p63 and p73 are not required for the development and p53-dependent apoptosis of T cells

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Summary

The recent discoveries of p63 and p73, homologs of the tumor suppressor p53, raised the possibility of a network of these family members governing cell cycle arrest and apoptosis in response to stress. However, mice lacking p73 show no tendency for spontaneous tumors, and mutations in p63 or p73 are rare in human tumors, rendering any obligate role of these genes in cell death and tumor suppression unclear. In an effort to reconcile these incongruent data, we examined the genetic interactions between p53, p63, and p73 in well-established paradigms of p53-dependent and -independent T cell death using primary, genetically defined lymphocytes. Our findings challenge the generality of the notion that p63 and p73 are required for p53 function or for apoptosis in T cells.

Introduction

p53 functions as a tumor suppressor and is mutated or dysregulated in virtually all human cancers. Mouse knockout studies have demonstrated critical roles for p63 and p73 in epithelial stem cell maintenance (Yang et al., 1999), neurogenesis, inflammation, and various homeostatic processes (Yang et al., 2000). The notion that these homologs also, along with p53, function as tumor suppressors is supported by a vast amount of data from cellular models that have implicated p73 and p63 in pathways leading to cell death (Agami et al., 1999; Gong et al., 1999; Irwin et al., 2000; Jost et al., 1997; Osada et al., 1998; Yang et al., 1998; Yuan et al., 1999). Related work on the mechanism of cell death following excessive T cell receptor (TCR) activation has argued for an essential role for p73 in this process (Lissy et al., 2000). Finally, analysis of genetically defined, E1A-programmed murine embryonic fibroblasts (MEFs) calls up functions for p63 and p73 in p53-dependent cell death (Flores et al., 2002). While together these data link p63 and p73 to tumor suppression, p63 and p73 are generally free of mutations in human tumors (Kaghad et al., 1997; Osada et al., 1998; Irwin and Kaelin, 2001), and p73 null mice show no obvious tendency to develop spontaneous tumors (Yang et al., 2000). The present work examines T cells engineered to have defined *p53*, *p63*, and *p73* genotypes for their ability to undergo various forms of p53-dependent and p53-independent cell death. The data derived from these studies argue against the generality of the concepts that p63 and p73 play essential roles in apoptosis and that p53 requires the functions of p63 and p73.

Results

To find systems to address the conflicting data surrounding the p53 homologs and cell death, we took advantage of adoptive transfer techniques in immune-deficient mice to generate lymphocytes of defined genotypes in a nominally wild-type background. Such reconstituted lymphocytes could then be probed for the specific requirement of each p53 homolog in well-defined paradigms of p53-dependent and -independent T cell death (Clarke et al., 1993; Lowe et al., 1993a).

As an initial step in these studies, we asked if the p53 homologs could be induced in wild-type thymocytes triggered by two major death-inducing signals. p53's requirement for cell death in thymocytes following ionizing radiation is well established, as is its dispensability for glucocorticoid-triggered thymocyte apoptosis (Clarke et al., 1993; Lowe et al., 1993a). As expected, p53 protein was induced in thymocytes treated with ionizing radiation but not in response to glucocorticoids (Supplemental Figure S1A at http://www.cancercell.org/cgi/content/full/6/1/85/DC1). However, neither treatment gave rise to detectable

SIGNIFICANCE

The advent of the p53 homologs p63 and p73 has triggered intense debate on whether and how they interact with p53 in tumor suppression. While p63 and p73 are spared mutations in tumors, myriad reports argue that p63 and p73 function autonomously or in conjunction with p53 to promote cell death. However, from experiments with T cells engineered for precise p63 and p73 genotypes, we now report that p53 acts alone in some forms of apoptosis, countering the notion that p63 and p73 largely mimic and facilitate the actions of p53. Our data argue that p53 suppresses cancer independently of p63 and p73 and that neither homolog is likely to mitigate or complement defects in the p53 gene.

