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References

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A Role for Perforin in Activation-Induced Cell Death

David Spaner,* Kaliannan Raju,* Laszlo Radvanyi,* Yunping Lin,* and Richard G. Miller*

The granule exocytosis pathway of T cell cytolysis is absent in mice whose perforin gene has been ablated by targeted mutagenesis. The ability of activated naive T cells to undergo apoptosis in vitro following reaggregation of the TCR complex with anti-TCR Abs via a Fas-independent pathway was found to be defective in the absence of perforin. Protection from death was most marked in CD8+ T cells. In wild-type cells, perforin was expressed at the same time that apoptosis occurred, and blockade of perforin expression by either incubation with perforin antisense oligonucleotides or with anti-IL-2 Abs resulted in increased viability of activated T cells. The role of perforin was not via perforin-dependent fratricidal killing. The results suggest a model in which perforin acts internally to cause a form of activation-induced T cell death distinct from that caused by members of the TNFR superfamily. The Journal of Immunology, 1998, 160: 2655–2664.

The role of perforin in the control of T cell cytolysis has recently been greatly clarified by studies in perforin knockout (pko) mice (1, 2). Two forms of cytolysis can be measured in short-term (<8-h) chromium release assays. The first is described by the granule exocytosis model and is absent in pko mice. Lytic granules in cytotoxic T cells house proteins such as perforin and granzyme family members. During killing, granular contents are extruded into the Ag-binding groove between the cytotoxic cell and the target cell. In response to pores in the membrane formed by perforin molecules, granzymes enter the target cell and induce apoptosis. The second kind of cytolysis is calcium independent, intact in pko mice, and results when Fas ligand on the cytotoxic T cell binds to the Fas receptor on the target cell.

Recently, it has been shown that some T cells die in a suicidal manner after activation, and the mechanisms of this activation-induced cell death (AICD) are being intensively studied (3). Evidence has been presented that some forms of AICD are controlled by interactions between members of the TNFR and TNF-ligand superfamilies, in particular the interaction between Fas and its ligand (4). These molecules clearly play a role in the deletion of superantigen-reactive T cells in vivo (5, 6) and in cell death resulting when the TCRs of previously activated T cell lines or clones are cross-linked (7–9). Less information is available regarding the control of AICD in primary activated T cells, which consist mainly of naive cells.

In this work, the role of perforin in the control of AICD was studied. The results indicate that, in addition to its role in killing by granule exocytosis, perforin is involved in an AICD pathway that occurs early after activation by Ag and is independent of pathways controlled by members of the TNFR family.

Materials and Methods

Animals

C57BL/6J (B6) (H-2b, Thy-1.2), C56BL/6J- bg/bg, and C57BL/6J- gld/gld mice were obtained from The Jackson Laboratory (Bar Harbor, ME). pko mice on the B6 background (1, 2) and C.B-17 scid mice were bred and maintained in the defined flora animal colony at Ontario Cancer Institute (Toronto, Canada). The original pko breeding pair was generously provided by Dr. H. Hentgartner (Zurich, Switzerland). OVA mice (10), which carry a transgenic TCR specific for amino acids 323–339 of chicken OVA presented by I-A^d (11), were obtained originally from Dr. D. Y. Loh (St. Louis, MO) and have been backcrossed at least six times onto BALB/c in Ontario Cancer Institute animal facility. OVA-scid mice were bred by crossing with C.B-17-scid/scid mice. C.B-17 is congenic to BALB/c, differing only in the region of the Ig heavy chain allele. BALB/c are IgHb and CB-17 are IgHc (12). F1 mice from the cross were interbred, and progeny selected for the presence of the transgene and the scid phenotype. The TCR can be identified by the Id-specific Ab, KJ1-26 (13). A high percentage of CD4^+ KJ26^+ PBL in the absence of circulating B or CD8^+ T cells indicated the presence of both the transgene and the scid defect. Offspring were derived by Caesarian section and foster-mothered under specific pathogen-free defined flora conditions. All work in this study was performed with such specific pathogen-free animals. 2C mice (14), which carry a transgenic TCR specific for the class I MHC molecule L^d, were also originally obtained from Dr. D. Y. Loh. They have been backcrossed onto B6 more than 10 times, and the expression of the transgene was determined using the mAb, IB2 (15), specific for the 2C TCR, in flow-cytometric analyses of peripheral blood.

