with or without addition of LPS. T cells were then purified and transferred to 96-well plates, and survival was measured after 20 h. Three independent experiments are shown. (*C*) Purified splenic T cells from *bcl-3*$^{-/-}$ mice were stimulated *in vitro* for 3 days with ConA, ConA plus LPS, ConA plus supernatant from unstimulated (SN C), or LPS-stimulated BMDDC (SN LPS). Cells were washed and transferred to 96-well plates, and survival was measured after 20 h. Three independent experiments are shown.

to but is not essential for adjuvant-mediated increase in T cell survival.

Bcl-3 Promotes T Cell Survival by Blocking the Activation of Bim. Bcl-3 most likely acts by regulating gene expression. We therefore compared the gene expression pattern between activated WT and *bcl-3*$^{-/-}$ T cells by microarray analysis. No difference was observed in known apoptosis-regulating genes, such as the pro- and antiapoptotic Bcl-2 family members. Bim is a known mediator of activated T cell death. The microarrays showed an increase in expression of *bim* mRNA by a factor of 1.6, and Western blotting indicated marginally increased levels of Bim protein in activated *bcl-3*$^{-/-}$ T cells (Fig. 3*E*).

Elevated Bim levels might sensitize cells to apoptotic stimuli. However, viable cells can harbor easily detectable levels of inactive Bim, which need to be activated to trigger apoptosis (14). Copurification of Bim with microtubules can be used to measure its activation status, because inactive Bim can be coprecipitated from a cell lysate together with large tubulin-containing com-

