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*J Immunol* 2001; 166:2311-2316; doi: 10.4049/jimmunol.166.4.2311
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Peripheral Deletion After Bone Marrow Transplantation with Costimulatory Blockade Has Features of Both Activation-Induced Cell Death and Passive Cell Death

Thomas Wekerle,* Josef Kurtz,* Mohamed H. Sayegh,† Hiroshi Ito,* Andrew D. Wells,‡ Steven Bensinger,‡ Juanita Shaffer,* Laurence A. Turka,§ and Megan Sykes3*§

Two major pathways of death of previously activated T cells have been described: activation-induced cell death can be triggered by restimulating activated T cells with high concentrations of Ag, is Fas-dependent, is not influenced by proteins of the Bcl family, and is blocked by cyclosporin A; in contrast, passive cell death is induced by the withdrawal of growth factors and activation stimuli, is Fas-independent, and is blocked by Bcl family proteins. We examined the role of these two forms of cell death in the peripheral deletion of donor-reactive host T cells after allogeneic bone marrow transplantation and costimulatory blockade with anti-CD154 plus CTLA4Ig in two murine models. The substantial decline in donor-reactive CD4 cells seen in wild-type recipients 1 wk after bone marrow transplantation with costimulatory blockade was largely inhibited in Fas-deficient recipients and in Bcl-xL-transgenic recipients. We observed these effects both in a model involving low-dose total body irradiation and a conventional dose of bone marrow, and in a radiation-free regimen using high-dose bone marrow transplantation. Furthermore, cyclosporin A did not completely block the deletion of donor-reactive CD4 T cells in recipients of bone marrow transplantation with costimulatory blockade. Thus, the deletion of donor-reactive T cells occurring early after bone marrow transplantation with costimulatory blockade has features of both activation-induced cell death and passive cell death. Furthermore, these in vivo data demonstrate for the first time the significance of in vitro results indicating that proteins of the Bcl family can prevent Fas-mediated apoptosis under certain circumstances. The Journal of Immunology, 2001, 166: 2311–2316.

Evidence support the conclusion that this deletion seen early (1 wk) after BMT occurs in the periphery and not in the thymus: 1) the speed of its occurrence makes it very unlikely to be caused by dilution of the peripheral repertoire through the output of newly developed, intrathymically deleted T cells; 2) a decline of CD8 PBL (bearing the same Vβ subunits), which can undergo deletion only in the thymus at the CD4+CD8+ double-positive stage but not in the periphery (because they do not react efficiently with superantigens presented by MHC class II), is not seen; and 3) the early deletion of donor-reactive CD4 PBL is also observed in thymectomized recipients.

Two broad categories of apoptotic cell death of lymphocytes are generally recognized (9). Activation-induced cell death (AICD) can be triggered by restimulating activated T cells with high concentrations of Ag (10). AICD is thought to play a role in the maintenance of self-tolerance by eliminating autoreactive lymphocytes (10, 11), is Fas (CD95)-dependent (10–13), can be promoted by IL-2 (14), and can be inhibited by cyclosporin A (CyA) (15). AICD in most cases cannot be prevented by proteins of the Bcl family (11, 13). When expressed as transgenes, Bcl-2 and Bcl-xL in large part seem to function interchangeably (16). In contrast, passive cell death (PACD) is caused by the withdrawal of growth factors and activation stimuli (11), which can be a consequence of a lack of costimulatory signals (9, 17). PACD is thought to play a role in terminating immune responses to foreign Ags, and can be prevented by the overexpression of Bcl-2 or Bcl-xL (11, 18) but is considered to be independent of the Fas pathway (11, 13, 19). However, in contrast to these seemingly distinct characteristics of AICD and PACD, several in vitro studies have indicated that Bcl proteins could inhibit Fas-mediated cell death (17, 20–23). Thus, considerable uncertainty persists regarding the precise relationship...
of the Fas-mediated and Bcl-inhibited cell death pathways. To better characterize the mechanism of peripheral deletion after costimulatory blockade and BMT, we examined this phenomenon using Fas-deficient (lpr) and Bcl-x<sub>L</sub>-transgenic recipients.

