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A Role for Perforin in Activation-Induced T Cell Death In Vivo: Increased Expansion of Allogeneic Perforin-Deficient T Cells in SCID mice

David Spaner,1*† Kaliannan Raju,* Brian Rabinovich,* and Richard G. Miller*

Despite defective granule exocytosis, T cells from mice whose perforin gene was ablated by homologous recombination (pko mice) caused a similar degree of graft-vs-host disease as normal T cells after injection into sublethally irradiated C.B-17 SCID mice. Moreover host spleens contained significantly greater numbers of T cells from pko mice than from wild-type mice following their i.v. injection. This increase could not be explained by persistence of host APCs that were not cleared by defective donor cytotoxic effector cells. The absence of functional perforin-dependent suppressor cells or an altered cytokine profile of donor T cells could also not account for the behavior of pko cells. Spontaneous and Fas-mediated apoptosis of in vivo activated donor T cells were independent of donor origin. However, pko T blasts exhibited less growth inhibition and cell death after reactivation in vitro. The results are compatible with a model of a defective activation-induced cell death (AICD) pathway, controlled by perforin, accounting for the increased expansion of alloreactive pko T cells. The Journal of Immunology, 1999, 162: 1192–1199.

A doptively transferred T cells can reconstitute the peripheral T cell pool of a lymphopenic host (1). The number of donor T cells in the host is the difference between the number of proliferating cells and the number that die by apoptosis. When donor T cells react against host Ags (2), as in graft-vs-host disease (GVHD), activation-induced cell death (AICD) (3) undoubtedly contributes to the latter number.

AICD is caused when previously activated T cells are subjected to strong restimulation through their TCR (3). AICD of T cells is thought to be controlled by members of the TNFR family of genes, one of whose members is Fas (4). Thus, the lymphoproliferative disorder in ppr/lpr mice, whose fas gene is inactivated by a retroviral insertion, is thought to be caused by the accumulation of activated T cells that cannot be cleared by cell death processes (5). CD8+ T cells especially are subject to AICD mediated by TNF-α in vitro (6) although TNFR1- (7) and TNFR2-deficient (6) mice are phenotypically normal.

Recently, we presented evidence that perforin, like TNF-α and Fas ligand, may be involved in autonomous AICD in vitro (8). The granule exocytosis (perforin-dependent) pathway is the major killing mechanism employed by CTLs (9), although TNF-α and Fas ligand, in addition to their role in AICD, are also used (10). Perforin is a 60-kDa protein that normally resides in lytic granules but can also be secreted directly (11). Granular contents are released from the CTL when it is stimulated through its TCR, allowing perforin to polymerize in the membrane of the target cell. Together with members of the granzyme family of proteins that also reside in the lytic granules (12), pores formed by perforin lead to the programmed cell death of the target. The ability of activated naive T cells to undergo Fas-independent apoptosis in vitro following reaggregation of the TCR complex was defective in the absence of perforin (8). The role of perforin was not via fratricidal killing and was independent of killing by TNF-α, although it appeared to mediate some of the effects of IL-2 on AICD (8, 13). In this study we show that alloreactive T cells from perforin knockout mice (pko) mice (14) expand to a significantly greater degree than wild-type T cells in an SCID mouse model of GVHD. The results suggest that this increased expansion is partly caused by defective AICD processes in T cells from pko mice.

Materials and Methods

Animals

C57BL/6J (B6) (H-2b, Thy-1.2), B6.PL-Thy-1cy (H-2b, Thy 1.1), and BALB/c (H-2k) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The pko mice (14) on the B6 background and C.B-17 SCID (H-2k, congenic to BALB/c) mice were bred and maintained in the defined flora animal colony at the Ontario Cancer Institute (Toronto, Canada). The original pko breeding pair was provided by Dr. H. Hentgartner (Zurich, Switzerland). Offspring were derived by cesarian section and foster-mothered under specific pathogen-free defined flora conditions. All work in this study was performed with such specific pathogen-free animals.

Abs, reagents, and cell lines

Anti-CD3 (145-2C11) (15) was purified by protein G column chromatography. Phycoerythrin (PE)- or FITC-labeled CD4 and CD8 Abs, 7-AAD, propidium iodine, and streptavidin-PE were purchased from Sigma (St. Louis, MO). The anti-FcyRIII-α Ab, 2.4G2, (16) was obtained from the American Type Culture Collection (Manassas, VA), and culture supernatants were prepared. Unconjugated anti-Fas Ab, PE- and FITC-labeled anti-Thy-1.1, Thy-1.2, H2D, H2k, IFN-γ, IL-2, TNF-α, IL-10, IL-4, CD3, CD69, CD44, and annexin V and biotinylated CD25 Abs were purchased from PharMingen (San Francisco, CA). Anti-CD4 and anti-CD8 tricolor Abs were purchased from Caltag (Burlingame, CA). The hamster anti-murine CD28 hybridoma (37.51) was a gift from Dr. James Allison (University of California, Berkeley, CA) (17), and antibodies were purified by protein G column chromatography.

