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Caspase Enzyme Activity Is Not Essential for Apoptosis During Thymocyte Development

Petra Doerfler, Katherine A. Forbush, and Roger M. Perlmutter

Caspases, a family of cysteine proteases, are critical mediators of apoptosis. To address the importance of caspases in thymocyte development, we have generated transgenic mice that express the baculovirus protein p35, a viral caspase inhibitor, specifically in the thymus. p35 expression inhibited Fas (CD95)-, CD3-, or peptide-induced caspase activity in vitro and conferred resistance to Fas-induced apoptosis. However, p35 did not block specific peptide-induced negative selection in OTI and HY TCR transgenic mouse models. Even the potent pharmacological caspase inhibitor zVAD-FMK (benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone) could not prevent peptide-induced deletion of OTI thymocytes, although it improved basal thymocyte survival in vitro. Moreover, the developmental block observed in rag-1−/− thymocytes, which lack pre-TCR signaling, was also not rescued by p35 expression. These results indicate that caspase-independent signal transduction pathways can mediate thymocyte death during normal T cell development. The Journal of Immunology, 2000, 164: 4071–4079.

Developing thymocytes sequentially rearrange their TCR β- and α-chain genes to generate an enormous repertoire of diverse TCR specificities, under circumstances in which each developing cell in general expresses only a single receptor species. Subsequent selection eliminates autoactive and nonfunctional cells from this repertoire (reviewed in Ref. 1). Programmed cell death (apoptosis) appears to be the default fate of thymocytes if they do not receive signals to proliferate and/or differentiate, thus eliminating nonfunctional cells (death by neglect). This is reflected at the earliest developmental stage when the expression of cytokine receptors and the pre-TCR (composed of a newly rearranged TCR β-chain and a surrogate TCR (pre-T) α-chain) is required for expansion and differentiation. In the absence of survival signals delivered via these receptors, the CD4+8− (double-negative, DN) cells undergo apoptosis (reviewed in Refs. 2 and 3). Thymocytes that advance beyond this regulatory checkpoint express low levels of the TCR as well as both CD4 and CD8 coreceptors (double-positive cells, DP). Such cells are subject to positive and negative selection within the thymus. DP cells that express TCRs of intermediate affinity for peptide ligands in the context of MHC molecules are positively selected to differentiate into CD4 or CD8 single-positive cells. Consequently, these cells up-regulate their TCR expression levels and eventually populate the periphery. In contrast, both very low as well as very high affinity interactions of the TCR with self-MHC/peptide ligand result in apoptosis. It is believed that most thymocytes express TCRs which cannot mediate signals that promote survival and differentiation. Hence, these cells die by neglect. On the other end of the spectrum, very high affinity TCR interactions with self MHC cause negative selection, i.e., the clonal deletion of these potentially autoactive cells (reviewed in Refs. 4 and 5).

To investigate the mechanisms responsible for negative selection, we sought to inhibit caspase-mediated apoptosis in vivo. Caspases, a family of at least 14 cysteine proteases, have been found to play a critical role in mediating apoptosis in species ranging from nematodes to mammals (reviewed in Refs. 6 and 7). In general, these proteases are constitutively expressed as inactive proenzymes, which become proteolytically activated during apoptosis. Based on sequence homologies and cellular function, caspases can be divided into two major subfamilies with close resemblance to either caspase-1 or to caspase-3. According to their respective function during apoptosis, they have also been categorized as initiator or effector caspases (7). Caspases are abundantly expressed in lymphoid cells, and their involvement in apoptosis suggested that they might play a role in negative selection. Indeed, studies performed using fetal thymic organ culture implicated caspase-3-related enzyme activation in thymocyte apoptosis (8). These authors reported that the generic pharmacological caspase inhibitor zVAD-FMK (benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone) inhibited deletion of thymocytes induced by either anti-CD3 Abs, the glucocorticoid dexamethasone, or antigenic peptide in vitro. Moreover, caspase-3 activation was detected specifically during apoptosis induced by TCR stimulation, but not during spontaneous cell death (9).

These studies leave open the mechanism whereby TCR stimulation might induce caspase activation and the importance of this process in vivo. It remains unclear whether caspase activation is actually required for negative selection to occur or whether it occurs as a secondary effect that accompanies negative selection-induced thymocyte death.

Gene disruptions of individual caspases yield phenotypes associated with the inflammatory response (caspase-1 and -11) or defects in cell- and/or stimulus-specific apoptosis (caspase-2, -3, -8,
and -9). However, none of these mice manifest abnormalities in thymocyte maturation (10–18), suggesting that several caspases may play redundant roles in thymocytes. We therefore pursued a strategy designed to block the function of most caspases expressed in the thymus.

