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Fas/Fas Ligand Signaling During Gestational T Cell Development

Martin Fleck,*† Tong Zhou,† Toru Tatsuta,†‡ Pingar Yang,† Zheng Wang,† and John D. Mountz‡†

Most thymocytes express high levels of Fas Ag (Apo-1/CD95); however, the role of Fas/Fas ligand-mediated apoptosis in thymocyte development remains unclear. During gestational development of thymocytes in C57BL/6(B6) +/+ mice, the highest levels of Fas ligand mRNA and Fas ligand protein expression were detected at gestational day (GD) 15, and there was a ninefold decrease in Fas ligand mRNA expression between GD 15 and 17 accompanied by a sixfold increase in Fas mRNA. Apoptotic thymocytes were first detected in the medulla at GD 15, and increasing numbers of cortical clusters and scattered, single apoptotic cells were present on GD 16 and 17. Thus, early apoptosis correlated with high expression of Fas ligand. High levels of Fas ligand mRNA were maintained throughout gestational development in thymocytes of Fas-deficient B6-lpr/lpr mice, but cortical clusters and scattered apoptotic cells were decreased relative to B6 +/+ mice before GD 17. Kinetic analysis of fetal thymic organ cultures treated with anti-Fas Ab demonstrated that thymocytes become sensitive to Fas-mediated apoptosis during the transition from the CD4+CD8− to the CD4+CD8+ phenotype. More mature CD4+CD8+ thymocytes and CD4+ and CD8+ thymocytes became resistant to Fas-mediated apoptosis after GD 17, despite high expression of Fas. However, low avidity engagement of the TCR on Fas-sensitive CD4+CD8+ thymocytes before GD 17 induced resistance to Fas-mediated apoptosis. The present results indicate that Fas plays a critical role in mediating apoptosis during early gestational thymocyte development and that thymocytes that receive a survival signal through TCR/CD3 become resistant to Fas-mediated apoptosis. The Journal of Immunology, 1998, 160: 3766–3775.

Fas (Apo-1/CD95) is a member of the TNF/nerve growth factor superfamily that can directly transduce an apoptotic death signal on trimerization with Fas ligand (CD95L) or Fas-specific Abs (1, 2). Fas is expressed on the surface of various cell types, including T cells, B cells, and macrophages, as well as cells of the liver, spleen, lung, testis, heart, brain, and intestine (1, 3). Fas ligand mRNA is present in immune cells, including T cells, B cells, macrophages, and NK cells and is expressed in non-immune sites, including the testis, eye, intestine, kidney, and lung (4–6).

Previous studies have indicated that Fas/Fas ligand-mediated apoptosis is critical to the maintenance of tolerance of peripheral T cells by mediating activation-induced cell death in an autocrine fashion (7–11), and that activation-induced cell death is significantly impaired in Fas-deficient lpr/lpr mice (12). It has been demonstrated that neonatal thymectomy inhibits the development of lymphoproliferation and prevents autoimmune disease in lpr/lpr mice, suggesting that defective Fas-mediated apoptosis of thymocytes is involved in the pathogenesis of the lpr disease phenotype (13–15). The data concerning the significance of Fas-mediated apoptosis during negative selection are conflicting. Several studies have indicated that the Fas/Fas ligand interaction is not involved in negative selection (11, 16–22), although modulation of apoptosis by Fas during negative selection of thymocytes has been described by other investigators (23–25). In addition, the extent of expression and function of Fas ligand in the thymus is controversial (4, 5, 26). Defective selection of CD4+ Dδ/HY T cells in the thymus of female mice has been reported by members of this research team (18, 27) and other investigators (28, 29), indicating modulation of apoptosis by Fas during the positive selection of thymocytes.