**Materials and Methods**

**Animals**

Female C57BL/6 (B6; I-E<sup>+</sup>), B6.lpr (lpr; Fas-deficient, I-E<sup>-</sup>), B10.A (B10.A; I-E<sup>+</sup>), and A.SW mice were purchased from Frederick Cancer Research Center (Frederick, MD) or from The Jackson Laboratory (Bar Harbor, ME). B6.Bcl-x<sub>L</sub>-transgenic mice (with transgenic expression of the long form of the Bcl-x gene (Bcl-x<sub>L</sub>) targeted to the T cell lineage (Ref. 24; B6.Bcl-x<sub>L</sub>; I-E<sup>+</sup>) and B6 nontransgenic littermates (B6, I-E<sup>-</sup>) were bred in the colony of Dr. L.A. Turka. Mice were maintained in a specific pathogen-free microisolator environment, as described (25).

**BMT protocols**

In the standard-dose BMT model, recipient mice were treated with 3 Gy total body irradiation (TBI) and were injected i.v. on the same day (day 0) with ~20 × 10<sup>6</sup> unseparated BM cells (BMC) harvested from fully MHC-mismatched B10.A donors. In the high-dose BMT model, recipients were injected i.v. with ~200 × 10<sup>6</sup> unseparated BMC harvested from B10.A donors. In both BMT models, a hamster anti-mouse CD154 mAb (MR1; 0.5 or 2 mg) was injected i.p. on day 0, and murine CTLA4Ig (0.5 mg) was injected i.p. on day +2. In the high-dose BMT experiment using B6.Bcl-x<sub>L</sub> recipients, rat anti-mouse B7.1 (1G10) and rat anti-mouse B7.2 (B7D2) mAbs (0.5 mg of each on day +2) were used instead of CTLA4Ig. CyA was administered s.c. at a dose of 20 mg/kg/d for the first 2 wk after BMT where indicated. CTLA4Ig was a gift of Bristol-Myers Squibb (Seattle, WA); the MR1 hybridoma was provided to us by Dr. Randolph I. Noelle; and the anti-B7.1 and anti-B7.2 mAbs were purchased from Bioexpress (West Lebanon, NH).

**Flow cytometric (FCM) analysis of TCR V<sub>β</sub> families**

PBL were stained with anti-V<sub>β</sub>5.1/2-FITC, V<sub>β</sub>11-FITC, and V<sub>β</sub>8.1/2-FITC mAb vs PE-conjugated anti-CD4 (all purchased from PharMingen, San Diego, CA). Two-color FCM analysis was performed on gated CD<sup>4</sup><sup>+</sup> cells. Background staining (as determined with nonreactive mAb HOPC-FITC) was subtracted from the percentage of cells staining with each anti-V<sub>β</sub> mAb. All p values were calculated using a two-tailed Student's t test.

**FCM analysis of thymocyte subpopulations**

Thymocytes were stained with anti-CD4-PE vs anti-CD8-CyChrome, and the percentages of CD4 or CD8 single-positive, CD4 CD8 double-positive, and double-negative thymocytes were determined by two-color FCM analysis.

**CFU assay**

CFUs were determined using a complete methylcellulose medium with recombinant cytokines (MethoCult GF M3434; Stem Cell Technologies, Vancouver, British Columbia, Canada) plus 1× penicillin/streptomycin plus 1× glutamine. BM from one tibia and one femur was flushed into IMDM with 2% FBS. RBCs were lysed in 2% acetic acid, and nucleated cells were counted. Cells were diluted to 1.33 × 10<sup>5</sup> cells/ml, and 1.5 ml of medium was placed into 35-mm petri dishes. After incubation for 7–8 days at 37°C with 5% CO<sub>2</sub>, the total number of colonies was counted on an inverted microscope.

**Results**

**Peripheral deletion of donor-reactive CD4<sup>+</sup> cells after BMT with costimulatory blockade in a model involving low-dose TBI**

Wild-type B6, Fas-deficient B6.lpr, Bcl-x<sub>L</sub>-transgenic B6 mice, and nontransgenic littermate controls were treated with 3 Gy TBI, 20 × 10<sup>6</sup> fully MHC-mismatched B10.A BMC, and costimulatory blockade consisting of one injection each of anti-CD154 plus CTLA4Ig. Seven days after BMT, the percentages of CD4 cells bearing certain V<sub>β</sub> subunits on their TCR were determined by two-color FCM analysis. The donor strain B10.A expresses I-E, which is required to present superantigens derived from mammary tumor virus (Mtv)-8 and -9 endogenous retroviruses encoded in the B6 background genome. Developing thymocytes whose TCR con-