Our studies make use of the baculovirus protein p35, which inhibits all caspase subfamilies through a stable interaction with their catalytic sites (19–23). In addition to its ability to protect baculovirus-infected insect cells from apoptosis, the ectopic expression of p35 blocks apoptosis involving diverse death signals, e.g., developmental death, growth factor withdrawal, or DNA damage, in many different cell types and species (reviewed in Ref. 24). These observations suggested that p35 might provide a potent tool to investigate mechanisms of apoptosis in thymocytes. In this study, we present results obtained from transgenic mice expressing the p35 protein under the control of the thymocyte-specific lck proximal promoter.

Materials and Methods

Transgenic p35 construct and mouse lines

The p35 cDNA (25) was amplified by PCR on linearized baculovirus DNA from Autographa californica nucleopolyhedrovirus (BaculoGold; Pharmingen, San Diego, CA) using the following primers (BamHI restriction sites are underlined; p35–3′ primer, 5′-GGG GGA TCC CCA TAG CAA AAT GTG TGT ATG TTT TCC GGT G-3′; p35-3′ primer, 5′-GGG GGA TCC TTA TTT AAT TGT GTT TAA TAT TAC ATT TTT GTT GAG-3′). The obtained 971-bp cDNA fragment was cloned into the BamHI site of the transgenic p1017 vector (26), placing its expression under the control of the lck proximal promoter. Transgenic mice were generated and propagated as described previously (27). For studies of negative selection or early thymocyte development, lck-p35 mice were also crossed with OT1 (28) or HY TCR transgenic mice (29) or Rag1−/− mice (purchased from The Jackson Laboratory, Bar Harbor, ME) (30), respectively. The presence of the respective transgenes was monitored by PCR strategies. All mice were housed under specific pathogen-free conditions.

Thymocyte stimulation assays in vitro

For CD3 and Fas (CD95) stimulation, 24-well plates were coated with 10 μg/ml anti-CD3e (145-2C11) or anti-Fas Abs (Jo2; Pharmingen). A total of 3 × 10⁶ thymocytes were seeded into Ab-coated or into control wells, and cultured in 1 ml medium (RPMI 1640 supplemented with 10% FCS, 2 mM l-glutamine, 50 μM 2-ME, and antibiotics) at 37°C for indicated periods. Thymocytes were cultured similarly for the lck proximal promoter. Transgenic mice were generated and propagated as described previously (27). For studies of negative selection or early thymocyte development, lck-p35 mice were also crossed with OT1 (28) or HY TCR transgenic mice (29) or Rag1−/− mice (purchased from The Jackson Laboratory, Bar Harbor, ME) (30), respectively. The presence of the respective transgenes was monitored by PCR strategies. All mice were housed under specific pathogen-free conditions.

Flow-cytometric analysis

A total of 5 × 10⁶ to 1 × 10⁷ cells were stained with saturating concentrations of Abs at 4°C for 30 min, using combinations of the following mouse-specific Abs: PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 (Becton Dickinson, San Jose, CA); biotinylated (BIO) anti-CD3ε, BIO anti-CD69, FITC anti-CD25, PE anti-CD44, and BIO annexin V (Pharmingen). Transgenic HY or OT1 TCR α-chains were detected with BIO T3.70 (gift from Dr. H. S. Teh, University of British Columbia, Vancouver, Canada) or BIO anti-Vo2 (Pharmingen), respectively. BIO Abs were visualized by tricolor-conjugated streptavidin (SA-TRI, Caltag). Data were collected on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Changes in mitochondrial transmembrane potential were visualized by staining with 3,3′-dipropylthiadicarbocyanine iodide (DiSC₃(5); Molecular Probes, Eugene, OR). In brief, cells were resuspended in 1 ml of staining medium (Hanks’ buffered saline, 10 mM HEPES, 1% BSA), and 1 μl DiSC₃(5) in DMSO was added for a final concentration of 40 nM. Cells were incubated for 20 min at room temperature in the dark, then washed and resuspended in staining medium, followed by data acquisition on a FACScan flow cytometer (Becton Dickinson) within the next hour. All analyses were performed using ReproMac 2.3 software (TrueFacts Software, Seattle, WA).

Protein extracts and Western blot analysis

Single cell suspensions from thymus or cultured thymocytes were washed in PBS twice and resuspended in hypotonic lysis buffer (10 mM HEPES, pH 7, 50 mM NaCl, 2 mM MgCl₂, 40 mM β-glycerophosphate, 5 mM EGTA) at 5 × 10⁶ cells/ml. Cells were disrupted by four alternating freeze-thaw steps, and the lysates were cleared by centrifugation at 20,000 × g for 4°C. Protein concentrations were determined by BCA protein assay (Pierce, Rockford, IL).

For Western blot analysis, protein extracts were separated on 12% SDS-PAGE gels, transferred onto polyvinylidene difluoride membranes (Amer sham Life Science, Arlington Heights, IL), and probed with polyclonal chicken anti-p35 Abs (1 μg/ml; provided by Dr. A. Niles, Promega, Madison, WI). Incubation with HRP- or alkaline phosphatase-conjugated anti-chicken IgY (1g-HP; 200 ng/ml; Promega) was followed by enhanced chemiluminescence or chemifluorescence (Amer sham Life Science) detection methods, respectively.