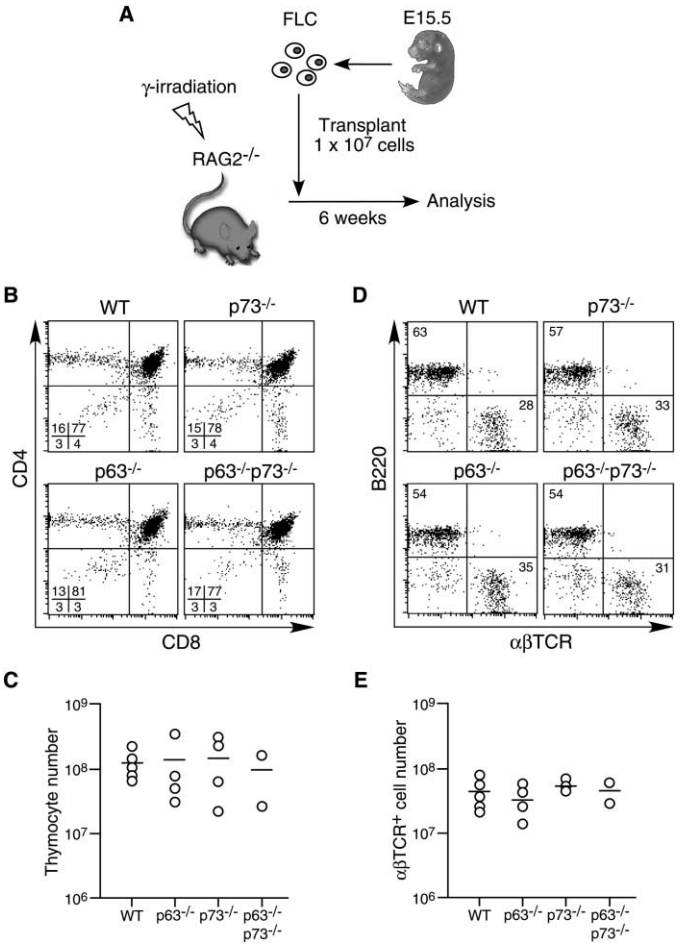

Figure 1. Reconstitution of T cell development in *RAG2*$^{-/-}$ mice

A: Overview of the experimental strategy. Fetal liver cells (FLC) from E15.5 embryos were injected intravenously into sublethally irradiated *RAG2*$^{-/-}$ mice (10^7 FLC per reconstitution). Cells from thymus and spleen were analyzed 6 weeks after reconstitution.
B: CD4 and CD8 expression in reconstituted thymocytes.
C: Absolute thymocyte number.
D: αβTCR and B220 expression in reconstituted spleen cells.
E: Absolute αβTCR$^+$ T cell number.
Bars in **C** and **E** indicate the average number of the cells.

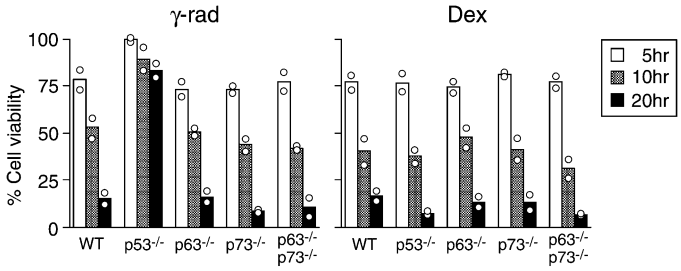

Figure 2. p53-dependent and -independent thymocyte death

Reconstituted thymocytes were treated with either 5 Gy γ irradiation (left panel) or 1 μM dexamethasone (right panel) for indicated times. The bars represent the average of two independent experiments (shown by white dots).

levels of p63 or p73 proteins (Supplemental Figures S1B and S1C at http://www.cancercell.org/cgi/content/full/6/1/85/DC1), despite our ability to detect transcripts encoding these proteins using reverse transcription polymerase chain reaction (RT-PCR) techniques (data not shown). It was possible, though, that p63 and p73 proteins were induced and active at levels below our detection sensitivity, necessitating a genetic approach to defining their role in T cell death.