Although >90% of thymocytes and almost 100% of CD4+CD8− thymocytes express abundant Fas Ag on the cell surface, anti-Fas Ab and soluble Fas ligand induce apoptosis predominantly in the CD4+CD8+ thymocyte subpopulation, whereas CD4+ and CD8+ thymocytes are less susceptible and CD4+CD8− thymocytes are resistant (30–35). To accurately correlate the timing of susceptibility to Fas-mediated apoptosis with the expression of Fas and Fas ligand in thymocytes, we analyzed apoptosis during fetal thymic development of B6 +/+ and B6-lpr/lpr mice in vivo and examined Fas-mediated apoptosis during fetal thymic organ culture (FTOC) of B6 +/+ thymocytes in vitro. Our data support the concept that Fas-mediated apoptosis occurs during early T cell maturation before expression of the TCR, and that TCR/CD3 signaling prevents Fas-mediated apoptosis in FTOC. Therefore, the Fas/Fas ligand interaction might lead to the elimination of “neglected” thymocytes and of thymocytes that do not receive a survival signal through TCR/CD3.

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3 Abbreviations used in this paper: FTOC, fetal thymic organ culture; 7-AAD, 7-aminocoumarine D; TdT, terminal deoxynucleotidyl transferase; TUNEL, JUPP nick end labeling.
Materials and Methods

Animals

Over an 8-h period, 10- to 14-wk-old female C57BL/6+/+ and C57BL/6-lpr/lpr mice (The Jackson Laboratory, Bar Harbor, MA) were mated with C57BL/6+/+ or C57BL/6-lpr/lpr male mice, respectively. A visible vaginal plug demonstrated successful mating and was used to establish day 1 of gestation. At days 15 to 18 of gestational age, mice were sacrificed and fetal thymi were obtained. 

Abs and reagents

Anti-Fas (clone Jo2), anti-Fas ligand (clone Kay-10) anti-CD4 (clone GK1.5), anti-CD8 (clone 53-6.7), anti-TCR (clone H57), anti-CD3 (clone 145-2C11) mAbs, and hamster IgG control Ab were purchased from Pharmingen (San Diego, CA) in either purified or conjugated form. Soluble Fas was prepared as previously described (36).

Fetal thymic organ culture

FTOC was conducted using thymi from fetal B6+/+ mice at day 16 of gestational age. Thymi were obtained under sterile conditions and two to three thymic lobes were placed on a nitrocellulose membrane (Millipore, Bedford, MA) supported by GelGlobe (Upjohn, Chicago, IL) in 24-well tissue culture plates that had been equilibrated with 1.2 ml of complete DMEM medium. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂. At the indicated timepoints, purified mAb specific for Fas (1 μg/ml), purified mAb specific for TCR (0.1-10 μg/ml), or control Ab were added to the cultures. Thymocytes were harvested 12 h after addition of Ab or at the end of the 6-day culture period and analyzed by flow cytometry. At each culture time point, thymi from three separate cultures were removed and analyzed. The error represents the mean ± SEM.