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2 Abbreviations used in this paper: GVHD, graft-versus-host disease; AICD, activation-induced cell death; pko, perforin knockout mice; PE, phycoerythrin; 7-AAD, 7-aminoactinomycin D; CM, complete medium; B6, C57BL/6J; PCD, programmed cell death.
The Journal of Immunology

1193

affinity chromatography (Pharmacia, Piscataway, NJ) in our laboratory. The hamster anti-murine TNF-α hybridoma, X6P-XT22 (18), was a gift from Dr. J. A. Abrams (DYNAZ Research Institute, Palo Alto, CA). As -cites was raised in pristane-treated SCID mice (19). Mouse IL-2 cDNA transfected X63Ag8–653 cells were a generous gift from H. Karasuyama (20). Supernatants from the cell line were titrated on CTLL-2 cells (ob tained from the American Type Culture Collection) and used as the source of IL-2 for cell cultures.

Brefeldin A was purchased from Sigma. P815 tumor lines were obtained from the American Type Culture Collection and maintained in exponential growth by serial passage in complete medium (CM; α-MEM, 10% FCS, 5 x 10^{-5} M 2- ME, 2 mM L-glutamine, and 15 mM HEPES, pH 7.3) at 37°C in an atmosphere of 5% CO₂.

**TCR complex religation of in vivo activated T cells**

A modification of our previously described assay for induction of AICD in vitro (21) was performed. Activated donor T cells were isolated from the spleens of SCID mice suffering from GVHD using Lympholyte separation medium (Cedarlane Laboratories, Hornsby, Canada). They were washed and resuspended at 4 x 10⁶ cells/ml in CM. Anti-CD3 antibodies were bound to 96-well Costar EIA plates (Cambridge, MA) by incubation of 100 µl of 10 µg/ml protein G-purified antibody in PBS for 3 h at 37°C. The plates were washed three times in PBS before use, and 100 µl of CM was added for 1 h at 37°C to block nonspecific binding. Then 100 µl of the activated cells were added and incubated at 37°C. Control wells were simply blocked with CM. IL-2 (25 U/ml) was added to all cultures.

After 48 h, 1 µCi of [³H]thymidine (2 µCi/ml) was added to the cultures for a subsequent 18 h. Cells were then harvested, and the incorporated radioactivity was measured in a beta scintillation counter.

**Mixed lymphocyte responses**

Responder cells were spleen cells diluted to 5 x 10⁶ cells/ml in CM. Stimulators were taken from the spleens of sublethally irradiated SCID mice with or without GVHD. Cells from BALB/c mice, also at 5 x 10⁶ cells/ml and given 2000 cGy of irradiation, were added to some cultures. In some cases, 1 x 10⁶ BALB/c spleen cells were plated onto a 96-well plate and allowed to adhere for 4 h at 37°C, and the nonadherent cells were then washed away. This procedure enriched for adherent cells (dendritic cells and macrophages), which were then used to stimulate. After a subsequent 90-min incubation and washing, purified T cells (5 x 10⁶ cells/well in CM) were plated over the adherent cells. T cells were purified by incubation with anti-mouse Ig magnetic beads (Advanced Magnetics, Boston, MA) at a 10:1 bead/cell ratio for 30 min at 4°C followed by adherence to plastic for 90 min. The MLRs were incubated for 72 h, and then 1 µCi of [³H]thymidine was added to the cultures for a subsequent 18 h. The cells were then harvested, and the amount of thymidine incorporation was measured in a beta scintillation counter.

**Redirected lysis assays**

P815 tumor targets in exponential growth phase were collected by centrifugation, resuspended in two drops of 100% FCS, and radiolabeled with 50 µl of Na₂⁵¹CrO₄ (7.14 mCi/ml; DuPont, New England Nuclear, Boston, MA) for 1 h. Chromium-labeled targets were washed three times with α-MEM and 1% FCS, and effector cells, purified from spleen cells using Lympholyte separation medium, and labeled target cells (2000/well), each in 100 µl of CM, were added at varying E:T cell ratios to individual wells of a U-bottom plate. Anti-CD3 antibody was then added at a final concentration of 1 µg/ml (15) before use and 100 µl of target cells (2 x 10⁶/ml in CM) were added to each well. The plates were centrifuged at 800 rpm for 5 min, and 100 µl of the supernatant was transferred to Fisherbrand flat glass tubes (Fisher Scientific, Pittsburgh, PA) and counted in a gamma counter (CompuCounter 1282, LKB, Stockholm, Sweden). Total release (TR) was measured by lysis of tumor targets with 1% acetic acid, and spontaneous release (SR) was measured in the absence of effector cells. The percent cytotoxicity was determined by the ratio (count per minute – SR)/(TR – SR) x 100%.

