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The Selective Increase in Caspase-3 Expression in Effector but Not Memory T Cells Allows Susceptibility to Apoptosis

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Caspases play a central role in T lymphocyte activation and death. We have demonstrated previously that caspase-3, an effector molecule for activation-induced cell death (AICD), is processed following T cell activation in the absence of apoptosis. We report in this study that caspase-3 mRNA levels were selectively increased in peripheral T cells, following Ag receptor-mediated activation. The up-regulation of caspase-3 mRNA was confined to cells in the early phases of the cell cycle (G0/G1) and was independent of IL-2 signaling. This increase led to the renewal of procaspase-3 as evidenced by a 6-fold up-regulation of the zymogen in nonapoptotic stimulated T cells. The increase of mRNA levels and of both the zymogen and the cleaved forms of caspase-3 was observed in in vivo stimulated Ag-specific effector, but not memory T cells, correlating with the enhanced susceptibility of effector T cells to AICD. Furthermore, we confirm that caspase-3 levels directly influence the sensitivity of activated T cells to apoptosis, as shown using T lymphocytes isolated from caspase-3 heterozygous and knockout mice. These findings indicate that the selective up-regulation of caspase-3 transcription is required to maintain the cytoplasmic levels of this protease, which control AICD and T cell homeostasis. The Journal of Immunology, 2004, 173: 5425–5433.

Mature T lymphocytes circulate through the blood and peripheral lymphoid organs in a resting state until they encounter APCs bearing the cognate peptide presented by MHC molecules (1). Engagement of the TCR/CD3 complex following a response to a pathogen results in T cell activation, an event characterized by the highly regulated expression of a large number of activation-specific genes, involved in cell cycle progression, proliferation, and apoptosis (2, 3). The majority of activated effector T cells are eliminated by activation-induced cell death (AICD), following clearance of the pathogen, a process that results from the interaction of death receptors with their specific ligands and the recruitment of the Fas-associated death domain adapter molecule (4). The latter interacts with the intracellular death domain of Fas via its own death domain and recruits procaspase-8 through death-effector domains found in both molecules (5–8). Aggregation of all three molecules (Fas, Fas-associated death domain, and procaspase-8) leads to the formation of the death-inducing signaling complex and subsequent cleavage of procaspase-8 to its active form (caspase-8) (9, 10). The active form of caspase-8 then initiates the proteolytic cleavage of caspase-3, which in turn cleaves caspase-6 and -7 and a multitude of cellular substrates leading to apoptosis (11).

Caspase-3 is also the point of convergence of the intrinsic apoptotic pathway initiated through the mitochondria, which further illustrates its central role in apoptosis. Following mitochondrial outer membrane permeabilization and loss of the mitochondrial transmembrane potential, cytochrome c is released from the intermembrane space and binds Apaf-1 (12). The interaction between Apaf-1, dATP, cytochrome c, and procaspase-9 in the cytoplasm leads to the formation of a multiprotein complex referred to as the apoptosome (13). That triggers the processing and activation of caspase-9, which then efficiently cleaves and activates caspase-3. Similar to apoptosis initiated through death receptors, the active form of caspase-3 then cleaves cellular substrates involved in cellular integrity and metabolism.

Surprisingly, cleavage of caspase-3 occurs in activated T lymphocytes in the absence of apoptosis and is required for T cells to enter the cell cycle (14–17). Indeed, inhibition of caspase-3 activity results in defective T cell proliferation following stimulation of naive T cells through the TCR, although the initial steps of T cell activation remain intact (16). Cleavage of caspase-3 thus appears

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About this article: AICD, activation-induced cell death; Ag, antigen; apopt, apoptosis; MHC, major histocompatibility complex; LCMV, lymphocytic choriomeningitis virus; LCMV-cl., LCMV-clone; MFI, mean fluorescence intensity; PL, propidium iodide; XIAP, X-linked mamalian IAP protein.
to be an integral component of the T cell activation process. The strength of TCR signaling also regulates caspase activation during T cell proliferation (18, 19). It was demonstrated that stimulation of naive CD4 T cells with high affinity ligands leads to caspase activation, whereas low affinity ligands fail to induce any caspase activation (19).

Members of the Bcl-2 family are up-regulated in memory T cells, while their levels are down-regulated in effector T cells, implying a role for these molecules in modulating the susceptibility of these cells to undergo AICD (20–23). Variability in the levels of expression of caspases could furthermore contribute to regulate the susceptibility of distinct T cell subsets to apoptosis. In support of this, experimental evidence is accumulating, showing that both mRNA and protein levels of caspase-3 have a profound effect on the onset of apoptosis in different cell types. For example, the down-regulation of caspase-1 and -3 basal expression observed in STAT-1 null cells leads to resistance to TNF-α-induced apoptosis (24). The lack of induction of caspase-2 and -3 gene expression in tumor cell lines correlates with resistance to etoposide-induced apoptosis (25). In line with these findings, it also was found that a majority of tumor cells isolated from breast cancer patients lack caspase-3 mRNA and protein expression, suggesting that absence of caspase-3 could play a role in tumor development (26). Finally, peripheral T lymphocytes isolated from mice, in which the caspase-3 gene has been inactivated by homologous recombination, are partially resistant to apoptosis following treatments with either anti-CD3 or anti-Fas Abs, suggesting that an intact pro-caspase-3 pool is critical for T lymphocyte homeostasis (27). In this study, we show that mRNA and protein levels of caspase-3 are significantly up-regulated following TCR stimulation, which allows the maintenance of adequate levels of the procaspase-3 pool required for the onset of AICD in effector T cells, but not in memory T cells.