**TBI is an essential component of the BMT model described above. Therefore, it was important to rule out the possibility that Fas deficiency or Bcl-x<sub>L</sub> overexpression indirectly influenced the peripheral deletion of CD4 cells by abrogating a participatory role for TBI. This becomes especially relevant in view of evidence that...**
Fas/Fas ligand interactions play a role in radiation-induced apoptosis (30). Likewise, Bcl-2-transgenic mice are more radioresistant than wild-type mice (31, 32). However, much less is known about the radiosensitivity of Bcl-xL-transgenic mice. Therefore, we compared irradiated Bcl-xL-transgenic and wild-type B6 mice. As shown in Table II, lymphocytes in Bcl-xL-transgenic mice were radiosensitive, but overall less so than those in wild-type mice, as demonstrated by the lesser decline in viable cells in thymus and spleen after irradiation with 3 Gy (p < 0.01 for comparison of the percent reduction in cell counts in these tissues in irradiated wild-type vs irradiated Bcl-xL-transgenic mice). BM and lymph node cell counts did not follow this trend, and CFUs were also comparable between the two groups. Of note, double-positive thymocytes were markedly less radiosensitive in Bcl-xL-transgenic mice than in wild-type mice (p < 0.05 comparing the change in percent double-positive cells after irradiation in wild-type vs Bcl-xL-transgenic mice). Thus, although the radiosensitivity of some cell populations differed between wild-type mice and Bcl-xL-transgenic mice, other cell types seemed to react similarly.

Peripheral deletion in a radiation-free model of high-dose BMT with costimulatory blockade

Because both lpr and Bcl-xL-transgenic mice react differently to radiation than wild-type mice, we next used a radiation-free model to evaluate mechanisms of peripheral T cell deletion after BMT with costimulatory blockade. We have recently demonstrated that early peripheral deletion of donor-reactive CD4+ T cells also occurs after a protocol involving high-dose BMT with costimulatory blockade without cytoreductive host conditioning (8). Wild-type B6, Fas-deficient B6/lpr, Bcl-xL-transgenic B6 mice, and B6-nontransgenic littermate controls were treated with 200 × 10^6 fully MHC-mismatched BMC and anti-CD154 plus CTLA4Ig (or anti-CD154 plus anti-B7.1 and anti-B7.2; Ref. 8). As in the experiments described above, the percentages of Vβ5+, Vβ11+, and Vβ8+ CD4+ PBL were determined 7 days after BMT. Similar to previous results, in wild-type B6 recipients the percentages of Vβ5+ and Vβ11+ (but not Vβ8+) CD4+ PBL declined by 81 and 79%, respectively, after high-dose BMT with costimulatory blockade compared with controls receiving BMT only without costimulatory blockade (p < 0.05) (Fig. 2A) (8). In contrast, Fas-deficient B6/lpr recipients showed no significant reduction (p > 0.05) in either Vβ5+ or Vβ11+ CD4+ PBL compared with normal B6/lpr mice (p < 0.005 for comparison of B6 and B6/lpr BMT recipients).

In an experiment to examine the role of Bcl-xL in the peripheral deletion of donor-reactive CD4+ cells, B6-nontransgenic littermate controls receiving high-dose BMT with costimulatory blockade demonstrated the expected reduction in Vβ5+ and Vβ11+ CD4+ PBL (58 and 59% reductions, respectively, compared with naive nontransgenic littermates; p < 0.05) (Fig. 2B). In contrast, transplanted Bcl-xL-transgenic recipients did not show a significant reduction in Vβ5+ or Vβ11+ CD4+ PBL (24 and 12% reductions, respectively, compared with naive nontransgenic litters; p = 0.05 for Vβ5, p = 0.3 for Vβ11). The difference in the extent of deletion between B6 and Bcl-xL-transgenic high-dose BMT recipients reached statistical significance for Vβ11 (p < 0.05; p = 0.1 for Vβ5).