Abs, reagents, and cell lines

Anti-CD3 (145-2C11) (14) was purified from culture supernatants by protein G column chromatography. Phytoerythrin- or FITC-labeled CD4 and CD8 Abs were purchased from Sigma Chemical Co. (St. Louis, MO). The anti-FcyRIII Ab, 2.4G2 (16), was obtained from American Type Culture Collection (ATCC, Rockville, MD) and used as a culture supernatant. Phycoerythrin- and FITC-labeled Thy-1.2, V88, and annexin-V Abs and bixin-labeled anti-Fas Abs were purchased from PharMingen (San Francisco, CA). 7-AAD and streptavidin-phyceroerythrin were purchased from Sigma Chemical Co. The anti-IL-2 Ab, 4B6.2 (17), was a gift of Dr. T. Mosmann (Edmonton, Alberta, Canada); the anti-TNF Ab, MP6-XT22 (18), was generously provided by Dr. J. A. Abrams (DNAX, Palo Alto, CA); and ascs from both were prepared in pristane-pretreated scid mice. Anti-perforin mAb ascites was purchased from Kamiya Biomedical Co. (Seattle, WA). The IB2 Ab. Id specific for the 2C transgene, was initially obtained from Dr. H. Eisen (Massachusetts Institute of Technology, Cambridge, MA) (15) and was purified and biotinylated in our laboratory.
Mouse IL-2 cDNA-transfected X63Ag8-653 cells were a generous gift of H. Karasuyama (Basel Institute Basel, Switzerland) (19). Con A was purchased from Pharmacia (Uppsala, Sweden).

CTLL-2 and P815 tumor lines were obtained from ATCC. P815 was maintained in exponential growth by serial passage in complete media (CM: α-MEM, 10% FCS, 5 × 10−3 M 2-ME, 2 mM l-glutamine) at 37°C in an atmosphere of 5% CO2. CTLL-2 was maintained identically except that the medium was supplemented with 25 μM IL-2.

TCR complex religation of primary activated T cells

Our assay for T cell activation and induction of AICD in vitro has been previously described (20). Briefly, spleen cells from B6 and pko mice were cultured at 2 × 10^5 cells/ml with 1 μg/ml of soluble anti-CD3 Ab. After 48 h, these primary activated blasts were isolated using Lympholyte separation medium (Lymphoretic, Burnaby, Ontario, Canada). The cells were washed, and resuspended at 5 × 10^5 cells/ml in CM. Anti-CD3 Abs were bound to 96-well EIA plates (Corning Costar, Cambridge, MA) by incubation of 100 μl of 2 μg/ml protein G (Pharmacia)-purified Ab in PBS for 3 h at 37°C or overnight at 4°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 non-specific binding. Then 100 μl of the primary activated blasts were added and incubated at 37°C. Control wells were simply blocked with CM. IL-2 (50 U/ml) was added to all cultures.

DNA analysis

The method of Telford et al. was followed (21). T cells (approximately 1 × 10^6) were washed and fixed in 70% ethanol at −20°C for several days at 10^6 cells/ml. The cells were then washed in Ca^2+-, Mg^2+-free PBS and resuspended in 1 ml of Ca^2+-, Mg^2+-free PBS with 0.1% Triton X-100, 0.1 mM EDTA, and 50 μg/ml RNase for 1 h at 37°C. This incubation period allows low m.w. DNA to escape through the permeabilized membranes (22). Cells were then washed and resuspended in staining buffer (Ca^2+-, Mg^2+-free PBS, 0.1 mM EDTA, 0.1% Triton X-100, and 50 μg/ml of propidium iodide (Sigma Chemical Co.)) at room temperature in the dark for 4 to 12 h. Cells were then filtered through nylon mesh and analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) using LYSIS II software.

Mixed lymphocyte responses

Responder cells were spleen cells diluted to 5 × 10^5 cells/ml in CM. Irradiated (2000 cGy) spleen cells from BALB/c mice at 5 × 10^5 cells/ml were used as stimulators. Cultures (200 μl of a 1:1 mixture) were then incubated for 72 h, and 1 μCi of [3H]thymidine (sp. act., 6.7 Ci/mmol) (Dupont NEN). Labeling was allowed to proceed for 1 h at 37°C. The reaction was stopped by adding 9 ml of CM. Both the labeled and unlabeled cells were centrifuged a second time using Lympholyte separation medium, and the interface layer was washed in 50 ml of α-MEM (24).

Immunofluorescence

Cell staining (in 100 μl vol) was performed after first blocking nonspecific binding by a 10-min incubation at room temperature with 10 μl of mouse serum (Cedarlane) and 40 μl of 2.4G2 culture supernatant. Cults (5 × 10^6) were then stained with the appropriate Abs for 20 min, washed, incubated with 7-AAD (Sigma) to identify dead cells, and then analyzed on a FACScan flow cytometer (Becton Dickinson) using LYSIS II software.