Analysis of cell-autonomous gene function in lymphocytes has been aided by the use of adoptive transfer in Rag2 null mice, as this approach circumvents any systemic defect imparted by the loss of a gene (Washburn et al., 1997). The adoptive transfer was achieved by injecting fetal hematopoietic cells from mice lacking one or more p53 family members into sublethally irradiated *Rag2*$^{-/-}$ mice (Figure 1A). T or B cells observed in *Rag2*$^{-/-}$ mice have the genotype of the donor animal, since the indigenous lymphocyte precursors fail to mature due to the lack of V(D)J recombination machinery, which is required to generate T cell receptor and immunoglobulin genes (Shinkai et al., 1992). Six weeks after the injection of donor cells into the *Rag2*$^{-/-}$ hosts, we examined two lymphoid organs for their reconstitution by donor cells. T cell development in the thymus was assessed by examining the expression of CD4 and CD8 antigens, markers of helper and cytotoxic T cells, respectively (Figure 1B). Significantly, the overall numbers of *p63*$^{-/-}$, *p73*$^{-/-}$, or *p63*$^{-/-}$*; p73*$^{-/-}$ thymocytes in the reconstituted thymi were similar to those reconstituted with wild-type cells, as were the percentages of cells that had differentiated into CD4/CD8 double-positive and CD4 and CD8 single-positive cells (Figures 1B and 1C). The analyses of T and B cells in splenocytes using αβTCR and B220, mature T and B cell markers, respectively, as well as the absolute numbers of αβTCR$^+$ T cells in the spleen of reconstituted *Rag2*$^{-/-}$ mice, revealed no obvious differences between wild-type donor cells and those derived from mice lacking one or more members of the p53 gene family (Figures 1D and 1E). Thus, the p63 and p73 genotypes of the hematopoietic donor cells did not affect T cell development, the number of thymocytes generated, or their migration to the spleen.

With the successful reconstitution of *Rag2*$^{-/-}$ mice by lymphocytes of defined genotypes, the requirement of p53, p63, and p73 in various models of T cell death could be determined. Thymocytes having *p53*$^{-/-}$, *p63*$^{-/-}$, *p73*$^{-/-}$, and *p63*$^{-/-}$*; p73*$^{-/-}$ genotypes were isolated from the reconstituted *Rag2*$^{-/-}$ mice and subjected to various stresses that are known to induce cell death in wild-type cells. Ionizing radiation was used to assess the involvement of p63 and p73 in p53-dependent cell death, and dexamethasone was used to test their role in a p53-independent form of cell death. Five hours after exposure to ionizing radiation, the *p53* null thymocytes appeared to be only marginally more resistant to cell death in comparison to wild-type, *p63* null, *p73* null, or the double *p63*/*p73* null cells (Figure 2, left panel). By 20 hr, however, the *p53* null cells proved to be remarkably resistant to cell death, whereas the other thymocytes having single or combined losses of the *p63* and *p73* genes were no more resistant than the wild-type cells (Figure 2, left panel). When thymocytes from the same reconstituted mice were tested for their response to dexamethasone, all proved equally sensitive over the time examined (Figure 2, right panel). These observations in thymocytes argue not only against required roles for p63 and p73 in p53-dependent apoptosis but also against

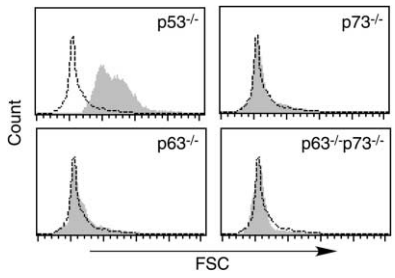

Figure 3. Forward scatter of reconstituted thymocytes

Cells from reconstituted thymi were analyzed by flow cytometry 6 weeks after transplantation. Shown is one representative data set out of two experiments with similar results. The dotted lines represent wild-type control thymocytes. Note that lymphoblastic appearance of thymocytes of the p53 null thymocytes is absent in cells lacking p63 and p73.

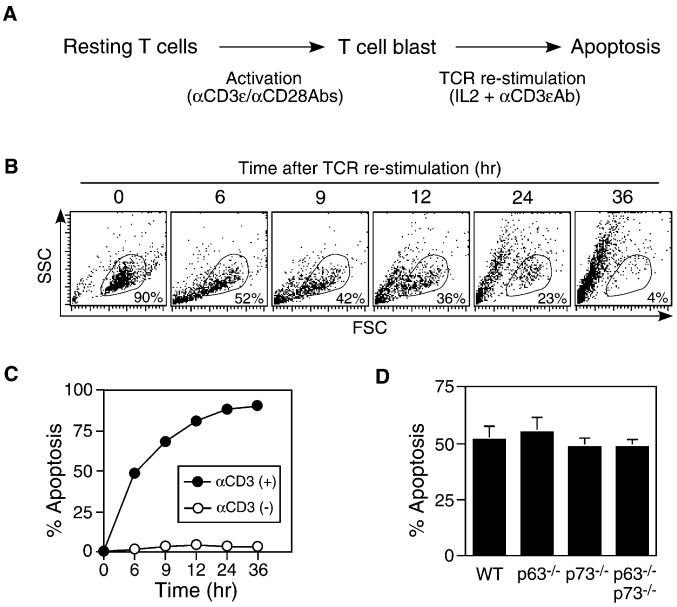

Figure 4. Activation-induced cell death in the purified $CD4^+$ T cells

A: Scheme of the activation-induced cell death (AICD) protocol, which consists of initial activation and secondary TCR stimulation.