**Immunofluorescence**

Non-specific binding was first blocked by a 10-min incubation at room temperature with 10 µl of 2.4G2 culture supernatant and 10 µl of mouse serum (Cedarlane). Cells (5 x 10⁶) were then allowed to react with pre-titrated doses of Abs, including annexin V, for 20 min, washed, incubated with 7-AAD to label dead cells, and analyzed on a FACScan flow cytometer (Becton Dickinson) using LYSIS II software.

**Production of GVHD**

C.B-17 SCID mice were irradiated with 275 cGy from a 137Cs gamma-ray source (Gammacell 40 Exactor, Nordion International, Kanata, Canada) on the same day as the injection. Inguinal lymph node cells were obtained
from donor mice, and varying numbers were injected into the tail veins of SCID hosts. Mice were examined daily and were sacrificed if moribund. Surviving animals were sacrificed by cervical dislocation, autopsies were performed, and cell suspensions were prepared.

**Intracellular cytokine staining**

The method of Ferrick et al. (22) was mainly followed. T cells were reactivated on plate-bound anti-CD3 antibodies along with 25 U/ml IL-2 as described above, and 5 μg/ml of brefeldin A was added after 18 h. The cultures were incubated for a further 4 h at 37°C. Cells were then harvested and washed, and nonspecific binding was blocked with 2.4G2 and mouse serum in a total volume of 90 μl. Cells were fixed in 75 μl of solution A (Caltag) for 30 min at room temperature. After washing in Ca²⁺-, Mg²⁺-free PBS, cells were stained at room temperature with the different combinations of cytokine-specific labeled Abs or isotype controls at previously optimized doses in 75 μl of solution B (Caltag) for 30 min. Cells were then washed and analyzed as described above.

**Statistical analysis**

The p values, comparing groups of responses, were obtained using Student’s t test.

**Results**

**Increased numbers of allogeneic T cells in SCID mice in the absence of perforin**

Sublethally irradiated lymphopenic C.B-17 SCID mice (H-2<sup>kl</sup>) injected i.v. with T cells from C57BL/6J (B6) (H-2<sup>b</sup>) mice rapidly develop an acute and lethal GVHD (23, 24). To study the role of perforin-mediated cytotoxicity in the pathogenesis of this model of GVHD, purified T cells from B6-pko mice were used as donor cells. Unexpectedly, mice undergoing GVHD caused by pko T cells accumulated significantly more donor T cells than mice injected with wild-type B6 cells (Fig. 1a) despite similar morbidity. This difference was not altered by the addition of normal T cells to those without perforin (Fig. 1a).

To determine the importance of irradiation for this effect, B6 wild-type and pko T cells were injected into unirradiated C.B-17 SCID mice. Again, an increased number of pko donor cells was noted by 1 wk after injection (Fig. 1a). A number of possible explanations for the increased numbers are considered below and eliminated, except for the last: that perforin plays a role in AIDC.

**Persistence of APCs does not account for the increased numbers of pko cells**

When Ag-reactive T cells become cytotoxic effectors, stimulatory APCs are killed, the antigenic stimulus is removed, and the immune response is limited (25). Thus, APCs could persist and continue to stimulate an immune response in mice receiving pko T cells if granule exocytosis-mediated killing was the only mechanism leading to destruction of APCs. It is not. Sublethal irradiation alone causes a rapid decline in the number of professional APCs (26), and 6 days after sublethal irradiation, spleen cells from C.B-17 SCID mice could not act as stimulators in a MLR (Table I, rows 1–3). Despite this similar decrease in APCs, pko T cells still increased in number compared with wild-type T cells in sublethally irradiated recipients. Using flow cytometry and antibodies against K<sup>d</sup> and I-A<sup>d</sup>, the results in Table I (columns 6 and 7, rows 5 and 6) confirmed that there was no preferential survival of host APCs when pko T cells were injected. In addition, the data in Table I show that adherent cells from the spleens of mice injected 10.5 days earlier with B6 or pko T cells could not stimulate fresh B6 T cells, suggesting that the absence of professional APCs caused by irradiation was maintained in both cases (Table I, rows 4–6). The ability to stimulate was rescued by the addition of fresh BALB/c (H-2<sup>kl</sup>) spleen cells, supporting a lack of stimulatory cells and not suppressor factors as being responsible for the inhibition of the MLRs (Table I, rows 7 and 8).