Thymocyte stimulation assays in vitro

For CD3 and Fas (CD95) stimulation, 24-well plates were coated with 10 μg/ml anti-CD3e (145-2C11) or anti-Fas Abs (Jo2; Pharmingen). A total of 3 × 10⁶ thymocytes were seeded into Ab-coated or into control wells, and cultured in 1 ml medium (RPMI 1640 supplemented with 10% FCS, 2 mM l-glutamine, 50 μM 2-ME, and antibiotics) at 37°C for indicated periods. Thymocytes were cultured similarly for the lck proximal promoter. Transgenic mice were generated and propagated as described previously (27). For studies of negative selection or early thymocyte development, lck-p35 mice were also crossed with OT1 (28) or HY TCR transgenic mice (29) or Rag1−/− mice (purchased from The Jackson Laboratory, Bar Harbor, ME) (30), respectively. The presence of the respective transgenes was monitored by PCR strategies. All mice were housed under specific pathogen-free conditions.

Results

Generation of transgenic mice expressing the caspase inhibitor p35

To directly express the p35 in immature thymocytes, we placed the p35 cDNA from baculovirus (25) under the control of the lck proximal promoter (26). Eight transgenic mouse lines were generated that exhibited a wide range of p35 expression levels, as determined by Northern and Western blot analysis (Fig. 1A, and unpublished data). We used these lines to establish a dose-response relationship, correlating p35 expression levels with caspase inhibition. Thymocytes were isolated from mice representing different transgenic lines or corresponding normal littermate controls (LMC) and stimulated with cross-linking anti-Fas (CD95) Abs in vitro for 4 h (Fig. 1B). Protein extracts from freshly isolated or cultured cells were analyzed for caspase activity using a synthetic tetrapeptide substrate (Ac-DEVD-AMC; Ref. 31) that is preferentially cleaved by caspase-3-related proteases, the main targets for inhibition by p35. The release of the fluorogenic compound AMC provides a measure of caspase activity. In agreement with the proposed stoichiometric inhibition of caspases by p35 (19), we observed a linear inhibition of caspase activity as a function of p35 expression, reaching maximal enzyme inhibition at ~1–2 ng p35 per 1 × 10⁷ cells. The residual background of substrate degrada tion, which is seen even at clearly saturating p35 expression levels, may be caused by proteasome-mediated cleavage (Nancy Thorn berry, personal communication).

The high p35 expression levels in lck-p35 transgenic thymocytes do not persist in peripheral lymphoid tissues (data not shown), which is in accord with the known down-regulation of endogenous lck proximal promoter activity in mature T cells (32). Analysis of freshly isolated tissues showed that thymus, spleen, and lymph nodes of lck-p35 transgenic mice were indistinguishable from normal control mice. Tissue size, cell numbers, and the relative representation of lymphocyte populations as determined by guest on April 23, 2017 http://www.jimmunol.org/ Downloaded from 4072
High p35 expression in thymocytes inhibits caspase-3-related enzyme activity and reduces apoptosis in vitro

Immature DP T cells, which represent the vast majority of thymocytes, undergo spontaneous cell death when cultured in single cell suspensions. Moreover, they are exquisitely sensitive to TCR stimulation with anti-CD3 Abs (a process that is thought to mimic negative selection in vitro) and to Ab-mediated cross-linking of Fas. Therefore, we tested the lck-p35 transgene for its ability to inhibit caspase activity and to rescue thymocytes under these apoptosis-inducing conditions in vitro.

Thymocytes from lck-p35 transgenic and LMC mice were isolated and cultured in the absence or presence of anti-CD3 or anti-Fas (CD95) Abs for 2, 4, and 24 h (Fig. 2). At each time point, flow-cytometric samples and protein extracts were prepared to determine the percentage of live DP thymocytes and the enzymatic activity of caspasases, respectively. The expression of p35 protein improved the survival of DP cells slightly (~7–10% more live cells) at all time points when cultured in medium only. In the presence of anti-CD3 Abs, a similar improvement of survival of DP thymocytes by p35 was observed at the 2- and 4-h time points; however, this protective effect was lost after 24 h. In contrast, p35 profoundly reduced apoptosis in anti-Fas-treated cells.

The corresponding protein extracts were analyzed for caspase activity. In control cell extracts, caspase-3-related enzyme activity transiently increased under all culture conditions. Strikingly, this enzyme activity was completely blocked by the presence of high levels of p35 (Fig. 2, lower panels). A dose-response relationship obtained with additional transgenic lines expressing low to intermediate levels of p35 underscored the causal link between the observed caspase inhibition and improved thymocyte survival, i.e., caspase inhibition by intermediate or low levels of p35 expression resulted in intermediate or weak protection from apoptosis, respectively (data not shown). Additional experiments focused on lines expressing very high levels of p35 (Fig. 1).