B: Forward scatter (FSC) and side scatter (SSC) analysis of wild-type $CD4^+$ T cells after the secondary stimulation for the indicated times. Viable cells were gated, and the percentages of those cells are shown on right bottom corner.

C: Annexin V staining. The same set of the cells in **B** was stained with FITC-conjugated Annexin V, and the percentages of Annexin V-positive cells were plotted over time (closed circles). The blasting T cells that were cultured in the absence of anti-CD3ε mAb served as negative control (open circles).

D: AICD in the purified $CD4^+$ T cells that were obtained from reconstituted *RAG2*$^{-/-}$ mice. The cells were assayed for apoptosis at 6 hr after secondary stimulation.

mutually compensating roles of p63 or p73 in either p53-dependent or p53-independent cell death.

Six weeks following reconstitution of the immune systems of the *Rag2*$^{-/-}$ mice, none of the *p63*$^{-/-}$, *p73*$^{-/-}$, and *p63/p73* null thymocytes showed a "blasting" appearance marked by enhanced cell size, which is common in *p53*$^{-/-}$ thymocytes and T cell lymphomas derived from *p53*$^{-/-}$ cells (Figure 3). This finding suggests that p53 functions independently of p63 and p73 to suppress the blasting phenotype, at least in a short-term experiment employed in this study.

In contrast to thymocyte cell death following DNA damage, cell death in mature T cells following strong T cell receptor stimulation proceeds in the absence of p53 activity (Boehme and Lenardo, 1996). However, recent data, primarily from the established Jurkat T cell line programmed with mutant versions of E2F-1 and p73, as well as unpurified spleen cells from adult *p73*$^{-/-}$ mice, have suggested a role for p73 in activation-induced cell death (AICD) (Lissy et al., 2000). To clarify and address the roles of p73 as well as p63 during the process of AICD in T cells in genetically defined cells, we purified peripheral $CD4^+$ T cells derived from *Rag2*$^{-/-}$ mice reconstituted with hematopoietic cells from *p63*$^{-/-}$, *p73*$^{-/-}$, and *p63/p73* null mice and analyzed those cells in a wild-type environment. Initially, AICD was induced in the purified wild-type $CD4^+$ T cells using a standard regimen of primary and secondary activation and monitored over time using forward- and side-scatter flow cytometer parameters (Figures 4A and 4B). This analysis indicated that 6 hr of TCR stimulation was sufficient to observe cellular changes consistent with apoptosis. These primary indicators were then confirmed by fluorescence-activated cell sorting (FACS) analysis of Annexin V staining and propidium iodide exclusion, which supported the observation that 6 hr of stimulation resulted in robust AICD (Figure 4C). When the same activation protocol was applied to purified $CD4^+$ T cells of the *p63*$^{-/-}$, *p73*$^{-/-}$, or *p63*$^{-/-}$*; p73*$^{-/-}$ genotypes for 6 hr, the overall percentages of AICD were very similar (Figure 4D), indicating that none of the p53 family members was required for the underlying processes of receptor-mediated T cell apoptosis.

Discussion

The Rag2 lymphocyte reconstitution system has revealed important features of cell-autonomous interactions among p53 family members that might have been obscured in syngeneic mice due to systemic defects. Overall, our data offer genetic evidence that p63 and p73 are dispensable for both radiation- and glucocorticoid-induced cell death in immature T cells and for AICD in mature T cells. These conclusions place important limitations on the generality of data implicating p73 and p63 in proapoptotic responses to cellular stress and for the role of these genes in p53-mediated cell death, at least as they apply to primary immature thymocytes and mature peripheral T cells. As much of the evidence in favor of a proapoptotic role for p73 and p63 has been derived from overexpression studies in transformed cell lines, the present data argue that further physiological experiments may be needed to validate the positive influence of the p53 homologs on cell death. Our finding that the combined loss of p63 and p73 fails to abrogate p53-dependent cell death (Figure 2, left panel) and blasting thymocytes due to a lack of p53 function (Figure 3) appears to contradict the conclusions from experiments performed with genetically defined MEFs (Flores et al., 2002). However, the MEFs that were employed in those studies were altered by stable expression of the E1A oncogene, a commonly used enhancer of apoptotic responses in MEFs (Lowe et al., 1993b). The mechanism by which E1A promotes cell death in response to stimuli remains incompletely understood but is thought to operate through mul-