Materials and Methods

Mice and viral infections

BALB/c (Charles River Laboratories, Wilmington, MA), C57BL/6, and B6.PL-Thy-1a/Cy mice (The Jackson Laboratory, Bar Harbor, ME); the 2C αβ TCR transgenic mice (H-2Kb) (28, 29); and the wild-type, heterozygous, and homozygous caspase-3 knockout mice (27) were used in our experiments. Lymphomatis choriomeningitis virus (LCMV) TCR-specific P14 transgenic mice (H-2b; Thy-1.2*) (22) were crossed onto B6.PL-Thy-1a/Cy (H-2b, Thy-1.1*) to generate Thy-1.1* P14 transgenic mice. To generate autologously infected and immune animals, 5 × 10^4 P14 splenocytes (Thy-1.1*) were adoptively transferred into normal (nonirradiated) C57BL/6 mice by i.v. injection. On the next day, chimeric mice were infected with 2 × 10^3 PFU of LCMV-Armstrong i.p. For LCMV reinfection, LCMV immune animals containing memory (CD44^high and CD62L^low) Thy-1.1* P14 CD8 T cells were infected with 2 × 10^5 PFU of LCMV-clone (LCMV-e1). 13 i.v., and the P14 CD8 T cells were examined 4 days later. All animal experiments were done with approved Institutional and Animal Care and Use Committee protocols.

Cell preparation, activation, and apoptosis assay

Total lymphocytes were isolated from lymph nodes and thymi of mice, cultured in six-well plates at 5 × 10^5 cells/well in the presence of 200 U/ml IL-2 (National Institutes of Health AIDS Research and Reference Reagent Program), and preincubated with 300 μg/ml 1-mimosine (Calbiochem, San Diego, CA), when indicated. Cells were stimulated with 10 μg/ml immobilized anti-CD3 Ab (145-2C11; from M. Julius, University of Toronto, Ontario, Canada). Lymph node T cells were cultured in 0.5 μM CFSE (Molecular Probes, Eugene, OR) for 10 min at 37°C in PBS. Labeling was quenched with FCS, and cells were washed in DMEM 10% FCS and seeded at 10^5 cells in 24-well cell culture plates in the presence or absence of coated anti-CD3. Lymph node T cells from 2C mice were stimulated with 1 μM P14 splenocytes in the presence of 200 U/ml IL-2 and APCs. T cell activation was assessed by monitoring the levels of CD69 and CD25 cell surface expression (BD Pharmingen, San Diego, CA) by flow cytometry. The number of live cells was determined by annexin V (AnV; BioSource International, Camarillo, CA) and propidium iodide (PI) staining, and analyzed using a BD Biosciences (San Jose, CA) FACSCalibur. The PC61 Ab (30) was used at a concentration of 50 μg/ml to block signaling through the IL-2R. Following 48 h of stimulation, T cells isolated from caspase-3 wild-type, heterozygous, or knockout mice were incubated in the presence of 1 μM etoposide (Calbiochem) or 50 μg/ml PC61 Ab to induce apoptosis over the course of 10 h. AnV– cells were negatively sorted on the AutoMACS using the Dead Cell Removal Kit (Miltenyi Biotec, Auburn, CA), and T cells were then isolated by positive selection using MACS CD90 (Thy-1.2) MicroBeads (Miltenyi Biotec).

Real-time RT-PCR assay

Reverse-transcriptase reactions were performed on 400 ng of total RNA, using ThermoScript One-Step RT-PCR with Platinum Taq (Invitrogen Life Technologies, Carlsbad, CA). A construct encompassing nucleotide sequences from caspase-3 or -8 and β-actin was developed to generate a standard curve for real-time PCR using LightCycler technology (Roche Diagnostic Systems, Somerville, NJ). Following the first round of amplification, PCR products were diluted 10-fold before on-line, nested real-time PCR using fluorescent probes. All samples were normalized to the relative levels of β-actin, and results are expressed as the fold increase in the relative levels of caspase-3 or -8 in stimulated cells relative to non-stimulated cells.