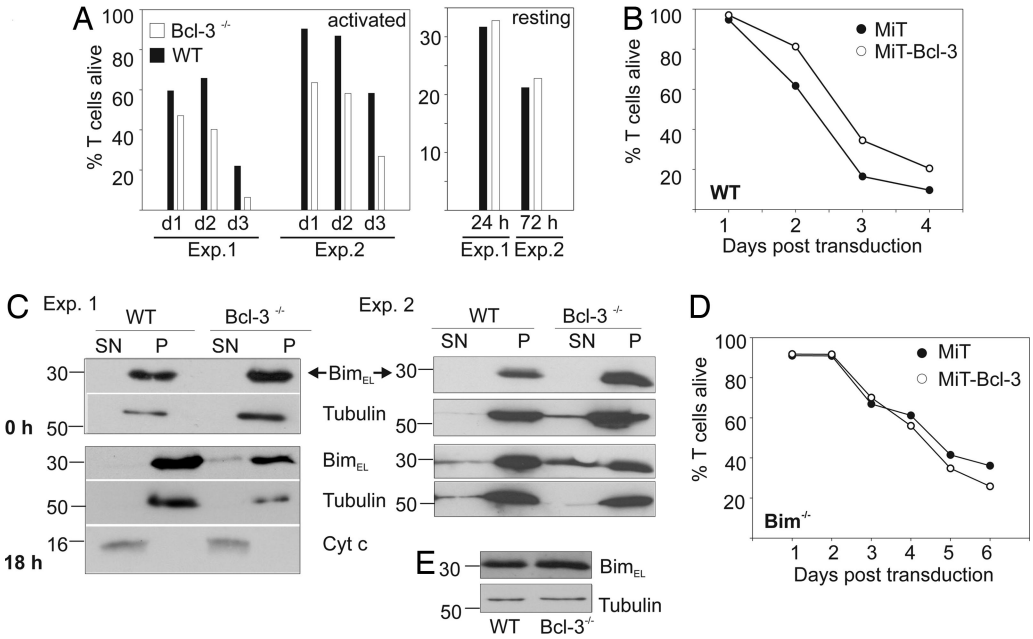

Fig. 3. Bcl-3 inhibits apoptosis of activated T cells by preventing the activation of Bim. (*A*) Activated Bcl-3-deficient T cells die more rapidly.(*Left*) Spleen cells from WT and *bcl-3*$^{-/-}$ mice were stimulated *in vitro* with ConA. On days 1, 2, or 3, T cells were purified, washed, and transferred into 96-well plates, and survival was determined 20 h later. Two independent experiments are shown. (*Right*) Resting T cells were purified and incubated in independent experiments for either 24 or 72 h. Another experiment was performed for 24-h incubation with similar results. (*B*) *Bcl-3* increases survival of activated T cells. Spleen cells from WT mice were stimulated *in vitro* for 2 days with ConA. Cells were then infected with empty control virus or retrovirus driving the expression of Bcl-3. One day later, cells were transferred to 96-well plates, and survival of transduced cells (identified by expression of the marker GFP) was measured at the times indicated (note the different time scales). Values are mean of triplicates in one experiment. Similar results were obtained in three separate experiments; one other experiment is shown in Fig. 8. (*C*) Increased release of Bim in *bcl-3*$^{-/-}$ T cells. Spleen cells from WT or *bcl-3*$^{-/-}$ mice were stimulated *in vitro* with ConA. On day 3, T cells were purified, washed, and 2.5×10^7 cells were prepared for subcellular fractionation (0 h, *Upper*). Another 2.5×10^7 cells were incubated for 18 h in the presence of the caspase-inhibitor z-VAD-fmk and then subjected to subcellular fractionation (*Lower*). Large complexes containing microtubules (after taxol polymerization) were precipitated by ultracentrifugation (P). Supernatants from this step (SN) were acetone-precipitated. Fractions were probed for Bim, tubulin (identifying microtubular fractions), and cytochrome *c* (as a loading control for the supernatant fraction). Only a small amount of released (activated) Bim$_{EL}$ is found in the SN fraction of WT T cells activated for 18 h, but this amount is increased in Bcl-3$^{-/-}$ samples. Data from two of three similar experiments are shown. (*D*) *Bcl-3* overexpression fails further to augment survival of Bim-deficient T cells. Cells were treated and the experiment was performed essentially as in *B* with the exception that *bim*$^{-/-}$ cells were used. Similar results were obtained in three separate experiments; one other experiment is shown in Fig. 9. (*E*) Bim expression is slightly elevated in Bcl-3-deficient T cells. Purified T cells from WT or *bcl-3*$^{-/-}$-mice were stimulated *in vitro* with ConA. On day 3, samples were analyzed for the expression of Bim. Staining with an antibody to tubulin was used as a loading control.

plexes. Upon its activation, Bim is released and can be recovered from the supernatant (14).

We compared the levels of microtubule-attached (inactive) and free (active) Bim. No free Bim was detected in T cells from WT or *bcl3*$^{-/-}$ mice that had been activated for 3 days with ConA (Fig. 3*C*). However, upon additional culture in the absence of growth factors [where abnormally increased apoptosis is seen in *bcl*-3$^{-/-}$ T cells and abnormally decreased apoptosis in *bim*$^{-/-}$ T cells (8)], small amounts of free Bim could be detected in samples from WT cells. This amount was clearly increased in cells from Bcl-3-deficient mice, indicating an accelerated activation of Bim in the absence of Bcl-3 (Fig. 3*C*).

This suggested that the function of Bcl-3 in the prevention of activated T cell death is the blockade of Bim activation. We further tested whether Bcl-3 was able to enhance the survival of activated T cells lacking Bim. Activated T cells from *bim*$^{-/-}$ mice showed enhanced survival in culture (8), although the cells eventually did die (Fig. 3*D*; compare to WT, Fig. 3*B*). However, in contrast to the results obtained with WT T cells (Fig. 3*B* and Fig. 9, which is published as supporting information on the PNAS web site), retrovirally mediated expression of Bcl-3 in Bim-deficient activated cells failed to improve their survival any further (Figs. 3*D* and 9). Bcl-3 thus exerts its antiapoptotic effect mostly, perhaps exclusively, by blocking the activation of Bim.