tiple pathways. For instance, the roles of Bax as a downstream target of p53 and in chemosensitivity were most clearly revealed in the MEF model employing an E1A background (McCurrach et al., 1997). Regardless, the apparent requirement of p73 and p63 in p53-dependent apoptosis in these E1A-expressing cells might be attributable to the influence of E1A rather than a reflection of the normal function of p63 and p73. More recently, p73 has been implicated in the apoptotic response to chemotherapy in a study that employed H-ras-expressing, p73 null MEFs (Irwin et al., 2003). Altogether, these data raise the intriguing possibility that oncogenic signals somehow render p53 pathways dependent on p63 and p73 function. As such, it will be important to understand how oncogenic factors might influence the functional interactions between p53, p63, and p73 and the extent of such phenomena. An alternative possibility that is raised by our work is that p63 and p73 are dispensable for p53-dependent cell death in physiologically relevant situations and that neither p63 nor p73 serves to functionally cover for p53 by triggering stress-related cell death. This latter issue is of considerable clinical significance, as much of the work on p73 and p63 has argued for their p53-like activities and therefore tumor suppressor functions. Additional experiments have produced data interpreted to support the concept that p73 and possibly p63 play important roles in the chemotherapeutic response (Irwin et al., 2003; Bergamaschi et al., 2003). Moreover, the analysis of radiation-induced neural apoptosis of mouse embryos also implicates p63 and p73 in p53-mediated cell death (Flores et al., 2002). These experiments support the requirement of p63 and p73 in p53-dependent cell death in the absence of oncogenes and therefore could be reconciled with the data that are presented here on T cells by invoking cell type specificity for p53 family interactions. It could also be argued that conclusions from the mouse knockout models are obscured by compensatory mechanisms that bring to bear new pathways in support of p53-dependent cell death.

It is also difficult to reconcile our data that p73 and p63 are dispensable for AICD in T cells and those implicating p73 in this process. AICD is the consequence of excessive T cell receptor activation and has been shown to be mediated by Fas ligand (Alderson et al., 1995). Systemic differences between our experiments and those of previous studies that linked p73 to AICD do exist; most notably, our analysis used purified T cells, whereas the other studies employed total splenocytes in a p73 null background. Our data on purified, p73 null $CD4^+$ T cells indicated that they were no more resistant than wild-type controls up to 24 hr after secondary TCR stimulation (Figure 4D and Supplemental Figure S2 at http://www.cancercell.org/cgi/content/full/6/1/85/DC1). Given the inflammatory defects observed in the p73 null mice (Yang et al., 2000), it is possible that cytokine abnormalities have confounded the previous analyses of AICD in p73 null mice (Lissy et al., 2000). Regardless, any requirement for p73 or p63 in AICD was not apparent from the present experiments on purified T cells of defined genotypes.

Finally, the dispensability of p63 and p73 for T cell death does not preclude interactions among p53 family members, which appear to be supported by evolutionary, genetic, and biochemical considerations (Yang et al., 2002). Phylogenetic analyses show the simultaneous appearance of p53 in chordates along with the putative dominant-negative versions of p63 and p73 (ΔN-p63 and ΔN-p73), suggesting the possibility that the dominant-negative species evolved to interfere with the transcriptional activities of p53. Given the high ratio of expression between ΔN-p63 and p53 in keratinocytes (unpublished data), the prospect exists that p53 activity is suppressed in the basal cells of many epithelial tissues. Indeed, mechanisms appear to exist in keratinocytes to reverse this ratio that involve p63 degradation and p53 accumulation in response to DNA damage (Liefer et al., 2000), which might contribute to epithelial cell tumors. Similar interactions between p53 and p73 have been deduced from the loss of sympathetic neurons in the p73 null mice, a phenotype attributed to the enhanced p53 activity in the absence of ΔN-p73 (Pozniak et al., 2000). Thus, it seems that the salient features of interactions between p53, p63, and p73 might be interfering rather than cooperative. Whether these three family genes participate in largely independent programs of tumor suppression, stem cell maintenance, and homeostatic control or interact in all three functions will have to await more detailed genetics and biochemistry.