Cell cycle analysis and FACS cell sorting

Cells were fixed and permeabilized in 70% ethanol, stained in 500 μl of PBS containing 300 μg/ml PI and 100 μg/ml RNase for 30 min at 37°C, and then analyzed by flow cytometry. When sorting for the different phases of the cell cycle (G0/G1, S/G2/M), cells were stained with the Hoechst 33342 dye (Sigma-Aldrich, St. Louis, MO) at a final concentration of 10 μg/ml for 2 h at 37°C, washed with cold PBS, and sorted on a FACSVantage cell sorter (BD Biosciences). Naive P14 mice or chimeric P14 mice were infected with LCMV, and T cell subsets were isolated by staining splenocytes with anti-CD8α and anti-Thy1.1 Abs at 8 (effector) or 14 days (memory) postinfection, followed by sorting using a FACSVantage cell sorter.

cRNA synthesis and DNA microarray hybridization

Isolated total RNA from P14 naive or day 8 and 40 P14 chimeric sorted cells was resuspended in 5 μl of diethyl pyrocarbonate water per 10^6 cells. cDNA was synthesized from total RNA of 10^6 cells using SuperScript Choice cDNA synthesis kit (Invitrogen Life Technologies) and an oligo(dT) primer containing a T7 promoter. Four hours in vitro transcription reactions using T7 RNA polymerase were used to amplify poly(A)+ RNA (referred to as cRNA) from the cDNA using the MEGAscript T7 kit (Ambion, Austin, TX). The cRNA was extracted, and a second round of double-stranded cdNA was synthesized from the cRNA using random and T7-oligo(dT)20 primers. A second round of cRNA synthesis was performed using biotinylated ribonucleotides, and 20 μg of biotinylated cRNA was fragmented and hybridized to the Affymetrix U74A chips (Affymetrix, Santa Clara, CA), according to manufacturer’s protocols, as previously described (31). Expression pattern clusters were defined using hierarchical tree and K-means clustering algorithms in J-Express v. 1.1 (32).

Detection of intracellular caspase-3 and flow cytometry

Mice adoptively transferred with Thy-1.1* P14 CD8 T cells were infected with LCMV, and on 4, 6, 8, 14, and 65 days postinfection (dpi) or 4 days following LCMV reinfection, the splenocytes were harvested and cells were stained with anti-Thy-1.1 Abs in staining buffer (PBS, 1% FCS) on ice for 30 min. The cells were washed, fixed, and permeabilized using the Cytofix/Cytoperm intracellular staining kit (BD Biosciences), as previously described (33). The cells were incubated with anti-caspase-3 and anti-cleaved caspase-3 Abs (Cell Signaling Technology, Beverly, MA) at 1/100 dilution in permwash for 30 min on ice. The cells were washed several times and then stained with FITC-conjugated anti-rabbit fragment Ab (Fab) (Caltag Laboratories, Burlingame, CA) for 30 min on ice. The cells were further washed several times and analyzed using a BD Biosciences FACSCalibur to monitor levels of caspase-3 on a per cell basis. Dead cells were gated out on the basis of forward/side scatter. For each sample, 10^5 events were collected.

Western blotting

Cells were washed twice in cold PBS and lysed in TBS containing 1 mM EDTA, 1 mM DTT, 0.2% Triton, 0.1% SDS, and the complete protease inhibitors mixture (Roche). A total of 30 μg of proteins was subjected to SDS-PAGE, and then transferred to polyvinylidene difluoride membranes

Authors’ contributions

W. R. performed experiments involving LCMV infection. S. P. and A. N. performed experiments involving caspase-3 and caspase-8. M. P. performed experiments involving cell cycle analysis. M. S. performed experiments involving RNA synthesis and DNA microarray hybridization. S. M. and D. R. performed experiments involving detection of intracellular caspase-3 and flow cytometry. J. W. and A. K. performed experiments involving Western blotting. J. T. performed experiments involving materials and methods. L. L. and H. T. performed experiments involving cell preparation, activation, and apoptosis assay. L. L. and S. P. performed experiments involving cell cycle analysis and FACS cell sorting. J. T. and A. N. performed experiments involving caspase-3 and -8. J. T. and A. K. performed experiments involving RNA synthesis and DNA microarray hybridization. All authors have contributed to the writing of the manuscript and edited the final version.

Figure legend

Figure 1. Caspase-3 expression is up-regulated in caspase-3 conditional knockout mice. Caspase-3 mRNA and protein expression was determined by real-time RT-PCR and Flow cytometry, respectively, in spleens and thymi from wild-type and caspase-3 heterozygous or knockout mice. Caspase-3 protein expression was determined by immunohistochemistry in thymi from wild-type and caspase-3 conditional knockout mice. (A) Real-time RT-PCR analysis of caspase-3 mRNA expression in spleens and thymi from wild-type and caspase-3 heterozygous or knockout mice. (B) Flow cytometry analysis of caspase-3 expression in spleens and thymi from wild-type and caspase-3 heterozygous or knockout mice. (C) Immunohistochemistry analysis of caspase-3 expression in thymi from wild-type and caspase-3 conditional knockout mice. (D) Western blot analysis of caspase-3 expression in spleens and thymi from wild-type and caspase-3 heterozygous or knockout mice.
Selective increase in caspase-3 mRNA levels is an early event (G_0/G_1) during T cell activation