Western blot analysis of perforin

Cells were harvested at various time points after TCR recross-linking, washed with PBS, and lysed in 50 to 100 μl of lysis buffer (50 mM HEPES, pH 6.8, 1% SDS, 150 mM NaCl, 50 mM NaF, 1 mM sodium vanadate, and 1 mM sodium pyrophosphate). The proteins in the lysate (50 μg/lane) were separated by SDS-PAGE and transferred onto nylon membranes using a semi-dry transfer apparatus (Hoeffer Scientific Instruments, San Francisco, CA). The membranes were blocked or Tris-buffered saline containing 5% skim milk, and incubated with antiperforin ascites (1/1000 dilution) in the same medium and 1% skim milk, according to the manufacturer’s instructions. After washing, the membranes were treated with secondary Abs conjugated to horseradish peroxidase, and the bands were visualized using an enhanced chemiluminescence kit (DuPont NEN).

Blockade of perforin expression by antisense oligonucleotides

Three sense oligonucleotides (5′-TGT GCC TGC AGC ATG GCC, 5′-ACC TCT CGC GCC TGC GCC, and 5′-CCA CGG AGG GTG GTG CCG GCC (sense 1, 2, and 3; positions 134–154, 296–316, and 483–503 on perforin mRNA, respectively)) and the corresponding anti-sense oligonucleotides (5′-GGC CAT GAT GCT GGC AGA ACA, 5′-GCC GGA GGC AGC AGA AGT, and 5′-CCC GCC CCA CAC CCT CCG TGG (antisense 1, 2, and 3)) were synthesized as phosphothioates. The sequences were chosen from the mouse perforin cDNA sequence in GenBank (accession number J04148) (25). They were added at the indicated concentrations during both initial activation and restimulation of splenic cultures with apoptosis measured by eosin uptake 48 h after reactivation.

Results

Decreased AICD in activated T cells from pko mice

A series of studies from our laboratory has shown that T cell blasts undergo programmed cell death when their TCR is reaggregated 48 h after initial activation (20, 26, 27). Activated wild-type and pko T cells were replated onto anti-CD3 Abs with 50 U/ml IL-2. Cell viability and DNA fragmentation were assessed 48 h later. As shown in Table I, the percentage of specific death caused by CD3 religation after 48 h was reduced significantly in cultures of activated pko T cells, while total cell numbers were little different (Table I). The changes in cell viability over time following CD3 religation in both cultures were also determined (Fig. 1, A and B). In both cases, control cells treated with IL-2 alone did not exhibit a significant drop in cell viability. However, in cultures of wild-type cells treated with anti-CD3, cell viability dropped to less than 2000 targets were added to the U-bottom plates in 100 μl of conditioned media. The plates were centrifuged at 600 rpm for 3 min and then incubated at 37°C for 4 h. Plates were then centrifuged at 800 rpm for 5 min, and 100 μl of the supernatant was transferred to Fisherbrand flint glass tubes (Fisher Scientific, Pittsburgh, PA) and counted in a gamma counter (CompuGamma Model 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. Percentage of cytotoxicity was determined by the ratio (cpm − SR)/(TR − SR) × 100%.

For the redirected lysis assays, 1 μg of anti-CD3 Ab was added to the P815 targets before addition of effectors (23).

FITC labeling of cells

Aliquots of the cells to be labeled were washed in PBS and resuspended at 20 to 50 × 10^6 cells/ml in PBS. FITC (Sigma Chemical Co.) was added (final concentration 30 μg/ml), and the cells were incubated at 37°C for 17 min. The reaction was stopped by adding 9 ml of CM. Both the labeled and unlabeled cells were centrifuged a second time using Lympholyte separation medium, and the interface layer was washed in 50 ml of α-MEM (24).

CONTROL OF T CELL DEATH BY PERFORIN
60% by 48 h (Fig. 1A), pko cells exhibited only a slight decrease in cell viability over the same time period. When the cultures were incubated further (for up to 60 h), protection from cell death was still observed in the pko cultures (data not shown), suggesting that a simple delay in cell death was not the cause.

Figure 1C showed that the number of T cells with hypodiploid DNA content 48 h after reactivation was decreased in the absence of perforin. Hypodiploid peaks are considered to be a quantitative measure of the number of cells undergoing apoptosis in a cell population (22). Therefore, primary activated pko T cells appeared to have a defect in their ability to undergo AICD.