Expression of transgenic TCRs early in thymocyte development reduces p35 expression levels

In a wild-type mouse with a normal mixed TCR repertoire, the number of negatively selected thymocytes is almost certainly very low (33). To facilitate the investigation of negative selection, we introduced the well-characterized HY and OT1 TCR transgenes onto a lck-p35 mouse background (28, 29). The HY TCR recognizes the male-specific HY Ag in the context of H-2D<sup>b</sup> MHC class I, causing positive selection of CD8<sup>+</sup> T cells in females and strong negative selection of DP thymocytes in male mice (29). TheOT1 TCR specifically recognizes the OVA peptide 257–264 in the context of H-2K<sup>b</sup> MHC class I (28). In the absence of peptide Ag, thymocytes are positively selected to the CD8<sup>+</sup> T cell compartment. However, the addition of the specific OVA peptide to these thymocytes in vivo or in vitro induces a concentration-dependent deletion of the DP compartment, thereby providing another model for negative selection (28).

We crossed the two mouse lines with highest p35 expression with HY and OT1 mice and analyzed transcript and protein expression levels in males and females from lck-p35 × HY and lck-p35 × OT1 litters. Both Northern and Western blot analysis revealed that mice with the single lck-p35 transgene maintained high p35 mRNA and protein expression. However, their lck-p35/HY and lck-p35/OT1 double transgenic littersmates had ~5-fold reduced p35 mRNA and protein expression levels (Fig. 3, and unpublished data). The same relative reduction was observed for both high p35-expressing lines and for additional lck-p35 × HY and lck-p35 × OT1 litters when low or intermediate p35-expressing mouse lines had been used for breedings (data not shown). These observations suggest that artificially early and high expression of a TCR reduces the activity of the transgenic lck proximal promoter at a much earlier step in thymocyte development than in normal thymi, in which high TCR expression is only achieved upon positive selection to single-positive T cells. Nevertheless, the previously established dose-response relationship (Fig. 1B) indicated that even these reduced p35 levels in the doubly transgenic mice would be sufficient to block caspase activity.

p35 expression fails to affect negative selection in male HY TCR transgenic mice

To study the deletion of DP thymocytes by endogenous Ag in the HY TCR transgenic mice, we compared HY mice with lck-p35/HY male litters. Fig. 4 depicts the CD4/CD8 flow-cytometric profile of an HY female control, showing the typical skewing of CD4<sup>+</sup>8<sup>+</sup> cells due to positive selection of HY TCR-expressing cells (upper left panel). As previously reported, this effect becomes more obvious if only cells that actually express the HY TCR are examined (as determined by staining with T3.70, an Ab specific for the Vα-chain of the HY TCR; upper right panel). In contrast, HY TCR-expressing thymocytes are autoreactive in male mice.
Consequently, the majority of DP thymocytes in male animals are eliminated and total thymocyte numbers are dramatically reduced (~10% of control). As some thymocytes can escape negative selection by replacing the transgenic TCR α-chain with a rearranged endogenous TCR α-chain (providing a different specificity), we again electronically gated on cells that are recognized by the T3.70 Ab to restrict the analysis to authentic HY TCR transgenic cells (Fig. 4, lower panels). Clearly, negative selection was not affected

**FIGURE 2.** p35 reduces apoptosis of thymocytes in vitro through the inhibition of caspase-3-related enzyme activity. Thymocytes from the two highest p35-expressing lines or from LMC were cultured in the absence or presence of cross-linking anti-CD3 or anti-Fas (CD95) Abs. At the indicated times, cells were harvested and split for flow-cytometric analysis and enzyme assays. Cells were stained with PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 Abs. Live cells were identified based on FSC and SSC properties. By means of electronic gates, the number of live DP thymocytes was determined for each sample and expressed as a percentage of corresponding freshly isolated cells (upper panels). Caspase-3-related enzyme activity (lower panels) in respective protein extracts was measured as described in Fig. 1. The data represent results for three individual lck-p35 transgenic mice (1 and 2 from the highest, and 3 from the second highest expressing line; see Fig. 1B) and mean values and SEs for four control mice analyzed in two independent experiments. Results are representative of four experiments.

**FIGURE 3.** p35 protein expression is reduced in TCR transgenic thymocytes. Female littermates of transgenic breeding sets for A, lck-p35 × HY, and B, lck-p35 × OT1 (five generations backcrossed onto the C57BL6 genetic background) were tested for p35 protein expression. Western blot analysis of thymocytes was performed as described in Fig. 1, except for visualizing and quantifying the p35 signals by enhanced chemifluorescence. Signal intensities were normalized to a p35-independent band (marked by an asterisk) and compared between transgenic mice, as indicated by numbers below the lanes (expression levels in lck-p35 single transgenic mice equal 100%).