Discussion

Costimulatory blockade can exert its effects through several mechanisms, with anergy (1), immune deviation (2), suppression (3), and apoptosis (4, 5) having been observed in various models. We have recently demonstrated that BMT under cover of costimulatory blockade (anti-CD154 and CTLA4Ig) leads to the peripheral deletion of donor-reactive host T cells immediately after BMT (6–8). This mechanism of peripheral deletion seems to provide an effective way to specifically eliminate T cells with a certain Ag specificity from an adult animal, and thus has potential for widespread clinical application. Here we describe studies in two BMT models that indicate that this form of peripheral deletion is: 1) largely dependent on the presence of functional Fas in the host; 2) preventable by the constitutive expression of Bcl-xL; and 3) not completely inhibited by CyA.

In theory, either AICD or PACD could be responsible for the deletion after BMT with costimulatory blockade. The injection of a large amount of Ag in the form of fully allogeneic BMC could lead to repeated activation of host T cells and subsequent AICD. In addition, several cell types with veto-like activity contained in the...
unseparated BM inoculum might induce Ag-specific apoptosis of host cells. Indeed, in the high-dose BMT studies, a slight reduction of donor-reactive CD4+ cells was observed in control mice receiving only the BM without costimulatory blockade, but this effect was not observed in a Fas-deficient control mouse (Fig. 2A and Ref. 8). Early deletion after BMT with costimulatory blockade was also observed to a similar extent when T cell-depleted BM was used (J.K. and M.S., unpublished data), suggesting that donor T cells are not required for the deletion and that they are not required as mediators of putative veto activity. Therefore, it is possible that the effect of anti-CD154 and CTLA4Ig is primarily to prevent the expression (35). Thus it seems possible that AICD can occur without preceding T cell expansion in the absence of an intact CD28 pathway, which would be the case in our model by day 2 when CTLA4Ig is administered.

PACD can be induced as a consequence of the absence of growth factors and activation stimuli due to a lack of costimulation signals (9, 17, 36). The CD28 pathway, which can be blocked by CTLA4Ig, is considered to provide the most important costimulatory signal for mature T cells by inducing IL-2 and other essential growth and activation signals (37). A hallmark of PACD is that it can be prevented by the overexpression of survival genes of the Bcl-2 family (4, 11, 18). In our experiments, peripheral deletion of Vβ6+ and Vβ11+ CD4+ cells was effectively prevented in Bcl-xL-transgenic recipients. Because the Bcl-xL transgene does not augment proliferation of T cells with or without costimulation (but in fact seems to delay cell cycle kinetics somewhat (Ref. 4, and A.D.W. and L.A.T., unpublished observation), it is unlikely that the powerful net effect on deletion observed in Bcl-xL-transgenic BMT recipients reflects increased proliferation rather than reduced deletion. Also, the lack of evidence for expansion before the deletion and the inability of CyA to prevent deletion completely is consistent with a major role for PACD. However, PACD is generally considered not to be mediated by Fas.

How can the observations that the extrathymic deletion in these BMT models is partly Fas-dependent but preventable by Bcl-xL expression be reconciled? First, Fas deficiency did not completely inhibit deletion in our studies, suggesting that there is a component of apoptosis that is Fas independent. This component of apoptosis could be due to PACD. However, because Bcl-xL is able to prevent deletion almost entirely, there seems to be a component that is

Table I. Influence of CyA on early deletion after BMT with costimulatory blockade

<table>
<thead>
<tr>
<th>CD4+ PBL</th>
<th>Vβ8</th>
<th>Vβ5</th>
<th>Vβ11</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBL6</td>
<td>17.24 ± 0.3</td>
<td>3.17 ± 0.4</td>
<td>5.28 ± 0.71</td>
</tr>
<tr>
<td>NBL10. A</td>
<td>16.8 ± 0.4</td>
<td>0.03 ± 0</td>
<td>0.04 ± 0</td>
</tr>
<tr>
<td>CyA + BM</td>
<td>17.32 ± 0.6</td>
<td>2.06 ± 0.7</td>
<td>4.1 ± 0.9</td>
</tr>
<tr>
<td>co.-bl. + BM</td>
<td>18.57 ± 0.7</td>
<td>0.86 ± 0.5</td>
<td>1.75 ± 1.1</td>
</tr>
<tr>
<td>co.-bl. + BM with CyA</td>
<td>18.54 ± 1</td>
<td>1.44 ± 1</td>
<td>3.19 ± 1.3</td>
</tr>
</tbody>
</table>

*Wild-type B6 mice were treated with 3 Gy TBI, 20 × 10^6 BMC, and costimulatory blockade with (co.-bl. + BM with CyA) or without the addition of CyA (co.-bl. + BM). A control group received 3 Gy TBI, 20 × 10^6 BMC and CyA (CyA + BM). The percentage of Vβ8, Vβ5+, and Vβ11+ CD4+ PBL, was determined by FCM analysis 1 wk after BMT. Combined results from two similar experiments (except control group) are shown as mean ± SD.