Experimental procedures

Mice and lymphocyte reconstitution

Knockout mouse lines that were used in this study were backcrossed over ten times on a Balb/c background. The generation of $p53^{-/-}$ (Jacks et al., 1994), $p63^{-/-}$ (Yang et al., 1999), $p73^{-/-}$ (Yang et al., 2000), and $RAG2^{-/-}$ (Shinkai et al., 1992) mice has been described previously. For reconstitution experiments, null embryos were derived from matings among $p53^{+/-} \times p53^{+/-}$ and $p63^{+/-}; p73^{+/-} \times p63^{+/-}; p73^{+/-}$ heterozygotes. For timed matings to generate staged embryos, the day of the vaginal plug was designated E0.5. At E15.5, fetal livers were removed, and the cells were dissociated and placed in culture (Roswell Park Memorial Institute [RPMI] medium supplemented with 10% fetal bovine serum), while genotyping was performed using tail DNA. The wild-type p53 allele was amplified using primers W5′ and W3′, whereas the knockout allele was amplified using primers M5′ and W3′ (Jacks et al., 1994). The wild-type p63 and p73 alleles were amplified using primers p63F and p63R and p73F and p73R, respectively, whereas knockout alleles were amplified using primers Neo and either p63R or p73R. The oligonucleotide sequences that were used were as follows. p63F, 5′-TTC TCA GAT GGT ACC GCT CC-3′; p63R, 5′-GGT GCT TTG AGG CCC GGA TC-3′; p73F, 5′-GGG CCA TGC CTG TCT ACA AGA A-3′; p73R, 5′-CCT TCT ACA CGG ATG AGG TG-3′; and Neo, 5′-GCT AAA GCG CAT GCT CCA GAC-3′. During the process of genotyping, 4- to 5-week-old $RAG2^{-/-}$ recipient mice received a single 250 cGy dose of total body γ irradiation (^{137}Cesium source). Six hours later, the viability of the fetal liver cells was examined by trypan blue dye exclusion (generally over 95% viable) and 1×10^7 viable fetal liver cells with the expected genotypes were injected i.v. into the recipient mice.

Immunoblotting

Thymocytes from 3-week-old wild-type mice were treated with 1 μM dexamethasone (Sigma) or 5 Gy γ irradiation. Immunoblotting was carried out as described (Lowe et al., 1993a) with a pool of anti-p53 monoclonal antibodies (mAbs) (PAb421 and PAb240; Oncogene Science), an anti-p63 mAb (clone 4A4), and an anti-p73 mAb (clone 11C12) raised against the carboxyl terminus of p73 (corresponding to aa 401-636 of the human transactivating isoform).

Flow cytometry

The following antibodies were used: fluorescein isothiocyanate (FITC)-anti-CD4 (clone GK1.5), phycoerythrin (PE)-anti-CD8 (clone 53-6.7), PE-anti-αβTCR (clone H57-597), and cychrome (Cyc)-anti-B220/CD45R (clone RA3-6B2) (all from BD Pharmingen). The labeled cells were analyzed on a FACSCalibur machine using the CellQuest software (BD Pharmingen).

Apoptosis assay

For thymocyte cell death, the reconstituted thymocytes were treated as described (Lowe et al., 1993a). The cell viability was determined by ApoAlert

Annexin V-FITC kit (BD Pharmingen) according to the manufacturer's protocol. For AICD (Boehme et al., 1995; Jones et al., 1990; Rocha and von Boehmer, 1991; Russell, 1995), $CD4^+$ single-positive T cells were purified from spleens using CD4 microbeads (Miltenyi Biotec) and were activated by placing on anti-CD3ε mAb (clone 145-2C11; BD Pharmingen)-coated plates in the presence of 4 μg/ml anti-CD28 mAb (clone 37.51; Southern Biotec) for 3 days. The activated T cells were then replated in the presence of interleukin-2 (IL-2) (50 units/10^6 cells) for 48 hr followed by centrifugation over Ficoll-Paque (Amersham) to remove dead cells. T cell blasts were stimulated by plate-coated anti-CD3ε mAb in the presence of IL-2 (100 units/10^6 cells) for the indicated times, and the percentage of apoptotic cells was determined as described above.

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