The use of primary MLR cultures to screen for the functional presence of surviving APCs may not detect nonprofessional APCs that can stimulate memory but not virgin T cells (27). Survival of cells that express only MHC class I molecules such as reticular cells in the secondary lymphoid organs of SCID mice injected with pko T cells may account for the observation of predominately CD8<sup>+</sup> T cell expansion. Coinjection of B6 T cells should then destroy such APCs and restore wild-type behavior to pko T cells.

**Table I. Ability of spleen cells from sublethally irradiated C.B-17 SCID mice with or without GVHD to stimulate an MLR<sup>a</sup>**

<table>
<thead>
<tr>
<th>Injected Cells</th>
<th>Time Postirradiation and/or Injection</th>
<th>Added BALB/c Spleen Cells (2.5 × 10&lt;sup&gt;6&lt;/sup&gt;/well)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Thymidine Uptake (cpm × 10&lt;sup&gt;2&lt;/sup&gt;)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
<th>K&lt;sup&gt;d&lt;/sup&gt;&lt;sup&gt;+&lt;/sup&gt; Cells (%)&lt;sup&gt;d&lt;/sup&gt;</th>
<th>IA&lt;sup&gt;d&lt;/sup&gt;&lt;sup&gt;+&lt;/sup&gt; Cells (%)&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>0</td>
<td>–</td>
<td>58.3</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>–</td>
<td>1 h</td>
<td>–</td>
<td>101.1&lt;sup&gt;e&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>–</td>
<td>6 days</td>
<td>–</td>
<td>–33.2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>B6 wild type</td>
<td>10.5 days</td>
<td>–</td>
<td>26.8</td>
<td>86.1</td>
<td>ND&lt;sup&gt;f&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>pko</td>
<td>10.5 days</td>
<td>–</td>
<td>–0.5</td>
<td>6.9</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>B6 wild type</td>
<td>10.5 days</td>
<td>+</td>
<td>51.4</td>
<td>176.2</td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>pko</td>
<td>10.5 days</td>
<td>+</td>
<td>98.6</td>
<td>114.4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> Varying numbers of spleen cells were prepared from unirradiated SCID mice and SCID mice irradiated 1 h and 6 days previously with 350 cGy and used to stimulate 5 × 10<sup>6</sup> B6 spleen cells in an MLR (rows 1–3). The responses using 2.5 × 10<sup>6</sup> spleen cells as stimulators are reported. Spleen cells were also taken from SCID mice injected with 2 × 10<sup>6</sup> B6 wild-type, pko, or BALB/c T cells, 10.5 days earlier (rows 4–8). Adherent cells from these spleens were used to stimulate 5 × 10<sup>6</sup> purified B6 wild-type T cells.

<sup>b</sup> Irradiated BALB/c spleen cells were added to the adherent APCs at the initiation of the cultures. Note that C.B-17 (Igh<sup>kg</sup>) and BALB/c (Igh<sup>rl</sup>) are Igk congenic mice and can be used interchangeably as sources of APCs (49).

<sup>c</sup> The difference between thymidine uptake in the stimulated culture and the sum of the uptakes in the wells with responders and stimulators alone. The number reported is the average of four different wells; in all cases the SEM was <10% of the average.

<sup>d</sup> To determine the number of host cells that could potentially act as APCs, spleen cell suspensions were stained with anti-K<sup>d</sup> or IA<sup>d</sup>-PE on day 8 after induction of GVHD and analyzed by flow cytometry. The total number of spleen cells were 2.2 × 10<sup>6</sup> and 4.8 × 10<sup>5</sup> for sublethally irradiated SCID mice injected with B6 or pko T cells, respectively.

<sup>e</sup> A release of cytokines, such as IL-1, in response to irradiation may account for the apparently greater stimulatory potency of spleen cells irradiated 1 h before use (50).

<sup>f</sup> ND, not done.
However, as shown in Fig. 1, a and b, the autonomous behavior of pko T cells in mixtures suggested that failure to clear either professional or nonprofessional APCs could not explain their increased cell numbers in vivo. A missing suppressor population does not account for the exaggerated responses of alloreactive pko T cells

The functional absence, in pko mice, of suppressor cells that use perforin to kill the cells they regulate (28) might cause exaggerated allogeneic T cell responses. In this case, a mixture of B6 T cells, containing the putative suppressor population, and pko T cells should proliferate normally in vivo. However, such a mixture of alloreactive cells expanded to a greater degree than B6 cells alone in sublethally irradiated SCID mice (Fig. 1a). In these experiments, only the total number of cells, but not the number in each component of the mixture, could be determined. To follow the fate of the cells in the mix, a congenic strain of B6 mice (Thy 1.1) was used. T cells from pko mice could be identified by an Ab against the Thy-1.2 allele. Because sublethally irradiated SCID mice generally became moribund around 2 wk after the transfer of allogeneic T cells, unirradiated hosts, in which the increased accumulation of pko T cells was also observed (Fig. 1a), were used. As shown in Fig. 1b, pko T cells expanded more than wild-type T cells independently of the initial Thy-1.2/Thy-1.1 ratio. The advantage was mainly for CD8+ cells.