*p < 0.01 compared to group receiving co.-bl. + BM. Co.-bl. denotes costimulatory blockade.

Table II. Effects of 3 Gy TBI on lymphocytes and hemopoietic cells of Bcl-xL-transgenic mice

<table>
<thead>
<tr>
<th></th>
<th>Thymus</th>
<th>CD4 SP</th>
<th>CD8 SP</th>
<th>DP</th>
<th>DN</th>
<th>Lymph Node</th>
<th>Spleen</th>
<th>BM</th>
<th>CFUs</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLB6 0 Gy</td>
<td>153.5 ± 19.1</td>
<td>27.9 ± 0.9</td>
<td>14.5 ± 0.2</td>
<td>93.3 ± 12.7</td>
<td>9.6 ± 0</td>
<td>2.6 ± 0.1</td>
<td>78.5 ± 12.0</td>
<td>18 ± 3.4</td>
<td>473 ± 4.2</td>
</tr>
<tr>
<td>[19 ± 1.4]</td>
<td>[10 ± 1.4]</td>
<td>[64 ± 2.8]</td>
<td>[6.5 ± 0.7]</td>
<td></td>
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<tr>
<td>NLB6 3 Gy</td>
<td>23.7 ± 2.4</td>
<td>10.3 ± 0.9</td>
<td>7.4 ± 1.2</td>
<td>0.2 ± 0</td>
<td>3.9 ± 1.6</td>
<td>0.4</td>
<td>12.9 ± 2.3</td>
<td>8.2 ± 1.2</td>
<td>167.3 ± 40.7</td>
</tr>
<tr>
<td>(15)*</td>
<td>[47.7 ± 4.1]</td>
<td>[34 ± 2.7]</td>
<td>[1 ± 0]</td>
<td>[17.7 ± 5.7]</td>
<td>[16]</td>
<td>(16)*</td>
<td>(45)*</td>
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</tr>
<tr>
<td>B6.Bcl-xL 0 Gy</td>
<td>116.5 ± 2.1</td>
<td>16.6 ± 4.1</td>
<td>8.2 ± 1.1</td>
<td>72.4 ± 4.4</td>
<td>12.4 ± 2.3</td>
<td>4.38 ± 0.2</td>
<td>40 ± 7.1</td>
<td>19.3 ± 1.4</td>
<td>442 ± 5.7</td>
</tr>
<tr>
<td>[15.5 ± 3.5]</td>
<td>[7.5 ± 0.7]</td>
<td>[68 ± 5.7]</td>
<td>[12 ± 2.8]</td>
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<tr>
<td>B6.Bcl-xL 3 Gy</td>
<td>38 ± 4.4</td>
<td>10.5 ± 1.8</td>
<td>7.4 ± 2.6</td>
<td>13.5 ± 1.5</td>
<td>4 ± 0.7</td>
<td>0.8</td>
<td>23.9 ± 1.0</td>
<td>4.1 ± 1.1</td>
<td>172.7 ± 31</td>
</tr>
<tr>
<td>(33)*</td>
<td>[30.3 ± 0.6]</td>
<td>[19 ± 4.6]</td>
<td>[39.3 ± 4.1]</td>
<td>[11.3 ± 1.2]</td>
<td>(18)</td>
<td>(60)*</td>
<td></td>
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<tr>
<td>BM CFUs</td>
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*p < 0.01 comparing percent change in cell counts in irradiated wild-type vs irradiated Bcl-xL-transgenic mice.