**The death pathway controlled by perforin affects mainly CD8+ T cells in unfractionated spleen cell cultures**

To determine whether reactivated CD4+ and CD8+ T cell blasts were affected similarly by the absence of perforin, triple staining for 7-AAD, annexin-V, and CD4 or CD8 was performed 21 h after reactivation. Phosphatidylserine molecules on the inner membrane of cells undergoing apoptosis flip to the outer membrane (28, 29), resisting the 7-AAD, annexin-V, and CD4 or CD8 was performed 21 h after reactivation. Phosphatidylserine molecules on the inner membrane of cells undergoing apoptosis flip to the outer membrane (28, 29), but the absence of perforin was significant only for CD8+ T cells. Despite the fact that these cells were CD4+ T cells, they expressed large quantities of perforin in response to activation by anti-CD3 Abs (data not shown), in agreement with recent studies showing that activation of CD4+ T cells in the absence of CD8+ T cells results in perforin expression (31, 32). As shown in Table II, two different antisense oligonucleotides, added at both the time of initial activation and recross-linking, increased the viability of the cells in a concentration-dependent manner. The corresponding sense oligonucleotides did not prevent cell death. These results implicated perforin directly in the apoptosis observed after reactivation.

**Perforin expression correlates with apoptosis**

Expression of perforin protein after initial activation was determined by Western blotting. As shown in Figure 3, perforin was expressed 24 h after reactivation and was present throughout the time period in which cells underwent apoptosis (lanes 2 and 5).

**Blockade of perforin expression by antisense oligonucleotides in wild-type T cells decreases apoptosis**

Although the development of T cells in pko mice is assumed to be normal (1, 2, 30), perhaps the molecular pathways that actually mediate AICD fail to develop when perforin is absent during ontogeny, accounting for the observed survival of pko T cells. To directly test the role of perforin in AICD of wild-type cells, the effect of antisense oligonucleotides that interfered with the translation of perforin mRNA was studied. Viability of reactivated B6 spleen cells was increased with antisense 3, but not the sense oligonucleotide (Table II). The experiments were repeated using spleen cells from OVA-scid mice as a monoclonal source of naive T cells. Despite the fact that these cells were CD4+ T cells. Despite the fact that these cells were CD4+ T cells, they expressed large quantities of perforin in response to activation by anti-CD3 Abs (data not shown), in agreement with recent studies showing that activation of CD4+ T cells in the absence of CD8+ T cells results in perforin expression (31, 32). As shown in Table II, two different antisense oligonucleotides, added at both the time of initial activation and recross-linking, increased the viability of the cells in a concentration-dependent manner. The corresponding sense oligonucleotides did not prevent cell death. These results implicated perforin directly in the apoptosis observed after reactivation.

**The effect of perforin is autonomous**

Nonspecific killing of bystander cells by perforin released after reaggregation of the TCR on activated T cells would be the most conventional explanation for the involvement of perforin in AICD. We first asked whether recross-linked cells could kill nonspecifically activated syngeneic T cells. As shown in Table I (lines 1 and 2, columns 7 and 8), control and recross-linked T cells were highly cytotoxic to P815 tumor cells in a redirected lysis assay. That this nonspecific cytotoxicity was due to perforin was revealed by its absence when pko T cells were evaluated in the same assay (Table I, lines 1 and 2, columns 7 and 8). Despite the presence of strong nonspecific cytotoxicity, 48-h activated Con A blasts were at best minimally killed by the recross-linked cells (Table I, line 2, column 9). These results suggested that the observed AICD was not just caused by the TCR recross-linked cells killing each other through nonspecific perforin release.

In another attempt to address the issue of whether perforin mediated suicide or fratricide, T cells from B6 or pko mice were activated for 48 h, labeled with fluorescein, and mixed with equal numbers of unlabeled cells of the opposite perforin-expressing phenotype. They were then replated on anti-CD3-bound plates. Forty-eight hours later, the recross-linked cells, along with control mixes grown only in IL-2, were harvested, and the number of viable cells of each type remaining in the cultures was enumerated by flow cytometry. If perforin controlled an autonomous death pathway, more pko cells than B6 cells should survive the TCR recross-linking step. If there was fratricidal killing of pko cells by B6, the number of surviving cells of each type should be approximately the same. As shown in Table III, pko cells had a survival advantage compared with B6 T cells following reactivation that was not observed in the control cultures and was quantitatively
Preferential protection from apoptosis of CD8\(^+\) pko T cell after TCR recross-linking. B6 spleen cells were activated with soluble anti-CD3 Abs, harvested after spinning over Lympholyte columns, and replated onto immobilized anti-CD3 on 96-well plates. Twenty-one hours after recross-linking, the contents of wells were resuspended and pooled so that the approximately 2 \(\times\) 10\(^6\) cells could be stained with annexin-V FITC, anti-CD4\(^+\) or CD8\(^+\) phycoerythrin, and 7-AAD. The percentage of 7-AAD\(^-\) cells that stained positively for annexin-V binding and surface expression of either CD4 or CD8 is reported as the average and SE of three independent experiments. The respective percentages of CD4 and CD8 cells in the cultures at the time of the annexin-V binding assay were: B6 control (33 \(\pm\) 2, 70 \(\pm\) 4), B6 recross-linked (27 \(\pm\) 5, 74 \(\pm\) 3), pko control (36 \(\pm\) 6, 66 \(\pm\) 6), and pko recross-linked (26 \(\pm\) 1, 72 \(\pm\) 1).