We investigated, using a real-time RT-PCR assay, the impact of T cell activation on the up-regulation of caspase-3 mRNA levels to test the hypothesis that transcriptional up-regulation of the caspase-3 gene is required to replenish the pool of procaspase-3, which is cleaved upon TCR triggering (14–17). Thymocytes and lymph node T cells from BALB/c mice were activated by cross-linking the TCR with anti-CD3 in the presence of APCs for 48 h. Live (>90% AnV\textsuperscript{-}) T cells were sorted for quantification of mRNA levels by real-time RT-PCR. In three independent experiments performed on live sorted T cells, caspase-3 mRNA levels increased as early as 6 h post-TCR stimulation, peaking (13-fold) at 48 h (Fig. 1A). The increase occurred in two steps with an initial 6-fold increase reached between 24 and 36 h at a time when most T cells are synchronized in the earliest phase of the cell cycle (89% G_0/G_1). A subsequent 2.2-fold increase occurred at 48 h when a large number of cells had entered the cell cycle (43% S/G_2/M). More than 90% of the cells showed the presence of the activation markers CD69 and CD25 at their cell surface, as determined by flow cytometry, demonstrating proper T cell activation (data not shown). This increase was selective for caspase-3 because caspase-8 mRNA levels remained unchanged. A similar, albeit lower (3-fold) up-regulation of caspase-3 mRNA levels was observed in sorted live (>90% AnV\textsuperscript{-}) thymocytes (Fig. 1A) and in murine T cell hybridomas.\textsuperscript{6}

To further confirm that the increase in caspase-3 mRNA levels occurs before entry into the S phase of the cell cycle, T cell proliferation was blocked using the late G_0 cell cycle inhibitor, l-mimosine. Expression of early markers of T cell activation remained intact after 48 h of stimulation in the presence of l-mimosine (>90% CD69\textsuperscript{+}/CD25\textsuperscript{+} cells) (Fig. 1B), although we observed a large decrease in the number of cells entering the S phase of the cell cycle (9% S/G_2/M) as compared with cells stimulated in the absence of the inhibitor (62% S/G_2/M) (Fig. 1C). Caspase-3 mRNA levels were up-regulated by 11-fold, at 48 h following anti-CD3 stimulation, a response that was slightly reduced to 7-fold in the presence of l-mimosine (n = 3) (Fig. 1C). Cells were then activated by TCR cross-linking in the presence or absence of l-mimosine for 48 h and sorted according to the G_0/G_1 and S/G_2/M phases of the cell cycle. Sorted stimulated cells showed a 15-fold (G_0/G_1) and 4-fold (S/G_2/M) induction in caspase-3 mRNA levels, relative to nonstimulated samples (Fig. 1D), while activation of T cells in the presence of l-mimosine confirmed that the bulk of the increase occurred in the G_0/G_1 phases (10-fold increase). These results demonstrate that the majority of the selective increase in caspase-3 mRNA levels is mediated early during T cell activation in the G_0/G_1 phases of the cell cycle.

IL-2-independent increase in caspase-3 mRNA levels in activated T cells

The IL-2 cytokine plays an essential role in promoting the early phases of T cell proliferation and also in enhancing cell death at the termination of an immune response (34). To determine whether the observed increase in caspase-3 mRNA levels was dependent on IL-2 signaling, lymph node T cells were preincubated with the PC61 Ab directed against the IL-2R \(\alpha\)-chain (IL-2R\(\alpha\)), which blocks signaling through this receptor (30) and therefore proliferation. Initially, we confirmed that at 48 h after TCR engagement T cells had undergone one to three divisions \((n = 3)\). However, the presence of the IL-2R\(\alpha\)-neutralizing Ab (PC61) completely blocked T cell proliferation, as we could not observe any T cells with low levels of CFSE (Fig. 1E). The levels of caspase-3 mRNA were then determined by real-time RT-PCR \((n = 2)\). Following 48 h of stimulation with anti-CD3, we observed a 10-fold increase in caspase-3 mRNA levels in cells treated with the PC61 Ab, compared with 15-fold in the absence of the IL-2R\(\alpha\) Ab (Fig. 1F). These results show that activation of T cells by anti-CD3 in the absence of IL-2 signaling still results in a significant increase in caspase-3 mRNA levels, formally demonstrating that the up-regulation of caspase-3 mRNA is independent of IL-2 signaling. Overall, these results demonstrate that TCR engagement results in a significant (at least 10-fold) increase in caspase-3 mRNA levels in peripheral T lymphocytes, within 24 h following TCR triggering and independently of IL-2 signaling.