**FIGURE 4.** p35 does not inhibit negative selection of male HY TCR-expressing thymocytes. Thymocytes from female and male HY or lck-p35/HY mice were stained with PE anti-CD4, FITC anti-CD8, and BIO T3.70 (anti-HY TCR α) Abs, followed by incubation with SA-TRI to visualize T3.70. Flow-cytometric profiles are shown as two-dimensional dot plots for the CD4/CD8 staining with each dot representing a live cell, as determined by FSC and SSC properties. Except for the first panel, an electronic gate was applied to restrict the analysis to cells expressing the authentic HY TCR (T3.70hi). Numbers in quadrants indicate percentages of cells within respective thymic subpopulations; numbers above the panels give the total number of thymocytes isolated from the individual animal. The figure shows 1 representative example of 11 paired analyses of HY and lck-p35/HY male mice (4–7 wk of age) and a female HY control mouse.
by the \textit{lck-}p35 transgene because neither the total cell numbers nor the relative representation of thymic subpopulations were changed. Positive selection in female HY TCR transgenic mice is likewise unchanged in the presence of p35 (data not shown).

\textit{p35 expression affects survival in vitro, but not negative selection in the OT1 TCR transgenic mouse model}

To examine the importance of caspases for thymocyte survival in more detail, we used peptide-mediated deletion of T cells in vitro as a surrogate for negative selection in vivo (28). Thymocytes from animals expressing the OT1 TCR, p35, or both were exposed to increasing amounts of OVA peptide presented by EL4 cells. Freshly isolated or cultured cells were stained with PE anti-CD4 and FITC anti-CD8 Abs and with BIO annexin V/SA-TRI and analyzed by flow cytometry, EL4 and apoptotic or dead cells were excluded according to FSC and SSC properties. The numbers of live DP thymocytes are expressed as percentage of corresponding fresh cells. The graph presents mean values and SEs obtained for 14 pairs of OT1 and \textit{lck-}p35/OT1 mice and for 7 pairs of \textit{lck-}p35 and LMC mice at each OVA peptide concentration.

As the difference in survival between OT1 and \textit{lck-}p35/OT1 thymocytes was small, we were interested to see whether it actually correlated with a difference in caspase enzyme activity. Therefore, we cultured the same set of thymocytes for 2, 4, or 20 h in the absence or presence of 1 nM OVA peptide, which is the minimal concentration to stimulate maximal deletion (Fig. 5A). Fig. 5B shows that caspase activity in both OT1 and \textit{lck-}p35/OT1 thymocytes increased in culture even in the absence of peptide stimulation (medium control). This correlates with the spontaneous death of a large subpopulation of cells with low CD4 and CD8 expression that is typically found in these mice (see Fig. 7). Nonetheless, p35 expression clearly reduced the magnitude of this spontaneous enzyme activation. OVA peptide stimulation increased caspase activity about 2-fold in both OT1 and \textit{lck-}p35/OT1 thymocytes but, as in the medium control, p35 expression suppressed the enzyme activity to \(\sim 50\%\) when compared with OT1 thymocytes. The persistently high level of p35 expression in the \textit{lck-}p35 control restricted caspase activity to background levels as before (Figs. 2 and 5B).

\textit{The potent pharmacologic caspase inhibitor zVAD-FMK does not block peptide-induced deletion of OT1 TCR transgenic mice}

Because the reduced levels of p35 in \textit{lck-}p35/OT1 transgenic thymocytes permitted some increase in caspase activity (Fig. 5B), it remained conceivable that negative selection and peptide-mediated deletion in vitro both require a threshold level of caspase activity. To test this possibility, we took advantage of the potent generic caspase inhibitor zVAD-FMK. In a pilot experiment, we determined that a concentration of 100 \(\mu\)M zVAD-FMK was sufficient to fully block caspase activity and prevent the loss of membrane polarity characteristic of apoptosis. Subsequent assays confirmed that this concentration also completely inhibits caspase-mediated oligonucleosomal DNA degradation following anti-Fas-, anti-CD3-, or dexamethasone-induced apoptosis of normal thymocytes (data not shown). zVAD-FMK does, however, not generally interfere with signal transduction pathways, as flow-cytometric analysis confirmed that all thymic subpopulations were capable of responding normally to peptide stimulation with respect to the induction of the T cell activation marker CD69 (unpublished data).

Fig. 6 documents the effect of zVAD-FMK on OVA peptide-induced thymocyte deletion in vitro. As in Fig. 5, the data represent the percentage of surviving DP thymocytes relative to that observed in freshly isolated cells. zVAD-FMK improves the overall survival of DP cells for both LMC and OT1 transgenic thymocytes by 20–30\% (Fig. 6, and unpublished data). However, the specific responsiveness of the OT1 TCR-expressing cells to OVA
peptide remains exactly the same. The shift of the peptide response curve occurred by the same magnitude at every OVA peptide concentration analyzed, suggesting that this difference is exclusively accounted for by the loss of spontaneous cell death.