To insure that the survival differences were not an artifact of the labeling procedure, experiment 3 was designed. Spleen cells from 2C mice, whose CD8\(^+\) cells could be identified by the 1B2 Ab, were activated and mixed with activated pko spleen cells. After recross-linking, the surviving wild-type cells were determined by the percentage of 1B2\(^+\) cells, and preferential survival of pko T cells was again observed.

The granule exocytosis pathway is defective in mice homozygous for the beige (bg) mutation (33), in this case due to a defect in granule formation (34) rather than the absence of perforin as in pko mice. If the observed cell death in the AICD assay used in this study was fratricidal and mediated by the granule exocytosis pathway, then reactivated T cells from bg/bg mice should also be protected from death. Figure 4A confirmed that redirected lysis of P815 cells by activated bg/bg T cells was approximately 10-fold lower than by activated wild-type B6 T cells. However, the number of apoptotic bg/bg T cells was even greater than wild-type cells 48 h after reactivation (Fig. 4B). This result indicates that AICD occurs in bg/bg T cells and is consistent with our hypothesis of an autocidal role for perforin.

**Role of IL-2 in perforin-dependent AICD**

It is known that IL-2 can program activated T cells to undergo AICD (35-37). We wondered whether defective IL-2 production, rather than the absence of perforin itself, might account for the decreased AICD observed with pko T cells. Accordingly, IL-2 production from pko and B6 wild-type T cells activated in an MLR was determined (Table IV). The pko T cells secreted approximately two- to threefold more IL-2 than wild-type T cells in the first 3 days of an MLR. Addition of wild-type T cells did not prevent the increased production of IL-2 (Table IV). Since pko T cells produce more IL-2 at short times after activation than wild-type T cells, it seems unlikely that the decreased death of pko cells is a direct effect of IL-2 levels.

A known function of IL-2 is to up-regulate the expression of perforin (38). We thus wondered whether IL-2 might exert its pro-apoptotic effect through perforin. By blocking IL-2 secretion throughout the culture period, the degree of apoptosis was decreased in wild-type B6 cells (Table V), consistent with previous results (35). IL-2 blockade of pko cultures did not increase further the viability of the remaining cells, suggesting that other apoptotic pathways were not blocked. Figure 3 confirmed the known dependence of perforin protein expression on IL-2, as perforin could not be demonstrated, or was markedly decreased, when anti-IL-2 ascites was added throughout the culture period (Fig. 3, lanes 2 and 5). These results suggested that changes in perforin may help to explain the pro-apoptotic effects of IL-2, at least in this short-term cell death assay.

**Fas-mediated death pathways are intact in pko mice**

There is much experimental evidence showing that Fas and its ligand cause the death of activated T cells in vivo and in vitro. Despite previously published results demonstrating normal Fas signaling in pko mice (2) and that the AICD assay used in these studies is Fas independent (5), the possibility that Fas defects could...
The death pathway controlled by perforin is independent of TNF-α

TNF-α has recently been shown to cause the death of reactivated CD8⁺ T cells by signals through the p75 TNFR (39, 40). These studies were performed using short-term T cell lines. Since the death pathway controlled by perforin in the AICD assay used in our current study seemed to mainly involve CD8⁺ T cells, the role of TNF-α was studied. Reactivation of pko T cells was performed in the presence of an anti-TNF-α mAb, and the degree of apoptosis was determined by eosin uptake 48 h following TCR religation. The logic is similar to that of gene complementation experiments. If TNF-α blockade prevented AICD in both pko and wild-type T blasts, it acts independently of the perforin-dependent death pathway. If TNF-α blockade protected wild-type cells, but not pko T cells from AICD, then the protection afforded by the perforin deficiency acts through TNF-α. As shown in Table VI, TNF blockade increased the survival of reactivated wild-type T cells, suggesting that TNF-α played a role in the apoptosis observed in the AICD assay used in this study. TNF-α blockade also increased the

Table II. Inhibition of AICD in vitro by perforin antisense oligonucleotides

<table>
<thead>
<tr>
<th>Source of Cells</th>
<th>Oligonucleotides</th>
<th>Concentration μM</th>
<th>Cell Viability (%)</th>
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</thead>
<tbody>
<tr>
<td>B6</td>
<td>Sense 3</td>
<td>10</td>
<td>60 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>62 ± 2</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>50</td>
<td>80 ± 2</td>
</tr>
<tr>
<td>Ova-SCID</td>
<td>Sense 1</td>
<td>10</td>
<td>52 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>54 ± 2</td>
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<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>64 ± 3</td>
</tr>
<tr>
<td>Ova-SCID</td>
<td>Sense 2</td>
<td>1</td>
<td>52 ± 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>50 ± 3</td>
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<td></td>
<td></td>
<td>25</td>
<td>74 ± 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>72 ± 1</td>
</tr>
</tbody>
</table>