Ex vivo and in vivo Ag-specific induction of caspase-3 mRNA levels is selective to effector T cells

Lymph node T cells from 2C mice expressing a transgenic TCR with specificity for the SYRGL peptide restricted by the class I H-2K\textsuperscript{b} molecule were activated with the SYRGL peptide to determine whether caspase-3 mRNA expression was up-regulated ex vivo following the specific interaction of a TCR with its cognate peptide/MHC complex. The levels of caspase-3 mRNA were monitored for 5 days following an initial stimulation with the SYRGL peptide, using the real-time RT-PCR assay. Transient up-regulation of caspase-3 mRNA levels was observed, with a peak occurring within 48 h after Ag-specific stimulation (15-fold increase), followed by a sharp drop and a return to steady state levels (3-fold relative to day 0) at 3 days poststimulation (n = 2, Fig. 2A). In contrast, caspase-8 mRNA levels remained unchanged, further confirming the selectivity of the increase in caspase-3 mRNA levels.

Experiments were also conducted in vivo to determine whether modulation of caspase-3 gene expression was selective to effector T cells because this subset is destined to undergo AICD. P14 mice expressing a transgenic TCR with specificity for the LCMV glycoprotein restricted by the class I H-2K\textsuperscript{b} molecule were thus used to compare caspase-3 mRNA levels among naive, effector, and memory CD8 T cells (33, 35, 36). Initially, Thy-1.1\textsuperscript{+} P14 splenocytes were adoptively transferred into C57BL/6 (Thy-1.2\textsuperscript{-}) mice. In vivo following the specific interaction of a TCR with its cognate peptide/MHC complex. The levels of caspase-3 mRNA were monitored for 5 days following an initial stimulation with the SYRGL peptide, using the real-time RT-PCR assay. Transient up-regulation of caspase-3 mRNA levels was observed, with a peak occurring within 48 h after Ag-specific stimulation (15-fold increase), followed by a sharp drop and a return to steady state levels (3-fold relative to day 0) at 3 days poststimulation (n = 2, Fig. 2A). In contrast, caspase-8 mRNA levels remained unchanged, further confirming the selectivity of the increase in caspase-3 mRNA levels.
memory T cells, relative to naive T cells (Fig. 2B). Interestingly, caspase-7 mRNA levels were also up-regulated in effector T cells (2.6-fold) relative to naive T cells. The increased levels of both caspase-3 and -7, compared with caspase-2, -6, -8, and -9, in effector T cells might be due to a general consequence of T cell activation on executioner caspases, suggesting that the expression of caspase-3 and -7, two proteases with redundant functions during apoptosis, may be controlled in a similar manner following TCR triggering. These results confirmed the transcriptional up-regulation of caspase-3 in effector T cells.

Procaspase-3 and activated caspase-3 levels are increased in effector T cells

We next sought to determine whether the increase in caspase-3 mRNA levels, observed in LCMV-specific effector CD8 T cells directly ex vivo, translates to an increase in protein levels. Mice and -8 mRNA levels from live (>90% AnV−) peripheral T cells and thymocytes was determined by real-time RT-PCR (n = 3). Proliferation was assessed by cell cycle analysis using PI and flow cytometry. The percentage of cells in S/G2/M phase at 24 and 36 h is representative of three independent experiments. B, T cells were stimulated for 48 h, in the presence or absence of L-mimosine (L-MIM), and activation was determined by surface expression of CD69 and CD25. C, The fold induction in caspase-3 mRNA levels of live (AnV−) activated T cells in the presence or absence of L-MIM was determined by real-time RT-PCR (n = 3). The percentage of proliferating cells is shown as S/G2/M, and is representative of three independent experiments. D, T cells activated for 48 h in the presence or absence of L-MIM were sorted for the G0/G1 and S/G2/M phases of the cell cycle, and the fold induction in caspase-3 mRNA levels was determined by real-time RT-PCR. E, Lymph node T cells were stained with CFSE, incubated in the presence or absence of the PC61 Ab, and stimulated for 48 h with anti-CD3. The degree of cell proliferation was determined by flow cytometry analysis (n = 3). F, The fold induction of caspase-3 mRNA levels from peripheral T cells stimulated in E was determined by real-time RT-PCR (n = 2).
were infected with LCMV, and after 4, 6, 8, and 65 dpi, splenocytes were isolated and stained with a caspase-3 Ab that recognizes both the pro-caspase and cleaved forms. Early after infection, on days 4 and 6, the relative expression of caspase-3 in LCMV-specific effector CD8 T cells (CD44^{high} and CD62L^{low}) had increased to nearly twice that found in naive CD8 T cells (68 and 61 mean fluorescence intensity (MFI) vs 25 MFI, respectively) or in LCMV-specific CD8 T cells at 8 or 65 dpi (32 and 30 MFI, respectively) (Fig. 3A). Thus, caspase-3 protein levels increase early during infection when the initial wave of T cell expansion occurs (37), but return to background levels after virus is cleared (at day 8). This low level of expression is maintained in resting memory CD8 T cells (CD44^{high} and CD62L^{high}) found up to 2 mo later (n = 3). As previously shown in CD8 T cells activated in vitro (Fig. 2A), the increased expression of caspase-3 at 4 and 6 dpi directly correlates with recent TCR triggering because virus is present at these times at high titers, but is cleared by day 8 (35).