Previous studies indicate that a partial down-regulation of CD4 and CD8 coreceptors, and thus the loss of bright DP thymocytes, not only occurs due to the induction of apoptosis in these cells, but also constitutes a part of the positive selection process before the selective up-regulation of either the CD4 or the CD8 coreceptor (34, 35). We therefore sought to confirm the cell death of DP and CD4low8low thymocytes by additional parameters. We analyzed changes in the mitochondrial transmembrane potential (Δψm), which occur at a very early stage of apoptotic cell death and can be visualized with Δψm-sensitive, lipophilic dyes such as DiSC3(5) or 3,3′-dihexyloxacarbocyanine iodide (DiOC6) (3, 36). Fig. 7 shows representative results for this analysis, using experimental conditions as described for Figs. 5 and 6. For clarity, the raw data are restricted to OT1 transgenic cells that were freshly isolated or incubated with 1 nM OVA peptide in the absence or presence of 100 µM zVAD-FMK. During apoptosis, thymocytes show a characteristic shift to lower FSC and higher SSC properties, which we exploited to determine the representation of live cells in all experiments described in Figs. 2–6. However, in cultures treated with zVAD-FMK, a significant proportion of cells displayed aberrant FSC and SSC properties (Fig. 7A, top panels). Analysis of mitochondrial conditions in cells cultured without zVAD-FMK confirmed that the FSC/SSC gate included almost exclusively cells with high Δψm, a characteristic of living cells (DiSC3(5)-bright, Fig. 7A). In contrast, in the presence of zVAD-FMK, one half of the population within the FSC/SSC gate manifested a low Δψm, indicating that more cells were committed to apoptosis than revealed by the FSC/SSC properties. Therefore, we compared the numbers of live DP and CD4low8low cells under all different conditions defined by either FSC/SSC or by high Δψm (Fig. 7B). Both methods of enumerating live cells performed equivalently in cells not exposed to zVAD-FMK, thus confirming the results for previous experiments with OT1 and lck-p35/OT1 transgenic cells. However, the comparison for zVAD-FMK-treated cells yielded much lower absolute numbers for total live cells and consequently for live DP and CD4low8low cells when based on Δψm. Moreover, by gating on DiSC3(5)-bright cells, the relative representation of CD4low8low cells was reduced. Additional characterization showed that, irrespective of culture conditions, all cells with high Δψm were negative for annexin V staining (which assesses membrane phospholipid polarity) (37) and uptake of 7-amino-actinomycin D (33% of these cells were annexin V positive, but still ~22% were 7AAD positive, confirming cell death within this population (data not shown). The restriction of the analysis to cells with high Δψm therefore provides a more accurate assessment of live cells in zVAD-FMK-treated cultures than the FSC/SSC gate (or annexin V). This becomes especially important in the case of CD4low8low cells, which might otherwise be overestimated and mistaken for intermediate cells during positive selection. Also for DP cells, the mitochondrial analysis indicates that the rescue by zVAD-FMK is less effective than was suggested by the analysis of FSC/SSC. These results are nevertheless consistent with those presented in Fig. 6, in that caspase inhibition by zVAD-FMK did not block peptide-mediated deletion of OT1 thymocytes in vitro.

In summary, the data obtained with zVAD-FMK confirm the observations with lck-p35 transgenic thymocytes and indicate that inhibition of caspase activity can reduce apoptosis due to lack of survival signals, but does not change the sensitivity of the OT1 TCR-expressing cells to negative selection.
FIGURE 8. p35 expression does not rescue the early developmental defect of rag1−/− thymocytes. Thymocytes of the indicated genotypes were stained for flow-cytometric analysis. CD4/CD8 and CD25/CD44 expression profiles (numbers indicate percentages of cells within subpopulations) are shown for representative examples of 11 rag1−/− and 12 rag1−/− lck-p35 transgenic mice analyzed between 5 and 8 wk of age.

p35 does not rescue immature thymocytes in the absence of pre-TCR signaling

The rescue of DP thymocytes from spontaneous cell death by p35 and zVAD-FMK in vitro suggested that p35 might also prevent apoptosis caused by the lack of survival signals during thymocyte development. For example, immature thymocytes die if they are unable to express a pre-TCR complex on the surface (reviewed in Ref. 3). In rag1−/− mice, thymocyte differentiation is blocked at the DN stage and total cell numbers are reduced to ~1% of those in normal thymus because of failure to rearrange TCR genes (30). We therefore assessed whether p35 expression could rescue rag1−/− thymocytes in vivo.

We crossed the high p35-expressing transgenic lines with rag1−/− mice and analyzed rag1−/− lck-p35 and control mice (Fig. 8). In heterozygous rag1-deficient mice, p35 expression did not affect thymocyte development. Similarly, p35 expression proved incapable of ameliorating the defects imposed by homozygous deletion of rag1 genes. Total thymocyte numbers in rag1−/− lck-p35 transgenic mice were as low as in rag1−/− mice (1.55 × 10⁶ vs 1.65 × 10⁸ cells), and no differentiation beyond the DN stage was detectable. The DN population can be further characterized by assessing the expression profile of CD25 and CD44 (39); cells mature from CD25−/−44− to CD25−/−44+ and subsequently extinguish CD25 expression. CD25 expression is normally lost upon successful signaling from the pre-TCR, leading to the appearance of CD25−/−44− cells that rapidly gain CD4 and CD8 expression (39). Fig. 8 clearly shows that in both rag1−/− and rag1−/− lck-p35 transgenic animals, the majority of thymocytes are CD25−/−44− (69% and 74%, respectively), and the distribution of cells in earlier stages is likewise indistinguishable. Consistent with the complete absence of DP cells in these mice, no CD25−/−44− thymocytes were detectable (Fig. 8). Importantly, mRNA analysis confirmed high expression of p35 transcripts in DN thymocytes from rag1−/− lck-p35 transgenic mice. In conclusion, p35 expression cannot rescue maturation of rag1−/− thymocytes.