* The standard AICD assay was performed in the presence of the indicated oligonucleotides added at the beginning of both initial activation and recross-linking. Their addition did not affect cellular proliferation (data not shown). Cell counts were performed 48 h after reactivation, and the percentage of dead cells was determined by eosin staining. The reported values are the average and standard deviation of four independent counts. Western blots for perforin expression were performed to determine the degree of inhibition by the antisense constructs and quantitated by densitometry. Perforin expression was 30% of controls after antisense treatment using cell lysates from OVA-scid cells, 24 h after reactivation.

Table III. Preferential survival of PKO blasts after reactivation

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Start (%)</th>
<th>Control (%)</th>
<th>Anti-CD3 (%)</th>
<th>Start (%)</th>
<th>Control (%)</th>
<th>Anti-CD3 (%)</th>
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<td>55</td>
<td>41</td>
<td>ND</td>
<td>45</td>
<td>59</td>
</tr>
</tbody>
</table>

* Forty-eight-hour B6 and pko blasts were harvested and isolated over lympholyte gradients and half were FITC-labeled as described in Materials and Methods (for experiments 1 and 2). The cells were counted and resuspended at 5 x 10⁶/ml in CM. Labeled and unlabeled cells of opposing perforin expressing phenotypes were mixed approximately 1:1 and then replated onto control and anti-CD3 bound wells of a 96-well plate. An aliquot of each mixture was saved so that the exact initial ratio of labeled and unlabeled cells could be determined. Forty-eight hours later the cells were harvested and counted, and approximately 5 x 10⁶ cells were washed in staining buffer. The cells were then analyzed by flow cytometry after gating out dead cells that took up 7-AAD. The percentage of live cells that stained brightly in the fluorescein channel (green) was determined and compared with the input value. Start = percentage of wild-type of pko cells before replating. Control = percentage of cells 48 h after replating in IL-2. Anti-CD3 = percentage 48 h after reactivation with anti-CD3.
cytometric determination of DC4-1L-2 produced by the unstimulated responder cells. The amount of IL-2 produced by the IL-2 measurement in four wells. The number in parentheses is the amount of value was determined from the calibration curve and is the average and standard error. The reported values were taken at 54 h in experiment 1 and 72 h in experiment 2. Each flow cytometric determination of DC4-1 cells, to ensure that the same starting numbers were being compared, was not performed routinely since previous work had shown that the phenotype of pko and B6 spleen cells was the same (1) and this finding was confirmed in our pko colony (unpublished results).

survival of reactivated pko blasts, and their previously demonstrated survival advantage was maintained compared with similarly treated wild-type cells. This result suggested that the death pathway mediated by perforin was independent of that mediated by TNF-α.

**Discussion**

The experiments described in this work suggest that perforin, in addition to its role in CTL and NK killing of target cells, is involved in the control of proliferation and death of activated T cells during an immune response. An autonomous role for perforin in the control of cell death is supported by the following data:

1) Reactivation of previously activated pko T cells caused less cell death, as measured by reduced eosin staining, hypodiploid DNA peaks, and annexin-V positivity (Table I and Figs. 1 and 2).

2) Death was not fratricidal since marked pko T cells behaved independently when mixed with wild-type T cells in vitro (Table III). Moreover, killing of chromium-labeled T cell blasts by reactivated T cells could not be detected (Table I).

3) Antisense blockade of perforin expression reduced the death of reactivated wild-type T cells (Table II).

4) The disproportionate number of CD4-1 and CD8-1 T cells that stained positively with annexin-V FITC (Fig. 2) after reccross-linking also supported an autonomous role for perforin. Fratricidal killing should not have discriminated between CD4-1 and CD8-1 T cells in cultures that involved both types of cells.

5) Reactivated T cells from bg/bg mice, in which perforin expression is normal despite significant defects in the granule exocytosis killing pathway, were not protected from apoptosis (Fig. 4).

Increased numbers of surviving cells could potentially also be explained by a faster doubling time of pko T cells. However, as shown in Figure 1A, the numbers of both B6 and pko T cells after reactivation were essentially the same for the first 30 h, suggesting that their doubling times were the same. Differences began to show up after this time, presumably reflecting the role of perforin in increasing the number of apoptotic cells.