Next, we examined whether caspase-3 expression increases upon secondary antigenic stimulation of memory CD8 T cells. LCMV immune animals were reinfected with a highly virulent strain of LCMV, LCMV-cl. 13, and 4 days later we observed that the secondary effector CD8 T cells had increased amounts of caspase-3 (52 vs 25 MFI in naive T cells), similar to that found in the primary effector T cells at 4–6 dpi (Fig. 3A). Thus, increased expression of caspase-3 in CD8 T cells normally occurs following T cell activation and effector T cell differentiation, confirming for the first time in an in vivo setting results obtained in a number of in vitro experimental systems (14–17).

Next, we examined whether effector T cells demonstrated increased levels of the cleaved forms of caspase-3 (p20 and p17), because they represent the T cell subset that is the most susceptible to apoptosis in contrast to memory T cells. Therefore, as described above, we stained live LCMV-specific CD8 T cells with an Ab that specifically recognizes the cleaved forms of caspase-3 after 4, 6, 8, 14, and 65 dpi. The expression pattern of cleaved caspase-3 was very similar to that observed for total caspase-3. The majority of effector CD8 T cells found early during infection, after 4 and 6 dpi, contained higher amounts of cleaved caspase-3 (137 and 90 MFI, respectively) than those found at 8, 14, and 65 dpi (65, 48, and 45 MFI, respectively) (Fig. 3). Moreover, the amount of cleaved caspase-3 increased in the secondary effector T cell population (96 vs 53 MFI in naive T cells) (Fig. 3A) when memory CD8 T cells...
were reactivated by reinfecting LCMV immune animals with LCMV-cl. 13. The levels of caspase-3 in naïve and memory CD8 T cells (at day 65 dpi) were near that of background based on staining with an isotype control Ab (28 and 35 MFI, respectively). The MFI of T cells stained with the isotype control was not affected by the activation state of the cells (ranged from 28 to 41 MFI). Interestingly, at about 6 and 8 dpi (primary infection), we observed the presence of a small subset of T cells (~1%) that expressed very high levels of cleaved caspase-3, referred to as cleaved caspase-3high effector T cells, and these cells are most likely actively undergoing apoptosis. (23) This population of cleaved caspase-3high effector cells peaks between 8 and 10 dpi (data not shown) (23) when the majority of effector CD8 T cell contraction occurs. Detection of apoptotic effector CD8 T cells directly ex vivo is difficult and generally underestimates the actual number of dying cells because these cells are rapidly engulfed by macrophages in vivo (38, 39). These results demonstrate that the up-regulation of caspase-3 expression (mRNA and protein) is confined to effector T cells, a subset destined to undergo AICD, suggesting that this selective increase is part of a homeostatic response critical for the regulation of effector and memory T cell numbers.

We further confirmed the increase of both the full-length and cleaved forms of caspase-3 in activated T cells by Western blot. Pro-caspase-3 levels were increased 6-fold in live (AnV−) activated mature T cells 36 h after TCR stimulation (Fig. 4A). Consistent with previous findings (14–17), caspase-3 was cleaved to its p20 and p17 form after 36 h in nonapoptotic mature T cells (85% AnV− cells) (Fig. 4A). In contrast, there was no increase in pro-caspase-3 levels and no processing to its cleaved form in live (88% AnV− cells) activated thymocytes, which had demonstrated a lower induction in caspase-3 mRNA levels (3-fold) when compared with mature T cells (13-fold) (Fig. 4). These results support the hypothesis that the increase in pro-caspase-3 expression allows the maintenance of constant levels of the proenzyme despite its cleavage upon activation and expansion of effector T cells.

**Sensitivity to apoptosis correlates with the levels of caspase-3**

The increase in caspase-3 expression during T cell activation suggests that caspase-3 plays a critical role in T cell homeostasis. Supporting this hypothesis are the findings demonstrating that caspase-3-deficient peripheral T cells are less susceptible to AICD (27). Therefore, it is likely that the increased expression of caspase-3 reported in this work is important for sensitizing activated T cells to apoptosis. To confirm this hypothesis, T cells were isolated from the lymph nodes of caspase-3 wild-type, heterozygous, and knockout mice and put in culture for 2 days in the absence or presence of anti-CD3. Following TCR cross-linking, T cells isolated from caspase-3 wild-type, heterozygous, and knockout mice demonstrated similar activation profiles (CD69+ and CD25+) and showed no defect in their proliferation (data not shown) (27). Western blot analysis using lysates from activated T cells isolated from either wild-type, heterozygous, or caspase-3 knockout mice confirmed the relative abundance of the proenzyme (Fig. 5A).