A Bim-Independent Apoptosis Pathway in Activated T Cells. The survival factors from adjuvant-stimulated DC appear to act by a process that is Bcl-3-independent (Fig. 2*C*). Because Bcl-3 appears to function by inhibiting Bim activation (Fig. 3 *C* and *D*), soluble factors may also act independently of Bim, and a Bim-independent pathway to activated T cell death may exist. We first compared the survival of activated T cells from *bcl-2*-transgenic and *bim*$^{-/-}$ mice. Bcl-2 can antagonize not only Bim but also other BH3-only proteins and therefore would inhibit Bim-dependent and -independent pathways. Bcl-2-overexpressing ConA-activated T cells clearly showed better survival than *bim*$^{-/-}$ T cell blasts, indicating the existence of a Bim-independent pathway (Fig. 4*A*). Furthermore, supernatants from LPS-stimulated DC enhanced survival also of *bim*$^{-/-}$ T cells (Fig. 4*B*). Previous studies have shown that Bim is required for the normal death of SEB-activated T cells *in vivo* and *in vitro* (4). We reproduced these results and detected almost no decline in the numbers of activated (TCRvβ8$^+$) T cells in the course of 10 days, when SEB was injected in Bim-deficient mice. However, coinjection of LPS caused a much stronger expansion and a persistence of TCRvβ8$^+$ T cells on a much higher level (Fig. 4*C*). These results indicate that apoptosis contributes to limit T cell expansion even in the absence of Bim, and that this apoptosis can be blocked by LPS-induced factors. Therefore, a second, Bim-independent pathway of activated T cell death must exist.

Activated Puma-Deficient T Cells Show Enhanced Survival. If other BH3-only proteins contribute to activated T cell death, Puma

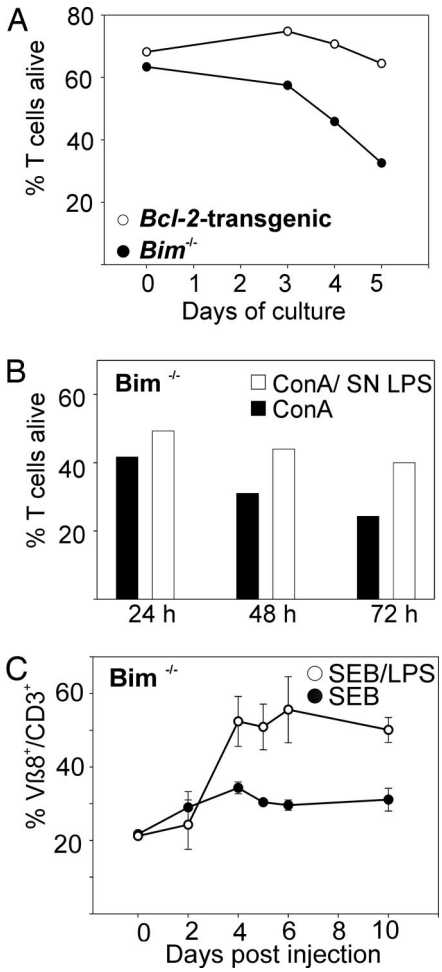

Fig. 4. Bim-independent pathways contribute to activated T cell death. (*A*) T cells were purified from spleens of either *bim*$^{-/-}$ or *vav-bcl-2*-transgenic mice and stimulated with ConA for 3 days. Cells were washed and incubated in 96-well plates for the indicated times before survival was measured. One of two separate experiments is shown. The second experiment is given as Fig. 10*A*, which is published as supporting information on the PNAS web site. Two further experiments investigating single time points were performed with similar results (not shown). (*B*) Purified T cells from spleen of *bim*$^{-/-}$ mice were stimulated for 3 days with ConA alone or in the presence of supernatant from LPS-stimulated BMDDC. T cells were washed, plated in simple medium (no growth factors or mitogens) and survival was measured at the indicated time points. One of two separate experiments is shown. The second experiment is given as Fig. 10*B*. (*C*) Six *bim*$^{-/-}$ mice were injected with SEB. Three mice received LPS 1 day later. The percentage of TCRvβ8^{+} T cells in peripheral blood was measured on the days indicated by immunofluorescent staining with surface marker-specific antibodies and flow cytometric analysis. Data are mean/SD from individual mice. Another experiment with one mouse for each treatment produced similar results.

was a promising candidate, because Puma-deficient thymocytes show increased survival in culture (15). Although the effect was smaller than in *bim*$^{-/-}$ mice, activated T cells from *puma*$^{-/-}$ mice clearly showed better survival than WT cells (Fig. 5). BH3-only protein Noxa was an additional candidate. We detected a small degree of up-regulation of *noxa* mRNA in T cells primed for apoptosis. This up-regulation was clearer in Bim-deficient T cells, presumably because these cells survive longer and can therefore still regulate *noxa* mRNA expression (Fig. 11*A*, which is published as supporting information on the PNAS web site). In accordance with this small up-regulation, noxa$^{-/-}$ T cells showed marginally improved survival (Fig. 11*B*).