**Equivalent activation of pko and B6 T cells in vivo**

Different levels of activation (29) of pko and B6 T cells in vivo could account for their different behavior in host SCID mice. However, the surface expression of CD25 (IL-2Ra), the early activation marker CD69, Fas, and CD44 and TCR density were identical in splenic donor T cells 4 days after injection (Fig. 2), suggesting that signaling deficiencies due to differences in the expression of activation-associated receptor complexes could not account for the increased expansion of pko cells.

**Equivalent cytokine profiles of in vivo alloactivated pko and B6 T cells**

The pko T cells can differentiate into Th1 or Tc1 and Th2 or Tc2 cells (30). T cells in acute GVHD differentiate mainly into Th1 or Tc1 cells that secrete IFN-γ (2). Could the different behavior of pko T cells be caused by their differentiation into cells of the Tc2 or Th2 phenotype? The pko or B6 T cells were harvested by density gradient centrifugation 6 days after injection into sublethally
irradiated SCID mice. Because the limited number of host SCID spleen cells is further reduced by sublethal irradiation, this simple procedure results in a population consisting of >95% donor T cells. Intracellular cytokine staining performed immediately ex vivo and after short term reactivation in vitro revealed that both pko and B6 T cells produced mainly IFN-γ and some TNF-α, but no IL-4 or IL-10 (Fig. 3, a–c). Thus, lack of perforin expression did not change the cytokine profile of in vivo activated pko T cells.

As a functional control that perforin was normally expressed in wild-type cells after in vivo activation, the purified T cells were used in redirected lysis assays against P815 targets. These results confirmed the perforin dependence of the redirected lysis assay and the functional expression of perforin in vivo, since pko T cells could not kill P815 targets compared with strong killing by in vivo activated B6 T cells (Fig. 3d).

Similar spontaneous and Fas-mediated apoptosis of allogeneic pko and wild-type T cells activated in vivo

Since the number of cells observed is the difference between the numbers of viable proliferating cells and dying cells, we wondered whether the increased expansion of pko T cells in SCID mice was associated with decreased apoptosis.

It has been previously shown that cultured T cells, activated in response to viral infections in vivo, undergo spontaneous apoptosis and exhibit increased AICD in response to subsequent reactivation in vitro (31–33). The capacity of alloactivated B6 and pko T cells to undergo spontaneous apoptosis and AICD was studied.

The number of T cells undergoing apoptosis in the spleens of mice with GVHD was determined by direct ex vivo staining with annexin V. Annexin V binds in a calcium-dependent manner to phosphatidylserine molecules that flip from the inner to the outer cytoplasmic membrane of cells undergoing apoptosis (34). 7-AAD is a nuclear dye taken up by apoptotic cells whose outer membrane is not intact. Cells that stain with annexin V but exclude 7-AAD have been shown to be in the early stages of apoptosis (34). As shown in Table II, annexin V bound T cells in the spleens of SCID mice suffering from GVHD, but did not distinguish between pko and wild-type T cells, suggesting that significant apoptosis accompanied in vivo alloactivation of either population. After density gradient separation, the viability of the cells was 80%. When placed in culture in the presence of IL-2, a significant number of cells died within 24 h, confirming that significant cell death processes were occurring. This number increased significantly from days 4–6 (Table II). Again, there was no difference between pko and wild-type T cells.

Spontaneous apoptosis presumably represents the outcome of a number of processes, including TCR reactivation by Ag in vivo, cytokine withdrawal, and Fas or TNFR signaling. The competence of the Fas receptor on in vivo alloactivated donor T cells was determined. Addition of anti-Fas antibodies resulted in equivalent increased death, suggesting that the Fas pathway was intact in pko T blasts. The increase in cell death within 24 h of treatment with anti-Fas antibodies increased from minimal to significant levels from days 4–6 after induction of GVHD, consistent with previous observations on Fas activation in vitro (35).