We next determined the sensitivity of activated T cells isolated from wild-type, heterozygous, or caspase-3 knockout mice to etoposide- or IL-2 withdrawal-induced apoptosis. T lymphocytes isolated from caspase-3 knockout or heterozygous mice were significantly (p < 0.05) more resistant to etoposide-induced apoptosis (70% ± 4 and 69% ± 2 AnV− cells, respectively) when compared with T cells isolated from wild-type mice (45% ± 7 AnV− cells). Similar results were obtained following IL-2 withdrawal-induced apoptosis (n = 3, Fig. 5B). Furthermore, nonstimulated samples, in which caspase-3 expression is not increased, were more resistant to etoposide- or IL-2 withdrawal-induced apoptosis when compared with anti-CD3-stimulated T cells (Fig. 5B). Therefore, the relative levels of caspase-3 in T cells correlate with their sensitivity to apoptotic stimuli, which suggests that the levels of this protease play an important role in controlling T cell number by eliminating the expanded population of effector T cells at the termination of an immune response.

**Discussion**

The execution of many death pathways requires the presence and activation of caspase-3. In this work, we studied the early expression of caspase-3 following TCR engagement. Our results demonstrate that caspase-3 mRNA and protein expression levels are selectively increased following TCR engagement. Interestingly, the increase in caspase-3 expression occurred early after TCR engagement, thereby suggesting that caspase-3 is an early regulator of T cell maturation and differentiation. The increase in caspase-3 expression was not due to the increase in the number of cells because inhibitors of T cell proliferation did not reduce the level of caspase-3 mRNA expression, and more importantly, the increase was exclusive to caspase-3 and -7. Our results suggest that the selective increase in the levels of caspase-3 before cell division ensures that each effector T cell will have sufficient amounts of the proenzyme to rapidly undergo apoptosis induced by extracellular and/or intracellular signals because caspase-3 is at the crossroad of both the extrinsic (death receptors) and intrinsic (mitochondria) pathways of apoptosis (40, 41). Furthermore, several reports have demonstrated that caspases are recruited to lipid rafts, following the clustering of the Fas receptor (42, 43). In that context, we have recently shown that caspase-3 colocalizes in lipid rafts with caspase-8 and its activity is required for complete caspase-8 activation following Fas cross-linking, demonstrating that caspase-3
plays a central role in the amplification of Fas signaling in T lymphocytes (43). Because caspase-3 also appears to be required in the initiation and amplification of apoptosis signals, the selective up-regulation in caspase-3 levels ensures the presence of sufficient amounts of the proenzyme to eliminate activated T cells at the termination of an immune response. These findings clearly establish the importance of maintaining minimal caspase-3 levels to ensure the elimination of effector T cells, which are the targets of AICD.

The up-regulation in caspase-3 gene expression following T cell activation was much more significant in peripheral T cells when compared with thymocytes, even though these cells up-regulate T cell activation markers following CD3 cross-linking (Fig. 1A). Thymocytes from caspase-3 knockout mice and wild-type mice were equally sensitive to the induction of apoptosis by anti-Fas, anti-CD3, ceramide, staurosporin, and dexamethasone (44). In contrast, peripheral T cells from caspase-3 knockout mice were shown to be less susceptible to AICD, anti-CD3-, anti-Fas-induced apoptosis (27), and etoposide- and IL-2 withdrawal-induced cell death (Fig. 5B). Thymocytes directly undergo apoptosis without cell division following TCR engagement, which could explain the lack of requirement for the up-regulation in caspase-3 levels in thymocytes to ensure cell death. In contrast, lymph node T cells proliferate in response to TCR stimulation, resulting in a requirement for caspase-3 up-regulation to replenish the cleaved caspase-3 following TCR triggering in proliferating effector T cells. The selective increase in caspase-3 mRNA levels is a general process resulting from TCR engagement, as these findings were reproduced in two different strains of mice, polyclonally activated T cells and Ag-specific T cells activated in vitro (2C TCR transgenic mice) or in vivo (P14 TCR transgenic mice).

It was shown recently that the selective expression of the IL-7R allows the survival and differentiation of effector T cells into memory T cells (23). However, nearly all cells expressing low levels of the IL-7R were positive for cleaved caspase-3. The pool of cleaved caspase-3, which is present in IL-7R low effector T cells, most probably emanates from the newly synthesized caspase-3, a result of the up-regulation of caspase-3 expression. These findings demonstrate a requirement to maintain high enough levels of the proenzyme to ensure the elimination of this T cell subset following T cell activation. Similarly, caspase-3 has also been shown to be a negative regulator of cell cycle progression in B cells and an essential component in the regulation of B cell homeostasis (45). Our findings clearly demonstrate that caspase-3 is an early marker whose presence could predict the fate of T cells following activation and consequently the success or failure of immune responses.