Discussion

The induction of apoptosis is a critical mechanism to delete non-functional or potentially harmful lymphocytes and to establish a functional immune system. During thymocyte development, apoptotic cell death clearly distinguishes negative from positive selection processes, suggesting that studies of apoptosis in this system should provide insights into the mechanisms of both negative selection and death by neglect. Caspase proteases have been found to play a major role in apoptosis, by cleaving and thereby inactivating proteins that are required for the structure and function of a cell, by inactivating endogenous inhibitors of apoptosis, or by activating proteins that subsequently contribute to the demise of the cell (reviewed in Refs. 6 and 7). However, thymocytes deficient for individual caspases do not manifest defects in TCR-mediated apoptosis (10–13, 15, 16, 18), except for caspase-9−/− thymocytes, which appeared less sensitive to this stimulus (17). It has therefore been assumed that the function of most caspases is redundant in thymocytes.

Several viruses encode proteins that inhibit caspase function (reviewed in Ref. 24). Most notably, the cowpox virus protein crmA and the baculovirus protein p35 function as pseudosubstrates, which become cleaved by caspas, but do not subsequently dissociate from the enzymes. CrmA, which inhibits caspases-1, -2, and -3, was shown to inhibit Fas-induced cell death, but not negative selection when overexpressed in transgenic thymocytes (40, 41). We decided to probe thymocyte apoptosis with p35, which inhibits caspases-1, -2, and -3, and subse-

p35 efficiently suppressed anti-Fas-induced cell death, but not negative selection when overexpressed in transgenic thymocytes.

Our results in vitro clearly show that the high p35 expression levels obtained in lck-p35 transgenic thymocytes effectively block the activity of caspase-3-related enzymes following apoptotic stimuli such as anti-Fas or anti-CD3 Abs, or the process initiated by prolonged cultivation of single cell thymocyte suspensions. However, this caspase inhibition only partially improved thymocyte survival. Consistent with previous studies in cell lines (42, 43), p35 efficiently suppressed anti-Fas-induced cell death, in which caspase-8 is known to play a primary, death-initiating role (reviewed in Ref. 44). In contrast, protection from anti-CD3-induced apoptosis and from spontaneous cell death was modest, implying the involvement of caspases that are not inhibitable by p35 and/or an additional, caspase-independent cell death mechanism under these conditions. In vivo, the number of thymocytes...
was unaffected by p35, indicating that death by neglect, which is thought to account for most of the observed cell death in the thymus (33), occurred normally.

*p35 expression does not rescue the early developmental block of rag1-deficient thymocytes*

To investigate the function of caspases during early thymic development, we analyzed the effect of p35 expression in *rag*-deficient mice. *Rag*<sup>−/−</sup> thymocytes lack a functional pre-TCR, which normally provides survival, expansion and differentiation signals at the DN stage. Signals that mimic pre-TCR stimulation fully restore thymocyte development from the DN to the DP stage (reviewed in Ref. 3). Surprisingly, the inactivation of the transcription factor p53, which plays a critical role in inducing apoptosis especially upon DNA damage, or the expression of a transgene encoding the anti-apoptotic molecule Bcl-2 enabled *rag*<sup>−/−</sup> thymocytes to distinguish CD25 expression and differentiate into intermediate CD4<sup>+</sup> or DP cells (45, 46). These experiments suggested that prolonged survival of DN thymocytes might permit them to continue an intrinsic differentiation program in the absence of pre-TCR signaling. However, the results for the Bcl-2 transgene were controversial (47).

Our results for *rag*<sup>−/−</sup> *lck*-p35 transgenic mice indicate that the developmental block in *rag*<sup>−/−</sup> thymocytes cannot be rescued by inhibiting caspase activity, and they underscore that death by neglect is not inhibited by p35 expression. These findings and the studies of Maraskovsky et al. (47) suggest that *rag*<sup>−/−</sup> thymocytes lack an essential differentiation signal from the pre-TCR that is also a prerequisite for survival and proliferation. It remains possible, however, that the results by Linette et al. (46), studying Bcl-2 overexpression, or by Jiang et al. (45) in p53-deficient thymocytes may reflect the importance of other, caspase-independent pathways in the cell death process.