Semiquantitative PCR analysis for Fas and Fas ligand mRNA

Total RNA was isolated from thymocytes of B6+/+ and B6-lpr/lpr mice at days 15 through 17 of gestational age using the guanidine isothiocyanate/acid phenol method. In all, 10 μg of total RNA were subjected to first strand cDNA synthesis in a total vol of 66 μl using the First-Strand cDNA Synthesis kit (Pharmacia-P-L Biochemicals, Milwaukee, WI). The primers used to amplify murine Fas were 5’ primer (sequence: CGCTGTTTCT CCTGCCGTGCA) and 3’ primer (sequence: AGAGITGTGGTGAC CCCCAT). The primers used to amplify murine Fas ligand were 5’ primer (sequence: GACCTGACAGACTACCTCAT) and 3’ primer (sequence: GGAAITCCTCGTGGCCCATGAT). The primers used to amplify murine β-actin were 5’ primer (sequence: GACCTGACAGACTACCTCAT) and 3’ primer (sequence: AGACAGCACTGTTGCTGGCAT). The amplification was performed in a final 50-μl reaction vol containing 1 × reaction buffer (Promega, Madison, WI), 1.5 mM of MgCl₂, 200 μM of dNTPs, 1 μM of each primer, and 2.5 U of Taq DNA polymerase (Promega) using a PerkinElmer Gene Amp PCR System 9600 (Norwalk, CT). Each cycle consisted of denaturation at 94°C for 1 min, annealing at 60°C for 1.5 min, and extension at 72°C for 1 min. For semiquantitative analysis of expression of PCR products for Fas and Fas ligand, various numbers of PCR cycles were performed followed by extension for 10 min. The RT-PCR-derived DNA fragments were subjected to electrophoresis on a 0.5% agarose gel. Gels were blotted and hybridized to a [32P]dCTP (Amersham Life Science, Arlington Heights, IL)-labeled cDNA for β-actin (control), Fas, or Fas ligand to verify specificity of each product. The amount of hybridized probe was quantified by the intensity (cpm) of each specific PCR product on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The intensity of the specific hybridization signal was plotted against the number of PCR cycles to ensure that comparisons were conducted in the linear range. The ratio of Fas or Fas ligand expression was determined relative to β-actin gene expression for four samples. The error represents the mean ± SEM. Significant differences in the expression patterns are marked by asterisks (p < 0.05, Student’s t test).

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL)

At least three thymi were obtained from each mouse strain at gestational days 15, 16, and 17 and analyzed. The method was modified slightly from that described previously (37). Briefly, snap-frozen sections of fetal thymic tissue were fixed in 10% formalin for 30 min. After thorough washing with deionized water, the slides were subjected to proteinase K digestion (10 μg/ml at room temperature for 7 min), and then incubated with freshly prepared TdT reaction mix (0.4 U/μl TdT, 10 nM digoxigenin modified-dUTP, and TdT buffer, which were purchased from Boehringer Mannheim, Indianapolis, IN) at 37°C for 60 min. The incorporated digoxigenin-dUTP was detected by incubation with alkaline phosphatase-conjugated anti-digoxigenin Ab at room temperature for 60 min, and positive reactions were revealed using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate. The percentage of apoptotic cells was estimated using a microscopic grid. At least 10 random independent fields of view on two to four serial sections from each thymus were analyzed to determine the percentage of apoptotic cells.

Immunohistochemical analysis

Immunohistochemical staining was conducted using a direct staining method. Briefly, sections from snap-frozen tissue were fixed in ice-cold acetone for 10 min. After washing, slides were incubated with 5% horse serum in PBS for 20 min and then stained with phycoerythrin-conjugated anti-Fas ligand Ab (PharMingen) for 30 min at room temperature. Sections were analyzed using a fluorescence microscope (magnification ×320).

Transmission electron microscopic analysis of apoptotic cells

Fetal thymic tissue was first fixed in 1% glutaraldehyde in 0.1% cacodylate buffer and postfixed with 2% phosphate-buffered osmium tetroxide. After routine dehydration with alcohol and propylene oxide, the tissue was embedded in Spurr low viscosity medium (38). Ultrathin sections (70–100 nm) were contrasted with uranyl acetate and lead citrate and analyzed using a Philips 300 electron microscope (Phillips, Mahwah, NJ).

Results

Detection of apoptosis in situ during gestational thymocyte development of B6+/+ and B6-lpr/lpr mice