1 p < 0.05 comparing percent change in thymocyte subpopulation in irradiated wild-type vs irradiated Bcl-xL-transgenic mice.
FIGURE 2. In a radiation-free model of high-dose BMT with costimulatory blockade, early peripheral deletion of donor-reactive CD4 cells is still inhibited in Fas-deficient and in Bcl-xL-transgenic recipients. A, B6 recipients of high-dose BMT with costimulatory blockade (group D; n = 7) showed a substantial reduction of the percentages of VB5+ and VB11+ (but not VB8+, data not shown) CD4+ PBL 1 wk after BMT compared with naive B6 (n = 2) (p < 0.02) or B6 controls receiving BM without costimulatory blockade (group C; n = 2) (p < 0.05). In contrast, B6.lpr recipients of high-dose BMT with costimulatory blockade (group B; n = 5) showed no significant reduction of VB5+ and VB11+ CD4+ PBL compared with normal B6.lpr (n = 2) (p > 0.05) or a control receiving BM without costimulatory blockade (group A). The difference in the percentages of VB11 and VB5 between B6.lpr and B6 BM recipients was statistically significant (p < 0.005 comparing groups D and B). B, B6-nontransgenic littermate controls receiving high-dose BMT with costimulatory blockade (group F; n = 4) showed the expected reduction of VB5+ and VB11+ CD4+ PBL 1 wk after BMT (p < 0.05 for comparison with naive nontransgenic littermate controls; n = 2). In contrast, Bcl-x-transgenic recipients of high-dose BMT with costimulatory blockade (group E; n = 3) did not show a substantial reduction of VB5 and VB11 (p = 0.3 for VB11 and p = 0.05 for VB5 compared with naive nontransgenic littermate controls). The difference in the percentage of VB11+ CD4 cells between B6.Bcl-x and B6 BM recipients was statistically significant (p < 0.05 comparing groups E and F; p > 0.05 for VB5). BM recipients were treated with 200 × 10^6 B10.A BMC and anti-CD154 plus CTLA4Ig (A), or anti-CD154 plus anti-B7.1 and anti-B7.2 (B), respectively. The percentages of VB5+, VB11+, and VB8+ CD4+ PBL were determined by two-color FCM analysis 7 days after BMT. Co-bl denotes costimulatory blockade.

simultaneously Fas-dependent and preventable by Bcl-xL. This form of apoptosis could be either AICD that can be overcome by Bcl-xL expression, or PACD that is Fas-dependent. An alternative explanation is that costimulatory blockade with anti-CD154 plus CTLA4Ig could lead to a novel form of apoptosis that is distinct from classical AICD and PACD. Some of our observations resemble results from in vitro studies describing apoptosis that occurs after TCR ligation without costimulation, is Fas-dependent, and is impeded by CyA (38). A role for T cell apoptosis by PACD and AICD, respectively, has been recently suggested in two transplant models involving peripheral tolerance induction (4, 5). However, considerable uncertainty still exists about the exact interrelationship between the Fas pathway and the Bcl-2/Bcl-xL pathways. Some evidence suggests that they are separate (9, 11, 13, 19, 39), whereas other studies argue that the Fas pathway intersects with the Bcl-2/Bcl-xL pathways. Fas-mediated apoptosis was partially inhibited by Bcl-2 and Bcl-xL, respectively, in vitro studies using murine and human cell lines (20, 21). To our knowledge, the studies presented here provide the first direct in vivo evidence that Bcl-xL expression can prevent Fas-mediated T cell death and that these two pathways do “overlap.”

In summary, the peripheral deletion of donor-reactive T cells in the early period after BMT with costimulatory blockade using anti-CD154 and CTLA4Ig is mediated in part by Fas and can be overcome by the constitutive expression of Bcl-xL. These data show for the first time: 1) that both Fas-dependent and Bcl-xL-reversable cell death play a role in peripheral deletion after BMT with costimulatory blockade, and 2) that the Fas and Bcl-xL pathways are not entirely separate but do intersect functionally in vivo. Thus these observations do not readily fit the criteria believed to distinguish AICD and PACD, but confirm in vivo the existence of a form of apoptosis in which the Fas-mediated and Bcl-xL-inhibited pathways intersect. This intersecting pathway may be important in the maintenance of self-tolerance by peripheral deletion of T cells encountering self-Ags in the absence of costimulation.

Acknowledgments

We thank Drs. Arlene Sharpe and Stephen Alexander for helpful review of the manuscript and Julia Lundell for expert secretarial assistance.

References