The appearance of the cleaved forms of caspase-3 early during an immune response does not immediately lead to apoptosis. Indeed, several groups have reported cleavage of caspase-3 during T cell activation and proliferation, in the absence of cell death (17, 46). In Jurkat T cells, the cleaved form of caspase-3 remains associated with the caspase inhibitor X-linked mammalian IAP protein (XIAP), until Smac/Diablo is released from the mitochondria (47). Interestingly, the ring finger motif of two inhibitors of apoptosis (IAP) family members (cellular IAP2 and XIAP) has been shown to contain ubiquitin ligase activity, which promotes ubiquitination of both the cleaved forms of caspase-3 (48, 49) and -7 (49). The ubiquitin ligase activities of both proteins lead to the degradation of the cleaved forms of caspase-3, thus enhancing their antiapoptotic function. Because the cleaved forms (p20 and p17) of caspase-3 disappear rapidly through XIAP- and cellular IAP2-mediated proteosomal degradation (48, 49), the transcriptional up-regulation of caspase-3 is most probably responsible for the replenishment and maintenance of adequate levels of the pro-caspase-3 pool. Furthermore, only caspase-3 and -7 mRNA levels were increased in activated effector T cells, whereas the expression of caspase-8 and -9 was not affected (Fig. 2B). Interestingly, all of these caspases, except caspase-8, can bind to members of the IAP

**FIGURE 5.** Caspase-3 expression correlates with sensitivity of T cells to apoptosis. A, Cell lysates of activated T cells isolated from the lymph nodes of wild-type (WT), heterozygous (HET), and knockout (KO) caspase-3 mice were analyzed by Western blot for the levels of procaspase-3. B, AnV cells of nonstimulated (NS) and anti-CD3 (α-CD3)-stimulated peripheral T cells from WT, HET, and KO mice were sorted after 2 days of culture. The cells were then treated with either 1 μM etoposide or 50 μg/ml PC61 Ab to induce apoptosis. The percentage of AnV cells was assessed by AnV/PI staining (n = 3).
family once activated. Therefore, the selective increase in caspase-3 and -7 expression may be related to their susceptibility to IAP-mediated degradation. Caspase-9 can also associate with IAPs; however, it is not activated following T cell stimulation, which may account for its lack of enhanced expression. In conclusion, the selectivity of caspase up-regulation may be the result of their activation status as well as their susceptibility to IAP-mediated degradation following T cell activation.

We ultimately measured caspase-3 expression observed in LCMV-specific effector CD8 T cells (Figs. 2B and 3) may be an important determinant of the natural process of effector T cell cell elimination. Salvesen and Dixit (50) suggested that a disruption in the balance of pro- and antiapoptotic proteins constitutes a major factor in regulating an apoptotic threshold. In agreement with this hypothesis, the imbalance between high levels of caspase-3 (Figs. 2B and 3) and the low levels of members of the Bcl-2 family in effector T cells (20–23) is likely to contribute significantly to their death by AICD. In contrast, Ag-specific memory CD8 T cells have elevated levels of Bcl-2 (20, 23) and reduced caspase-3 expression as compared with effector T cells (this study). This exquisite balance between proapoptotic and antiapoptotic molecules contributes to the survival and persistence of memory T cells.

Several signaling pathways mediated through the TCR have been suggested to potentially regulate caspase expression levels. Previous reports have demonstrated a role of STAT signaling in the basal constitutive expression of caspase-1, -2, and -3 (24). Furthermore, activation of STAT1 through the IFN-γ receptor led to increased expression of caspase-1, -3, and -8 and sensitized cells to apoptosis (51–54). Moreover, IFN-γ has been recognized to be required for AICD of activated T cells by controlling T cell numbers at the termination of an immune response through the increase in caspase-3 and -8 gene expression (55). Furthermore, the E2F-1 transcription factor that plays a critical role in cell cycle entry and T cell proliferation has been shown to be a mediator of AICD (56). Interestingly, several studies have demonstrated a potential role of this transcription factor in the induction of caspase-3 mRNA levels (57, 58). Whether any of these transcription factors are directly responsible for the increase in caspase-3 mRNA levels during T cell activation remains to be determined.

Based on the results presented in Fig. 3, it appears that the increased expression of cleaved caspase-3 occurs in two incremental steps: early after T cell activation, the expression of cleaved caspase-3 increases to an intermediate level in effector T cells that are becoming apoptotic. The significance of the intermediate level of cleaved caspase-3 found in effector T cells early during infection is not clear, but perhaps a low level of caspase-3 activity is nonlethal and is important for effector T cell function, expansion, or differentiation, as has been previously suggested (14–17, 59). Although other factors regulating apoptosis are involved in mediating death of activated T cells, our results support a model whereby the selective up-regulation of caspase-3 levels upon engagement of the TCR contributes significantly in tilting T cell homoeostasis toward apoptosis during an immune response and the subsequent elimination of effector T cells.

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References