Fetal thymi of B6+/+ and B6-lpr/lpr mice were obtained at days 15 to 17 of gestational age. At least three different pregnant mice were sacrificed at each time point. The number and distribution of apoptotic thymocytes was detected in situ by TDT labeling of DNA strand breaks (TUNEL) in three to five thymic lobes at each time point (Fig. 1). Thymocytes undergoing apoptosis were observed at day 15 of gestational age in B6+/+ mice (Fig. 1). The apoptotic thymocytes appeared to be confined to scattered individual thymocytes (Fig. 2A), mainly located in the more mature medullary region of the thymus (41). In contrast, only a few or no apoptotic thymocytes could be detected in thymic tissue of B6-lpr/lpr mice obtained at the same gestational age. On gestational day 16, clusters of apoptotic cells were present in the cortex and the region of the corticomedullary junction in B6+/+ mice, whereas a few single apoptotic thymocytes were detected in B6-lpr/lpr mice. Larger clusters of apoptotic cortical thymocytes and increased numbers of scattered single apoptotic cells were found at day 17 of gestational age in thymic tissue of both B6+/+ mice and B6-lpr/lpr mice (Figs. 1 and 2B). The number of apoptotic thymocytes at each time point estimated by cell counting in representative regions is shown in Figure 3.

Expression of Fas and Fas ligand mRNA in gestational thymocytes of B6+/+ and B6-lpr/lpr mice

There was a sixfold increase in Fas mRNA expression in fetal thymic tissue of B6+/+ mice from gestational days 15 to 17 as estimated by semiquantitative PCR (Fig. 4A). In contrast, Fas
mRNA expression remained at low levels in B6-lpr/lpr mice (Fig. 4B) and was approximately 1 to 2% of that observed in B6 +/+ mice at gestational day 17, which is consistent with our previous report that the levels of Fas expression in adult B6-lpr/lpr mice are reduced to 1 to 5% of those in B6 +/+ mice (42). The expression of Fas ligand mRNA was highest at gestational day 15, followed by...
by a ninefold decreased expression by gestational day 17 (Fig. 4C). Thus, in B6 +/+ mice, there was an inverse correlation between the expression of Fas and Fas ligand during gestational days 15 to 17. Thymocytes of B6-lpr/lpr mice continued to express high amounts of Fas ligand mRNA throughout (Fig. 4D), with the expression of Fas ligand mRNA in B6 +/+ mice at day 17 of gestational age being approximately 8% of that observed in B6-lpr/lpr mice.

Increased expression of Fas ligand at day 15 of gestational age (Fig. 4E) compared with gestational day 18 (Fig. 4F) was also determined by immunohistochemical staining. There was a focal and scattered distribution of Fas ligand in the cortex and medulla during fetal thymic development, which correlated with the foci of apoptotic thymocytes.

Anti-Fas Ab-induced apoptosis in thymocytes in situ during FTOC

The data thus far indicate that Fas-mediated thymocyte apoptosis occurs on gestational days 15 and 16. To determine the location and frequency of Fas apoptosis-sensitive thymocytes at gestational day 16, FTOC was conducted for 12 h in the presence or absence of anti-Fas Ab. Approximately 7% of the B6 +/+ thymocytes cultured with control Ab exhibited characteristic DNA strand breaks, as indicated by TDT labeling, compared with approximately 30% of thymocytes in the anti-Fas Ab-treated FTOC (Fig. 5A). Electron microscopic analysis confirmed that thymocytes were undergoing a typical apoptosis process as a consequence of the anti-Fas Ab treatment, demonstrated by membrane blebbing, cell shrinkage, and nuclear condensation (Fig. 5B). These results indicate that on gestational day 16, there are numerous foci of Fas apoptosis-sensitive thymocytes throughout the entire thymus.