![FIGURE 3. Cytokine profile and functional perforin expression of pko and wild-type T cells activated in vivo.](http://www.jimmunol.org/content/1196/9/1196/F1d)

The cytokine profile and functional perforin expression of pko and wild-type T cells activated in vivo. The capacity of donor T cells from sublethally irradiated SCID mice 6 days after injection to produce IFN-γ and IL-4 (b) or IL-10 and TNF-α (c) was determined by staining intracellular cytokines and flow cytometric analysis. Cells were stained after an 18-h incubation on plate-bound anti-CD3 antibodies in the presence of IL-2. The isotype controls are shown in a. Similar profiles were seen in cells that were directly stained without stimulation after isolation ex vivo (data not shown). This intracellular cytokine assay system has been used to demonstrate IL-4 and IL-10 expression in reactivated BALB/c T cell blasts in vitro (K. Raju, unpublished observations) so that the failure to observe IL-4 and IL-10 expression by in vivo activated T cells of B6 origin is not an artifact. d. At the same time as the spleen cells were purified for the cytokine analyses, redirected lysis assays on P815 target cells were performed. Significant cytotoxicity was shown by B6 T cells (open squares), while almost none was shown by the pko cells (closed squares). This experiment has been repeated three times with similar results.
**Table II. Spontaneous and Fas-mediated apoptosis of in vivo alloactivated T cells**

<table>
<thead>
<tr>
<th>Donor</th>
<th>Day</th>
<th>Annexin V+</th>
<th>CD4+ (%)</th>
<th>CD8+ (%)</th>
<th>Spontaneous Death (%)</th>
<th>Fas-Mediated Death (%)</th>
<th>Specific Fas-Mediated Death (%)</th>
</tr>
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<tbody>
<tr>
<td>B6</td>
<td>4</td>
<td>26.5 ± 5.6 (n=2)</td>
<td>49.5 ± 2.5 (n=2)</td>
<td>22.5 ± 3.6</td>
<td>22.5 ± 1.8</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>pko</td>
<td>4</td>
<td>39 ± 0.5 (n=2)</td>
<td>51.0 ± 0.5 (n=2)</td>
<td>17.7 ± 3.8</td>
<td>19.5 ± 7.4</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>B6</td>
<td>6</td>
<td>69 ± 7.4 (n=4)</td>
<td>89.5 ± 4.3 (n=4)</td>
<td>40.2 ± 4.6</td>
<td>59 ± 2.6</td>
<td>18.8</td>
<td></td>
</tr>
<tr>
<td>pko</td>
<td>6</td>
<td>65.8 ± 5.7 (n=4)</td>
<td>87.5 ± 3.0 (n=4)</td>
<td>38.8 ± 2.7</td>
<td>55 ± 6.8</td>
<td>16.2</td>
<td></td>
</tr>
</tbody>
</table>

*At 4 and 6 days after injection into sublethally irradiated SCID hosts, spleen cell suspensions were made and stained with annexin V-FTTC, CD4- or CD8-PE, and 7-AAD. The percentage of annexin V+ cells that excluded 7-AAD is reported as the average and SE of the results from at least three separate experiments. The percentage of CD8+ T cells at the initiation of culture was always similar for both B6 wild-type and pko donor cells. To determine the susceptibility of the plated cells to Fas-mediated killing, anti-Fas Ab (1 μg) was added to some wells at the initiation of culture, and the number of live and dead cells was counted after 24 h. Specific Fas-mediated killing was calculated by subtracting the percentage of cells that spontaneously died.*

**Decreased activation-induced death of alloactivated pko T cells**

Six days after injection into sublethally irradiated SCID mice, density gradient-purified B6 and pko T cells were reactivated on plate-bound anti-CD3 Abs in the presence of IL-2. As shown in Table III (rows 1 and 2), after 70 h, specific death in the cultures from pko T cells, as determined by manual counting on a hemocytometer, was lower than that in the cultures from B6 T cells. This implicated a role for perforin in AICD and was the first evidence of an underlying cause of the different behaviors of pko and wild-type T cells in GVHD. TNF has been implicated in causing AICD of T cells (35), mainly CD8+ T cells in vitro (6) via a separate pathway than that involving perforin (8). To determine whether altered responses to TNF-α accounted for the differences in specific death observed with reactivated wild-type and pko T cells, anti-TNF-α antibodies were included in the reactivation cultures. As shown in Table III, lines 3 and 4, TNF blockade decreased the amount of specific death observed, but the survival advantage of pko T cells was maintained, implicating a role for perforin in AICD distinct from the role played by TNF-α.

**Decreased proliferation of reactivated wild-type T cells compared with pko T cells initially activated in vivo**

T cell hybridomas (36) and in vitro proliferating T cells (21) undergo growth inhibition when reactivated with mitogenic stimuli in the presence of IL-2. The results shown in Fig. 4 and Table IV confirmed that in vivo alloactivated T cells also were growth inhibited after reactivation in vitro. Strikingly, pko T cells were significantly less inhibited than wild-type T cells.