Like Bim, Puma therefore plays a role in the initiation of

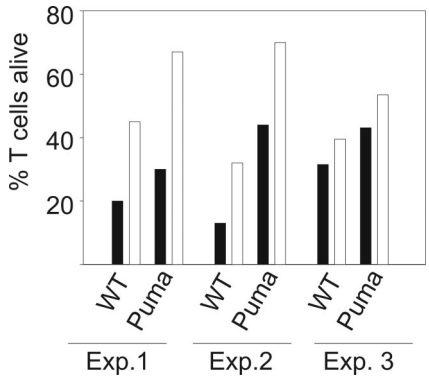

Fig. 5. Role of Puma in activated T cell death. Purified T cells from WT or from *puma*$^{-/-}$ mice were stimulated with ConA (filled bars). Some aliquots were activated in the presence of supernatant derived from LPS-stimulated BMDDC (open bars). On day 3, T cells were washed and plated in medium (no added growth factors), and survival was measured after 20 h.

apoptosis in activated T cells. Puma is known to be transcriptionally activated by the tumor suppressor p53 (16–18), and Puma-deficient cells are resistant to p53-dependent apoptotic stimuli, such as γ-irradiation or treatment with etoposide (15, 19). We therefore analyzed survival of p53-deficient activated T cells. However, these T cells showed no increased survival compared to WT T cells (data not shown), demonstrating that Puma activation in activated T cells occurs through a p53-independent mechanism.

Soluble Factors That Mediate Survival. We tested whether the prosurvival effect of DC-derived supernatant is also seen in Puma-deficient T cells. Activated *puma*$^{-/-}$ T cells also survived longer when activated in the presence of supernatant from LPS-stimulated DC than those activated by mitogens alone (Fig. 5). We tested individual cytokines for their ability to prime T cells during mitogenic stimulation for survival. IL-1, IL-7, and IL-15 showed this activity in WT T cells (Fig. 6) whereas TNF, IFN-α, IL-4, IL-10, IL-12, and IL-16 failed to improve T cell survival (data not shown). Perhaps surprisingly, IL-1, IL-7, and IL-15 had similar activities in WT, *bcl-3*$^{-/-}$, *bim*$^{-/-}$, and *puma*$^{-/-}$ T cells (Fig. 12, which is published as supporting information on the PNAS web site). This suggests that, during activated T cell death, a pathway is activated that can initiate the activation of both Bim and Puma. The presence of adjuvants during T cell activation, however, inhibits the activation of this pathway(s).

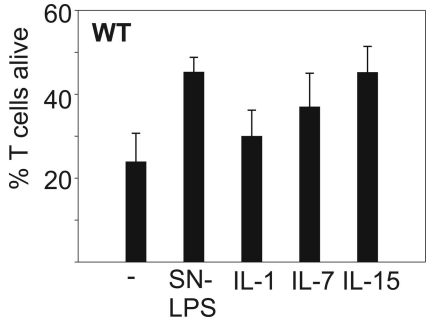

Fig. 6. IL-1, IL-7, and IL-15 prime T cells for enhanced survival. Purified T cells were stimulated with ConA, either alone or in the presence of BMDDC-LPS-derived supernatant or the recombinant cytokines IL-1, IL-7, or IL-15. On day 3, T cells were washed and transferred into simple medium (no added growth factors). After 20 h, survival was measured. Data are mean/SD from three independent experiments.