Effect of anti-Fas Ab on thymocyte maturation in FTOC

Previous reports by us and other investigators indicated that immature CD4+CD8+ thymocytes that express low to intermediate levels of CD3 complexes represent the major thymocyte population that is deleted by Fas-mediated apoptosis (25, 33, 35, 43). To determine the effect of anti-Fas Ab treatment on thymocytes at an early developmental stage, we added anti-Fas Ab at the indicated time points of FTOC and then analyzed thymus 12 h later for anti-Fas-induced apoptosis using 7-AAD, CD4, and CD8 labeling as described (Fig. 6). There was a gradual increase in the total number of 7-AAD−CD4+CD8+ thymocytes from ~20 × 10^3 to 50 × 10^3 cells per thymic lobe during FTOC days 16 to 18 followed by a decrease during FTOC days 19 to 21. Incubation with anti-Fas Ab did not affect the initial increase or later decrease in the number of these CD4+CD8+ thymocytes during the 6 days of FTOC compared with the control FTOC. Initially, a population of CD4+CD8+ thymocytes was present that was highly susceptible to anti-Fas Ab-induced apoptosis (days 16–17). By days 18 to 19 of FTOC, most thymocytes had differentiated into the CD4−CD8+ phenotype, and were now resistant to induction of apoptosis after incubation with anti-Fas Ab for 12 h. By days 20 to 21 of FTOC, most of the CD4+CD8+ thymocytes were resistant to anti-Fas-induced apoptosis. Treatment with anti-Fas Ab had no effect on apoptosis of CD4− or CD8+ thymocytes. These results indicate that newly generated CD4+CD8+ thymocytes are highly sensitive to Fas-mediated apoptosis; however, by day 18 of FTOC, most CD4+CD8+ thymocytes are no longer susceptible to Fas-mediated apoptosis.

Development of anti-Fas apoptosis-resistant thymocytes in FTOC

The presence of anti-Fas Ab at gestational day 16 completely suppressed the development of thymocytes during 6 days of FTOC (Fig. 7A). In contrast, there was a progressive development of CD4−CD8+ thymocytes followed by development of CD4+ and CD8+ positive thymocytes in the presence of control Ab. Inhibition of thymocyte development was due to Fas-mediated apoptosis, since addition of soluble Fas (1 µg/ml), which can neutralize anti-Fas Ab, also blocked the ability of anti-Fas Ab to induce apoptosis.

To further investigate the developmental time point when thymocytes are sensitive to Fas-mediated apoptosis during the maturation of CD4−CD8+ thymocytes, anti-Fas Ab was added at different days during FTOC, and the thymi were analyzed 6 days after initiation of the culture on day 22 of FTOC. The addition of anti-Fas Ab to FTOC at day 16 of gestational age completely prevented development of thymocytes during the following 6 days of culture (Fig. 7, B and C). Most of the thymocytes remaining after 6 days of culture with anti-Fas Ab were CD4−CD8+ and Fas−. In contrast, there was a progressive increase in the survival of thymocytes after 6 days in culture if the addition of anti-Fas Ab was delayed until days 17 to 20 of fetal age. Thus, a transition in Fas sensitivity occurred before or during the progression of thymocytes from the CD4−CD8+ to the CD4−CD8+ phenotype corresponding to day 17 of FTOC. By day 18 of FTOC, some of the thymocytes had differentiated into anti-Fas apoptosis-resistant thymocytes, and 14% of the thymocytes survived to the end of culture. Addition of anti-Fas Ab at days 20 to 21 did not significantly inhibit development of these thymocytes, indicating that most of these Fas− thymocytes have become Fas-apoptosis resistant. This resistance to Fas-mediated apoptosis was not attributable to decreased Fas expression in that, by day 20, approximately 82% of the thymocytes exhibited high expression of Fas Ag but were totally refractory to Fas Ab-induced apoptosis even after incubation with Fas-specific Ab for 48 h (treated on day 20 and assayed on day 22) (Fig. 7C).

Engagement of TCR rescued double positive thymocytes from anti-Fas-mediated apoptosis

The results thus far demonstrate that Fas-mediated apoptosis in the thymus is limited to newly generated, early stage CD4+CD8+ thymocytes. It has been shown that, in thymocyte development, high avidity binding of the TCR/CD3 complex leads to clonal deletion,
whereas low avidity binding leads to survival (44–48). To determine whether TCR/CD3 signaling might be responsible for the transition in Fas sensitivity that occurs during the CD4⁺CD8⁺ stage of thymic development, we performed FTOC that was initiated in the presence of anti-Fas Abs, and in the presence or absence of different concentrations of anti-TCR Abs (Fig. 8). Abs were added at the time of the initiation of culture and the results were analyzed after 6 days of culture. Treatment with anti-Fas Ab (1 μg/ml) alone prevented the development of CD4⁺CD8⁻, CD4⁺, and CD8⁺ single positive thymocytes and reduced the total number of thymocytes to 12% of the medium control (Fig. 8A). Treatment with anti-TCR Ab (10 μg/ml) alone reduced the total