**Caspase inhibition represses spontaneous cell death in vitro, but not peptide-specific negative selection in vitro and in vivo**

Evidence for caspase activation during anti-CD3 Ab or peptide-induced thymocyte deletion implied that caspases might be required for negative selection (8, 9). However, our results show that caspase inhibition by p35 or zVAD-FMK reduced spontaneous cell death of OT1 thymocytes in medium without peptide, but the relative dose response to deleting OVA peptide was indistinguishable from controls for either caspase inhibitor. Moreover, p35 expression did not prevent the deletion of DP thymocytes in the HY TCR model for negative selection by an endogenous Ag. Although thymocytes can be detected in male HY mice that have replaced the transgenic HY TCR α-chain by a rearranged endogenous Ag, although thymocytes can be detected in male HY mice that have replaced the transgenic HY TCR α-chain by a rearranged endogenous TCR α-chain of a different specificity and thereby escaped negative selection, the percentage of these cells is not increased by p35 (data not shown). In summary, these data indicate that negative selection is not prevented by the inhibition of caspase activity.

During the preparation of this manuscript, Izquierdo and colleagues (48) reported their studies of p35-expressing mouse lines that were generated using a similar transgenic construct. Their p35 protein expression levels appear comparable with our high-expressing mouse lines, as judged from signals on Western blots using p35 Abs from the same source. Consistent with our results, Izquierdo et al. (48) demonstrated that the p35 transgene did not perturb T lymphocyte development and homeostasis, but conferred resistance to anti-Fas-induced apoptosis. They also find less significant protection from CD3 cross-linking, dexamethasone, UV, and several additional inducers of apoptosis. A moderate degree of protection from spontaneous cell death by p35 in vitro could be dramatically improved by the addition of zVAD-FMK, indicating residual caspase activity in the p35 transgenic cells. However, their effort to investigate the effect of p35 on negative selection led them to a different conclusion. Apoptosis of DP cells was reduced in the F5 TCR transgenic model and upon exogenous superantigen stimulation in the presence of p35 when low to moderate Ag concentrations were applied. This result suggested to the authors that p35 could block negative selection. However, they report that p35 expression did not inhibit thymocyte deletion induced by high Ag concentrations and/or by chronic Ag treatment, nor was negative selection by endogenous superantigen blocked (48).

In view of these and our own results, we believe that the inhibition of caspase activity by p35 or by zVAD-FMK can reduce the spontaneous apoptosis of thymocytes, providing an advantage for survival even in the presence of deleting Ags. Nonetheless, the relative response to negative selection remains intact. In *lck*-p35 transgenic mice, persistent TCR activation by endogenous or chronic Ag stimulation probably causes the down-regulation of the *lck* proximal promoter and consequently reduced p35 expression levels, but even a persistently high concentration of zVAD-FMK only affects spontaneous cell death, not negative selection.

The relative improvement of thymocyte survival by zVAD-FMK, which we observe for every given peptide concentration, may be consistent (although less pronounced) with the results by Clayton et al. (8), in which zVAD-FMK protected DP cells in FTOC from apoptosis induced by a single dose of anti-CD3 Ab, dexamethasone, or specific Ag peptide. However, in the absence of a titration of the apoptotic stimuli in FTOC, it is difficult to judge whether these results reflect an intrinsic difference between FTOC and adult thymocytes.

**Implications of the results for caspase inhibition for the mechanism of TCR-induced apoptosis**

Caspase inhibitors block Fas-induced cell death and suppress morphological hallmarks of apoptosis. However, evidence is accumulating that a variety of apoptotic stimuli induces alterations in mitochondrial function that suffice to cause cell death in the absence of caspase activation (reviewed in Ref. 49). For example, the recently cloned mitochondrial apoptosis-inducing factor (AIF) induced the loss of plasma membrane polarity, nuclear chromatin condensation, and DNA degradation to large (~50-kb) fragments despite caspase inhibition (50). Moreover, the mitochondrial release of cytochrome c, an important electron carrier in the respiratory chain, can be observed upon UV or staurosporin-induced apoptosis even if caspase activity is blocked (51). The resulting disruption of the mitochondrial respiratory chain may, per se, be sufficient to cause cell death. Very recent studies indicate that activation-induced cell death of peripheral T cells cannot be prevented by caspase inhibitors, while protection of these cells from reactive oxygen species inhibited apoptotic changes, e.g., the loss of the mitochondrial transmembrane potential (52).

In summary, we have shown that the caspase inhibitors p35 and zVAD-FMK may delay, but do not block the TCR-induced loss of the mitochondrial transmembrane potential and subsequent apoptosis. Although we cannot formally exclude residual caspase activity even under conditions when no enzyme activity was measurable, the caspase-dependent morphologic characteristics of apoptosis, such as plasma membrane depolarization and oligonucleosomal DNA fragmentation, were efficiently suppressed. Although caspase activation may normally contribute to apoptosis in negative selection and death by neglect, our results clearly indicate that other caspase-independent pathways are sufficient to cause thymocyte death upon TCR stimulation and to eliminate nonfunctional or autoreactive T cells.
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