Discussion

It had previously been shown that Bim is a critical initiator of activated T cell death, whereas Bcl-3 is a negative regulator of this process, but the interrelation of these two factors has been unexplained. Our data show that Bcl-3 inhibits Bim activation. Moreover, our work shows that Puma contributes significantly to activated T cell death, and that Noxa may play a marginal role.

During infections, T cells are stimulated not only through the TCR but also by DC-derived adjuvant-induced signals. DC-derived adjuvant-induced soluble factors are produced that could affect T cell survival (2). Because, at the end of an immune response, these factors disappear, their withdrawal from T cells is likely to trigger T cell death. Certain cytokines, particularly those that signal through the common γ chain [IL-2, IL-4, IL-7, and IL-15 (20)], as well as type I IFN, promote survival of activated T cells (21). Our results describe a priming effect for IL-1, IL-7, and IL-15, cytokines known to be produced by adjuvant- (LPS) stimulated DC. Survival effects therefore can be conveyed by cytokines that are present either during activation (and their continued presence may not be required) or by cytokines that are present after T cells have been activated.

The mechanisms by which these factors enhance T cell survival are not clear. In the mitochondrial pathway, possibilities include: (*i*) down-regulation of both Bax and Bak (which we did not detect), (*ii*) induction of the antiapoptotic family members (which we did not observe for Bcl-2 or Bcl-X_L), or (*iii*) a change affecting the regulation of BH3-only proteins.

Indeed, two BH3-only proteins, Bim and Puma, contribute to the regulation of activated T cell death, Bim (refs. 3 and 4 and this study), and Puma. Noxa may play a marginal role. Bim activation can involve transcriptional induction by the Forkhead transcription factor FOXO3a (22, 23), JNK-dependent regulation (24, 25), and Erk-mediated phosphorylation that primes Bim for proteasomal degradation (26). However, transcription is clearly not the only mechanism for regulating Bim activity. Bim appears to be bound by the dynein light chain DLC1/LC8 to cytoskeletal structures (14). Stimuli that cause Bim-dependent apoptosis have been shown to trigger the release of Bim, allowing its translocation to mitochondria (14, 27). We observed that, in live T cells, Bim is almost exclusively found in the fraction containing microtubules, whereas a portion of Bim is released during apoptosis induction. Why others observed that in T cells Bim is localized on the mitochondria, complexed to the antiapoptotic proteins Bcl-2 and Bcl-X_L (28), is unclear.

An antiapoptotic activity of Bcl-3 has been demonstrated (10). It was speculated that Bcl-3 might function by regulating the expression of mitochondrial transporters and channels (2). We found that the amount of free Bim was increased in activated T cells from *bcl-3*$^{-/-}$ mice that underwent apoptosis, suggesting that Bcl-3 functions by inhibiting Bim activation. This observation is supported by the finding that forced expression of Bcl-3 keeps activated WT T cells alive but fails to enhance the survival of Bim-deficient T cells. Bcl-3 thus seems to be a regulator of Bim (but not Puma) activity. Bcl-3 overexpression is found in some forms of lymphoma, suggesting a contribution to lymphoma formation (29). Furthermore, Bim can be a suppressor of c-myc-induced lymphoma in mice (30). The Bcl-3-Bim pathway may therefore also be relevant to the formation of hematological malignancies.

Although the activation of Bim is clearly an important event in the initiation of activated T cell death, Puma also appears to have a significant role in this process. The two BH3-only proteins seem to be regulated by similar pathways in activated T cells. Thus, DC-derived factors inhibited both Puma and Bim, because they can block apoptosis of T cells lacking either Bim or Puma.

Puma is a transcriptional target of the tumor suppressor p53 and a mediator of p53-dependent apoptosis (15, 19), but it can also be induced by p53-independent apoptotic stimuli, such as serum withdrawal or treatment with glucocorticoids (18). IL-2 withdrawal in T cells can also up-regulate Puma expression by FOXO3a (31). Although *noxa* is primarily known as a p53 target gene, it can also be induced by p53-independent stimuli (32, 33). The data of this study suggest that growth factor withdrawal can also be a stimulus for the activation of both Puma and Noxa in T cells.

Our study thus identifies interrelations in the pathway to apoptosis of activated T cells. These results have implications for autoimmunity, T cell memory, and T cell homeostasis.