FIGURE 4. Expression of Fas, Fas ligand, and β-actin in fetal thymocytes determined by PCR. Semiquantitative analysis of Fas and Fas ligand mRNA expression in B6 +/- and B6-lpr/lpr gestational thymus. Semiquantitative PCR was conducted using Fas, Fas ligand, and β-actin-specific primers as described. After amplification, DNA was blotted and hybridized to probes specific for mouse Fas, mouse Fas ligand, or mouse β-actin, and the intensity of the PCR product was quantitated using a PhosphorImager. The results are representative of those obtained using at least five fetal thymi on three different gestational days. The intensity (log cpm) of each specific PCR product is normalized to the value for the β-actin PCR product. Significant differences in the expression patterns are marked by asterisks (p < 0.05, Student’s t test). A, Expression of Fas mRNA in B6 +/- mice. B, Expression of Fas mRNA in B6-lpr/lpr mice. C, Expression of Fas ligand mRNA in B6 +/- mice. D, Expression of Fas ligand mRNA in B6-lpr/lpr mice. E, Immunohistochemical staining of Fas ligand expression in fetal thymic tissue of B6 +/- mice at gestational day 15 (original magnification, ×320). F, Expression of Fas ligand in fetal thymic tissue of B6 +/- mice at gestational day 18 determined by immunohistochemistry (original magnification, ×320).
number of thymocytes to approximately 30% of the medium control, and reduced the number of CD4^+CD8^- thymocytes by approximately 60%, presumably due to TCR/CD3 signaling-mediated deletion (49). On treatment with both anti-Fas and anti-TCR Ab, the depletion of CD4^+CD8^- thymocytes was decreased compared with that observed in the cultures treated with anti-Fas Ab alone, indicating that Fas-mediated apoptosis was inhibited by the addition of anti-TCR Ab (Fig. 8). Lower concentrations of anti-TCR Ab (0.1 μg/ml) resulted in decreased TCR-induced deletion, representing less inhibition of Fas-mediated apoptosis. Thus, TCR engagement provides a survival signal for immature CD4^+CD8^- thymocytes by rendering the thymocytes insensitive to Fas-mediated apoptosis.

**FIGURE 5.** Apoptosis in situ after treatment with anti-Fas Ab during FTOC. On gestational day 16, FTOC was established and treated with control Ab or anti-Fas Ab (1 μg/ml) for 12 h. Thymus was examined using TUNEL (A) and by electron microscopy analysis (B). The percentage of the thymocytes undergoing apoptosis was determined by quantitating the percent of thymocytes undergoing apoptosis relative to the total number of thymocytes as determined by TUNEL staining.

**FIGURE 6.** Effect of anti-Fas Ab treatment on the development of thymocytes in FTOC. Thymi were obtained from B6 +/- mice at day 16 of gestational age and placed in FTOC. Anti-Fas Ab (1 μg/ml) or control Ab (hamster IgG) were added at initiation of culture (day 16) or at the indicated timepoints until day 21. Thymi were harvested 12 h after addition of anti-Fas Ab and analyzed by flow cytometry after labeling with 7-AAD and anti-CD4 and anti-CD8 Ab (control: open bars, anti-Fas Ab: hashed bars). Values represent the average of three cultures. The SE is indicated.
apoptosis. In contrast, thymocytes that do not receive an appropriate TCR engagement signal during early development remain sensitive to Fas-mediated apoptosis.