Increased costimulation in the spleens of mice injected with pko T cells may have accounted for their increased proliferation after reactivation despite the evidence in Table I that the Ag-presenting capability was independent of donor origin. To control for this possibility, reactivation was performed in the presence of plate-bound anti-CD28 Abs (37) (vertical lines, groups 2 and 3). Again, T cells lacking perforin were significantly less growth inhibited. Blockade of TNF-α with specific Abs to remove a death pathway separate from that mediated by perforin decreased the growth inhibition after reactivation, but did not change the proliferative advantage displayed by pko T cells (horizontal bars, groups 4 and 5). In fact, provision of both costimulation and TNF-α blockade was able to completely reverse the growth inhibition of pko but not wild-type T cells (vertical bars, groups 4 and 5).

**Table III. Decreased AICD of alloactivated pko T cells is independent of TNF-α**

<table>
<thead>
<tr>
<th>Donor Cells</th>
<th>TNF Blockade</th>
<th>Specific Death (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6</td>
<td>−</td>
<td>61 ± 2.4</td>
</tr>
<tr>
<td>pko</td>
<td>−</td>
<td>39 ± 2.5</td>
</tr>
<tr>
<td>B6</td>
<td>+</td>
<td>35 ± 1.1</td>
</tr>
<tr>
<td>pko</td>
<td>+</td>
<td>26 ± 1</td>
</tr>
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</table>

*Density gradient-separated donor T cells in CM plus IL-2. 6 days after injection into sublethally irradiated SCID mice, were plated into 96-well plates that had either been precoated with anti-CD3 Abs or simply with FCS for control. The numbers of viable and dead cells were enumerated after 70 h by manual counting and eosin uptake. In some wells, anti-TNF-α ascites (1/100 dilution) was included at a dose previously shown to block the effect of TNF-α in this AICD assay (8). The average and SE of the results from three separate counts are reported and are representative of four separate experiments.

**FIGURE 4.** Increased proliferation of reactivated pko T cells initially activated in vivo. Six days after initial injection, B6 and pko T cells were purified from SCID spleens and plated at a density of 4 × 10^5 cells/ml into individual wells of a 96-well plate along with 25 U/ml of rIL-2. The wells had been precoated with 1 μg of anti-CD3 and anti-CD28 Abs, anti-CD3 only, or serum only. After 48 h, [3H]thymidine was added, and the amount of thymidine uptake was determined in a gamma scintillation counter 18 h later. The average and SE of four measurements are reported. In groups 4 and 5, anti-TNF-α ascites (1/100 final concentration) was also added throughout the reactivation period. The data are representative of seven different experiments on both days 4 and 6 after initial injection and three experiments involving TNF blockade on day 6. 1, Control B6 spleen cells; 2, B6 GVHD; 3, pko GVHD; 4, B6 GVHD and anti-TNF; 5, pko GVHD and anti-TNF.
Table IV. *Increased in vitro proliferation of in vivo alloactivated pko T cells*

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Day</th>
<th>Donor Cells</th>
<th>Anti-TNF (cpm)</th>
<th>IL-2 (cpm)</th>
<th>Anti-CD3 (cpm)</th>
<th>Anti-CD28 (cpm)</th>
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<td>2,402</td>
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<td>4</td>
<td>Control</td>
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<td>B6</td>
<td>16,712</td>
<td>2,783</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
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<td></td>
<td>B6</td>
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<td>pko</td>
<td>2,194</td>
<td>560</td>
<td>ND</td>
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</tr>
</tbody>
</table>

*On the indicated day after injection, in vivo-activated B6 and pko T cells were reactivated in vitro as described in Materials and Methods; subsequent proliferation was determined by thymidine uptake 48 h later. The results of each individual experiment are reported, and Expt. 4 is displayed graphically in Fig. 5.

The average of four separate measurements is reported. The SE of the measurements was always <10% of the average.

Discussion

In this study, alloreactive T cells from pko mice were shown to accumulate significantly more than from wild-type mice in both sublethally irradiated C.B-17 SCID mice (Fig. 1a). This increased expansion did not seem to be due to persistence of host APCs that survived, as a result of the defective pathway of cellular cytotoxicity in pko T cells, and continued to stimulate donor cells (Table I).

The mixing experiments in vivo (Fig. 1) also argue against APC persistence being responsible for the increased expansion. Wild-type B6 T cells should have helped to clear APCs, but did not decrease the observed increased accumulation of donor cells in the mix. Functionally defective suppressor T cells that acted via a perforin-dependent mechanism (28) could have allowed the hyperexpansion of pko T cells. Again, restoration of normal expansion in vivo by a mixture of wild-type and pko T cells, which should have provided putative suppressor cells, was not observed (Fig. 1b).