The cell death pathway controlled by perforin mainly seems to involve CD8-1 T cells when unfractionated spleen cells are activated. This result fits intuitively with the known distribution of perforin expression, which is mainly confined to CD8-1 T cells and NK cells. However, some types of human and murine CD4-1 T cells, especially Th2 cells, express mRNA for perforin (38). Recent studies have also shown that the context of initial activation may determine the expression of perforin by CD4-1 cells. Allo-reactive CD4-1 T cell lines derived by stimulation of whole spleen cell suspensions did not express perforin compared with similarly reactive lines derived by first purifying CD4-1 T cells (31, 32). Certainly the CD4-1 OVA-scid cells expressed perforin after activation by anti-CD3 Abs (Table II) perhaps because of the absence of CD8-1 T cells in this system.

The increased amount of IL-2 production noted in MLRs with pko T cell responders can be explained in terms of a defect in AICD. Sarin et al. (41) have shown previously that protease inhibitors can block AICD and also increase IL-2 production by T cell lines. Their explanation is that cells that would otherwise die continue to produce IL-2 in response to Ag. This explanation may also apply to the finding of increased IL-2 production from pko mice in vitro (Table III). Sad and Mossman (42) have also noted increased production of cytokines by pko T cells.

The in vitro assay used in these experiments may have emphasized the role played by perforin in AICD. Recently, both Fas-Fas ligand and TNF-TNFFR (p75) interactions have been implicated in the control of AICD of T cell hybridomas and primary activated T cells expanded in IL-2 for more than 2 days (3, 40). The paradigm that has emerged suggests that Fas-Fas ligand interactions cause the death of CD4-1 T cells, and TNF-p75TNFR interactions kill CD8-1 T cells. However, the assay used in this study involved direct reactivation of primary activated T cells without prior expansion in IL-2. The period of expansion in IL-2 may be an important step that allows for the full development of the death machinery controlled by members of the TNFR family (4). There are important quantitative and kinetic differences between the two types of assays. As illustrated in Figure 5B, the death of short-term T cell lines mediated by TNFR-family members is more destructive (higher percentage of dead cells) and much faster (by 24 h) compared with the death of primary activated T cells.

Fas-Fas ligand interactions do not seem to be important in the type of AICD involving primary activated T cells without expansion in IL-2. T cells from young (6- to 8-wk-old) lpr mice are not protected from cell death in this assay (5), and susceptibility to the Fas death signal requires 4 days of expansion after initial activation and occurs later than the AICD studied in this investigation (4, 43).

In a similar fashion, blockade of TNF signaling throughout the culture period increased the viability of B6 blasts, but never to the same degree as pko blasts (Table VI). This result suggests...

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**Table IV. Increased IL-2 production by T cells from pko mice stimulated in a mixed lymphocyte response**

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Source of T Cells</th>
<th>IL-2 (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B6</td>
<td>9.6 ± 1.3 (0)</td>
</tr>
<tr>
<td></td>
<td>pko</td>
<td>20 ± 1.0 (1.6)</td>
</tr>
<tr>
<td>2</td>
<td>B6 + pko</td>
<td>29.6 ± 1.2 (4)</td>
</tr>
<tr>
<td></td>
<td>B6</td>
<td>12.8 ± 1.3 (1.6)</td>
</tr>
<tr>
<td></td>
<td>pko</td>
<td>35.2 ± 4.7 (1.0)</td>
</tr>
<tr>
<td></td>
<td>B6 + pko</td>
<td>35.2 ± 4.0 (1.4)</td>
</tr>
</tbody>
</table>

* Mixed lymphocyte responses were set up using B6 and pko cells as responders and irradiated BALB/c spleen cells as stimulators. In the mixing experiments, B6 and pko cells were mixed in a 1:1 ratio and all cultures contained the same total number of responders. At 1, 2, or 3 days after the start of each MLR, supernatants were harvested and assayed for the presence of IL-2 as described in Materials and Methods. The reported values were taken at 54 h in experiment 1 and 72 h in experiment 2. Each value was determined from the calibration curve and is the average and standard error of the IL-2 measurement in four wells. The number in parentheses is the amount of IL-2 produced by the unstimulated responder cells. The amount of IL-2 produced by the stimulator cells alone was 0 for experiment 1 and 1.2 U/ml for experiment 2. Flow cytometric determination of DC4-1 cells, to ensure that the same starting numbers were being compared, was not performed routinely since previous work had shown that the phenotype of pko and B6 spleen cells was the same (1) and this finding was confirmed in our pko colony (unpublished results).