Discussion

The present study demonstrates that both Fas and Fas ligand are expressed in the thymus at an early stage of thymocyte development. Fas ligand mRNA was detected at high levels at gestational day 15 and was down-modulated on the following days in B6 mice, whereas B6-lpr/lpr mice continued to express high levels of Fas ligand. The expression pattern of Fas ligand in thymic tissue of B6 mice could be confirmed by immunohistochemical analysis using an anti-Fas ligand Ab. At this early stage of T cell development, before expression of CD3 or TCR is completed, Fas ligand may be expressed either as a membrane-bound molecule on T cells or by thymic stromal cells and may act as a signaling molecule for further thymocyte development. Semiquantitative PCR analysis revealed an approximately sixfold increase of Fas expression in B6 mice during gestational days 15 to 17. This is in agreement with a previous report of Fas mRNA expression in gestational thymocytes at day 16.5 (50). The inverse relationship between the patterns of expression of Fas and Fas ligand in B6 mice and the rapid and dramatic changes in their expression emphasize the physiologic importance of this pathway during gestational thymocyte development.

Histologic examination of FTOC cultured in the presence of anti-Fas Ab at gestational day 16 demonstrated functional Fas expression in approximately 30% of the thymocytes, as these cells...
underwent apoptosis and apoptotic thymocytes were scattered throughout the thymus. Moreover, kinetic analysis of Fas-mediated apoptosis during FTOC of B6 +/+ mice indicated that immature, newly generated CD4⁺CD8⁻ thymocytes were highly sensitive to Fas-mediated apoptosis. However, a transition in Fas sensitivity occurred before or during the progression of thymocytes from CD4⁻CD8⁻ to CD4⁺CD8⁻ phenotype at gestational day 17. This finding was further substantiated by the subsequent development to more mature CD4⁺CD8⁻, CD4⁺, and CD8⁺ T cells, which were resistant to anti-Fas Ab-induced apoptosis despite high Fas Ag expression. A similar transition from Fas apoptosis sensitivity to resistance occurs during maturation of CD4⁺CD8⁻ thymocytes in vivo since all CD4⁺CD8⁻ thymocytes express Fas, but only newly generated CD4⁺CD8⁻ thymocytes are sensitive to Fas apoptosis. A comparable phenomenon of Fas-apoptosis resistance after Ag receptor engagement has been observed in B cells (51). The clear separation between early stage

Fas-sensitive and late stage Fas-resistant CD4⁺CD8⁺ thymocytes in the present experiments may be also due to the analysis of thymocyte development using in vitro organ culture. This allowed the analysis of CD4⁺CD8⁺ thymocyte development without the complicating effect of new thymic emigrants from the bone marrow to replenish the pool of CD4⁺CD8⁻ thymocytes and early stage CD4⁺CD8⁻ thymocytes, resulting in a gradually diminished rate of transition of CD4⁺CD8⁻ thymocytes to CD4⁺CD8⁺ thymocytes. Depletion of the pool of CD4⁺CD8⁻ thymocytes may account for the decrease in CD4⁺CD8⁻ thymocytes at late time points as well as the lack of Fas-mediated apoptosis effect on CD4⁺CD8⁻ thymocytes at the end of the culture period. Our interpretation of these data is that there is a window of susceptibility during thymocyte development from gestational days 15 to 17 during which induction of apoptosis by Fas signaling can result in substantial apoptosis.