Methods

Reagents and Antibodies. ConA was from Amersham Pharmacia Bioscience. FITC-coupled antibodies to NK 1.1, B220, I-A/I-E, and TCRvβ8; CD90, antigen-presenting cell-coupled antibodies to CD3; and unconjugated antibodies to actin, CD28 (37.51), CD3 (clone 145–2C11), and CD16/CD32 were from BD Biosciences PharMingen. Anti-FITC-Ig-coupled beads were from Miltenyi Biotec (Bergisch Gladbach, Germany), and Apyrase, Taxol, LPS, SEB, Staurosporine, Etoposide, Taxol, Polybrene, and anti-α-tubulin and anti-Bim antibodies were from Sigma-Aldrich. Recombinant human IL-6, IL-10, IL-15, IL-1-α, IL-16, and IL-7 were from PeproTech (Rocky Hill, NJ). Ethidium monoazide bromide was from Invitrogen. Ionomycin was from Calbiochem. Z-VAD-fmk and dexamethasone were from Bachem.

Mice. C57BL/6 mice were purchased from Harlan-Winckelmann (Borchen, Germany). The strains *vav-bcl-2* (34), *bcl-3*$^{-/-}$ (35), and *bim*$^{-/-}$ (ref. 8; housed at the Institute for Medical Microbiology) and *puma*$^{-/-}$ (ref. 15; Institute for Pathology, Innsbruck, Austria) and *noxa*$^{-/-}$ mice (ref. 15; Walter and Eliza Hall Institute) have been described. The *vav-bcl*-2 transgenic, *noxa*$^{-/-}$ and *puma*$^{-/-}$ mice were generated on C57BL/6 background. The bcl-3$^{-/-}$ and bim$^{-/-}$ were generated on a mixed C57BL/6 $\times$ 129SV background (using 129SV ES cells) and were backcrossed onto the C57BL/6 background for 12 (*bcl-3*$^{-/-}$) or 14 (*bim*$^{-/-}$) generations. Mice were kept under specific pathogen-free conditions.

Cell Preparation, Stimulation, and Analysis. T cells were purified from spleen by negative selection with magnetic beads. Survival of CD3$^+$ cells was measured by flow cytometric analysis by using forward and side light-scatter criteria as well as staining for ethidium monoazide bromide or with annexin V/propidium iodide (see *Supporting Text*, which is published as supporting information on the PNAS web site, for details). Bone-marrow-derived DC (BMDDC) were generated as described (ref. 36; see *Supporting Text*).

Western Blot Analysis. T cells were extracted in lysis buffer (20 mM Tris/HCl, pH 8.8/10 mM NaCl/1 mM EDTA) supplemented with 0.5% Igepal CA-630/0.1% SDS/complete protease inhibitor mixture (Roche, Mannheim, Germany). Lysates were boiled in Laemmli buffer. Equal amounts of proteins were loaded onto SDS polyacrylamide gels. Blots were developed by using the enhanced chemiluminescence system (PerkinElmer).

Subcellular Fractionation. Subcellular fractionation was performed essentially as described (14). The protocol is also given in *Supporting Text*.

Retroviral Transduction. The MiT and MiT-*bcl-3* retroviral vectors were kindly provided by Phillippa Marrack (National Jewish Medical and Research Center, Denver; ref. 10). Retrovirus was produced and cells were infected according to published protocols (see *Supporting Text*).

***In Vivo* and *ex Vivo* T Cell Stimulation Experiments.** Forty micrograms of SEB (Sigma-Aldrich) was injected i.p. per mouse. Fifty

micrograms of LPS was injected the next day. Blood was taken, red blood cells were lysed, and cells were stained with antigen-presenting cell (APC)-conjugated anti-CD3 mAb and anti-Vβ8-FITC antibodies and analyzed by flow cytometry. In some experiments, mice were killed, and single-cell suspensions from spleens were prepared. Cells (3×10^5) were plated in flat-bottom 96-well plates and incubated for 20 h. Survival of the T cells was measured by staining with APC-anti-CD3 antibodies and light scatter characteristics as above.

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