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**Table V. Effect of IL-2 blockade on AICD**

<table>
<thead>
<tr>
<th>Source of Cells</th>
<th>IL-2 Blockade</th>
<th>Cell Viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Expt. 1</td>
</tr>
<tr>
<td>B6</td>
<td>–</td>
<td>54 ± 4</td>
</tr>
<tr>
<td>B6</td>
<td>+</td>
<td>76 ± 2</td>
</tr>
<tr>
<td>pko</td>
<td>–</td>
<td>72 ± 3</td>
</tr>
<tr>
<td>pko</td>
<td>+</td>
<td>73 ± 2</td>
</tr>
</tbody>
</table>

* The previously described short-term AICD assay was performed in the presence of either anti-IL-2 ascites (added 24 h after the initial activation and at the start of reccross-linking) or exogenous IL-2 (50 U/ml) added at the same time points. Anti-IL-2 was used at a final concentration of 1:40, and no free IL-2 could be detected throughout the reactivation period, as shown by the inability of the culture supernatant to support the growth of CTLL-2 cells (data not shown). The results of two independent experiments are shown and reported as the average and standard errors of four cell counts.
that TNFR signaling is involved in the death of primary activated T cells not expanded in IL-2, but the death pathway involving perforin is independent of TNF-α. The roles of other members of the TNFR superfamily (such as NGFR (nerve growth factor receptor), 4-1BB, Ox40, CD27, CD30, the TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) receptor, and CD40) (44) in this death pathway remain to be determined.
In addition to routing to the lytic granules, perforin can be implicated in lymphopenia and immunodeficiency. Perforin in lymphocytes of transgenic mice may result in profound cellular damage that activates the effector mechanisms of apoptosis. Perforin can be found at multiple sites outside of the nuclei of lymphocytes in transgenic mice. They also predict that constitutive overexpression of perforin in the secretory pathway may mediate the AICD studied in this work, plus the demonstrated independence from Fas and TNF mechanisms, suggests that there are other mediators of early cell death still to be described. Possible candidates include other members of the TNFR superfamily.

Further evidence that the death pathway controlled by perforin is distinct from that controlled by Fas is provided by the phenotype of mutant mice deficient for both perforin and functional Fas ligand. These mice develop more severe lymphoproliferative disease than Fas ligand-deficient gld/gld mice (45). This observation is more consistent with a second pathway of AICD controlled by perforin than the possibility that the observed defects in AICD in pko mice act through Fas.

IL-2 is another mediator of AICD of T cells. Again, many of its known in vitro effects on T cell death have been studied in short-term cell lines (35) rather than in primary cultures, as we have done in this study. IL-2 most likely is involved in multiple processes that cause AICD. The results of Table V suggest that one of these processes is its control of perforin expression.

The findings in this study suggest that a hitherto unknown mechanism of AICD has been revealed by pko mice. The mechanism has not been determined in this work, but is currently under investigation in our laboratory. Our current hypothesis is that perforin acts internally on intracellular membranes, including mitochondria, the nucleus, or the endoplasmic reticulum, causing damage that activates the effector mechanisms of apoptosis. Perforin may act internally in concert with granzyme family members as when killing CTL targets. Consistent with this idea is the serine proteases of granular origin have been found in the nuclei of lymphokine-activated killer cells (46). These hypotheses strongly predict that perforin can be found at multiple sites outside of the granules after re-cross-linking and should be demonstrable by cell fractionation experiments and immunofluorescence or confocal microscopy. They also predict that constitutive overexpression of perforin in lymphocytes of transgenic mice may result in profound lymphopenia and immunodeficiency.

Perforin has recently been shown to have two routes of secretion (47). In addition to routing to the lytic granules, perforin can be secreted directly. Since this second route is intact in bg/bg mice, perforin in the secretory pathway may mediate the AICD studied in this investigation.

The results in this study suggest that there are multiple levels of control on AICD in T cells. One level may be controlled by perforin, mainly in CD8$^+$ T cells, and fairly early after activation. At later times after activation, pathways controlled by members of the TNFR superfamily, such as Fas and p75 TNFR, may become dominant in the control of immune responses. This later dominance of the TNFR-controlled pathways may explain the fact that pko mice do not develop a lymphoproliferative syndrome, as seen in lpr and gld strains of mice. However, the importance of the perforin-controlled death pathway in vivo may be illustrated by the accelerated disease seen in gld-pko mice described above (45) and the hyper-expansion of (mainly) CD8$^+$ cells in pko mice infected with non-cytopathic viruses (30), or when T cells from pko mice are injected into sublethally irradiated CB-17 scid mice (D. Spaner, manuscript in preparation). The fact that pko T cells are not completely protected from death in the assay used in the studies described in this work, plus the demonstrated independence from Fas and TNF mechanisms, suggests that there are other mediators of early cell death still to be described. Possible candidates include other members of the TNFR superfamily.

### References