Previous studies have demonstrated that rearrangement of the TCRβ locus begins as the cells differentiate to the CD25⁺ stage and that the production of the β-chain of the TCR on double negative thymocytes is required for prepositive selection and further maturation (48, 49, 52, 53). It has been proposed that only a small number of CD4⁺CD8⁻ thymocytes with productive TCRβ rearrangement are selected for further maturation characterized by the loss of CD25 expression, subsequent expression of CD4 and CD8 coreceptors, and rapid proliferation, whereas large numbers of CD4⁺CD8⁻ thymocytes with nonproductive rearrangement of the TCRβ gene die by apoptosis (44–49). Alternatively, it has been proposed that the number of apoptotic thymocytes is low in early gestational development as only a few apoptotic cells have been detected in fetal thymic tissue (54). Using the TUNEL method for detection, we found apoptotic thymocytes at gestational day 15 in B6 +/+ mice and increasing numbers of apoptotic thymocytes on the following days. Using this technique, we observed significantly lower levels of apoptosis in B6-lpr/lpr mice compared with B6 +/+ mice only on gestational days 15 and 16, but not on gestational day 17. These results are consistent with our previous report of an increase in the percentage of the CD4⁺CD8⁻ IL-2Rα⁻ subpopulation of thymocytes in Fas mutant lpr/lpr mice (43), and that the accumulation of peripheral CD4⁺CD8⁻ B220⁺ T cells might result from a Fas-related apoptosis defect in early thymocyte development (55).

The finding of apoptotic cells at gestational day 15 is novel and indicates that Fas plays a role in deletion of thymocytes at a very early stage of thymocyte development. These thymocytes do not express CD3 or TCR, so the basis for the selection is unknown. Notably, the cells undergoing apoptosis at gestational day 15 were located in the thymic medulla, whereas apoptosis at later stages was detected mainly in the cortex and the corticomediulary junction. This difference in anatomic sites may reflect activation of different apoptosis pathways (41). Previous reports have demonstrated that early thymocytes are dependent on several cytokines, including IL-1, IL-2, IL-7, and TNF-α (56–59), and the prevention of apoptosis by endogenous steroids has been described for thymocytes at an early stage of FTOC (60). Furthermore, deprivation of cytokines has been demonstrated to render activated T cells susceptible to Fas-mediated apoptosis (61, 62). Therefore, it appears possible that cytokine deprivation or lack of endogenous steroids may contribute to the induction of Fas-mediated apoptosis in B6 +/+ mice on gestational day 15.

The grouping of apoptotic thymocytes in cortical clusters at gestational days 16 and 17 suggests the elimination of thymocytes that developed from a single precursor clone. Such clonal elimination may be the result of a prepositive selection event in the thymus that

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**FIGURE 8.** Induction of thymocytes resistant to anti-Fas apoptosis by TCR signaling. Thymi were obtained from B6 +/+ mice on day 16 of gestational age and placed in FTOC in complete medium containing control Ab, different concentrations of purified mAb specific for TCR (0.1 μg/ml-10 μg/ml), or purified hamster mAb specific for Fas (1 μg/ml), or both. Six days later, the thymi were harvested. A, Flow cytometric analysis was performed as described, and the percent of thymocytes expressing CD4 and CD8 is indicated. B, The dose response of the thymocytes to different concentrations of mAb specific for TCR and mAb specific for Fas was estimated using the same procedure except that different doses of Abs specific for TCR were used in the absence (white bars) and presence (hashed bars) of 1 μg/ml of mAb specific for Fas.
invokes apoptosis after 1 to 2 days of thymic development but does not require functional TCR/CD3 expression (52).

A synergistic effect of Fas and TCR/CD3 signaling resulting in apoptosis has been described in late CD4+CD8+, CD4+ tert, and adult αβ thymocytes using high concentrations of TCR/CD3 Abs (63, 64). The present results indicating decreased apoptosis in the presence of anti-TCR plus anti-Fas Ab in thymocytes do not contradict these findings, as the antagonistic effect was observed in a different experimental system using low concentrations of anti-TCR Abs in cultures of intact thymus and at an earlier stage of development. One possible mechanism for the decreased apoptosis is that TCR engagement in early thymocytes might activate downstream inhibitors of Fas-mediated apoptosis, including Bcl-2 and Bcl-x (65). We propose that inhibition of Fas-mediated apoptosis by low affinity anti-TCR signaling on CD3+CD8+CD4+ thymocytes might be one event associated with survival of thymocytes during positive selection.

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