The perforin deficiency may have caused alloreactive pko T cells to differentiate into another functional phenotype in vivo (with different activation requirements), accounting for their behavioral differences from wild-type cells. Fig. 3 showed that, despite the absence of perforin-dependent redirected lysis of P815 cells, the cytokine profile of pko T cells was the same as wild-type T cells on day 6 and exclusively of the Th1/Tc1 type (38).

Alloreactive T cells, isolated from the spleens of sublethally irradiated SCID mice and reactivated in vitro on plate-bound anti-CD3 Abs in the presence of IL-2, proliferated significantly less than cells grown only in IL-2 as measured by [3H]thymidine uptake (Fig. 4). The pko T cells were two- to threefold more resistant to this reactivation-mediated growth inhibition, although they appeared to be equivalently activated in vivo, as measured by CD25, CD69, and CD44 up-regulation and TCR down-regulation (Fig. 2). Since alloreactive donor T cells are presumably subject to multiple encounters with host Ag in vivo, this resistance of pko T cells to reactivation-mediated growth inhibition, with or without costimulation, may help to explain their increased expansion in vivo.

What are the mechanisms that partially protect activated pko T cells from growth inhibition after reactivation? Apoptosis and growth arrest may be interrelated (39, 40). Recently, we showed that mitogen-activated pko T cells were protected from death in a short term in vitro AICD assay (8). This assay, which has been previously described (21, 41), begins with a 2-day activation of primary spleen cell cultures. After isolation on density gradients, T cell blasts are reactivated on plate-bound anti-CD3 antibodies in the presence of IL-2. The reactivated cells are growth inhibited, T cell blasts are reactivated on plate-bound anti-CD3 antibodies in the presence of IL-2. The reactivated cells are growth inhibited, and >50% die after 48 h. This death, although Fas independent (42), is partially mediated by TNF-α (8). However, pko T cells were significantly protected from death despite blockade of TNF-α by specific Abs. In mixing experiments with wild-type T blasts in vitro (and similarly in vivo; Fig. 1b), pko T cells preferentially survived, suggesting that the cell death pathway mediated by perforin was autonomous and not due to an absence of fratricidal killing (43, 44). This interpretation was further supported by the finding that T cells from beige mice, which express perforin but are defective in granule exocytosis killing, were not protected from death in this short term assay. We speculated that perforin may leak out of granules or transport vesicles during synthesis and cause apoptosis, with or without the cooperation of granzymes, after reactivation.

Could this perforin-dependent AICD pathway, defined in vitro, play a role in the AICD observed during GVHD in SCID mice? This question is difficult to answer conclusively because a number of AICD pathways are operating simultaneously in vivo and can mask the effect of perforin. The in vitro assay was able to uncouple the perforin-dependent pathway from the Fas-dependent pathway as it was performed before the Fas receptor became competent to transmit a death signal (35). In vivo, susceptibility to Fas killing and spontaneous apoptosis is time dependent and increases from days 4–6 after injection of donor cells (Table II). Decreased growth inhibition of alloreactive T cells was constant on days 4 and 6 (Fig. 4), suggesting that another mechanism is involved. TNF-α does account for some of the observed apoptosis, since TNF-α blockade decreased cell death and increased proliferation after reactivation in vitro (Table III and Fig. 4). However, the survival advantage of pko T cells remained in the presence of TNF-α blockade.

We speculate that a defective perforin-dependent AICD pathway can account for the preferential expansion of pko T cells in GVHD that was not decreased by addition of wild-type B6 T cells (Fig. 1) and their decreased growth inhibition (Fig. 4) and AICD (Table III) after subsequent reactivation in vitro. The significant amount of cell death seen in response to reactivation in vitro after 24 h is probably mediated by Fas and possibly TNF-α, since it occurs earlier than the death mediated by perforin in vitro.

AICD has previously been shown to accompany a normal immune response in vivo. Cell death has been shown to take place in staphylococcal enterotoxin B-reactive V88+ T cells (45), activated transgenic CD8+ αβ-T cells (46), and γδ-T cells (47). Apoptosis...
also accompanies T cell activation in response to viral infections (31, 48), including HIV (32). Our results generalize this finding to an allogeneic response and GVHD.

Acknowledgments

We thank H. Hengartner for the provision of perforin knockout mice. We also thank J. Allison for providing the 37.51 anti-CD28 hybridoma, H. Karasuyama for mouse IL-2 DNA-transfected cells, and J. Abrams for the MP6-XT22 anti-TNF-α hybridoma.

References