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Fas Apoptosis Inhibitory Molecule Regulates T Cell Receptor-mediated Apoptosis of Thymocytes by Modulating Akt Activation and Nur77 Expression*

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Fas apoptosis inhibitory molecule (FAIM) has been demonstrated to confer resistance to Fas-induced apoptosis of lymphocytes and hepatocytes *in vitro* and *in vivo*. Here, we show that FAIM is up-regulated in thymocytes upon T cell receptor (TCR) engagement and that *faim*$^{-/-}$ thymocytes are highly susceptible to TCR-mediated apoptosis with increased activation of caspase-8 and -9. Furthermore, injection of anti-CD3 antibodies leads to augmented depletion of CD4$^+$CD8$^+$ T cells in the thymus of *faim*$^{-/-}$ mice compared with wild-type control, suggesting that FAIM plays a role in thymocyte apoptosis. Cross-linking of the TCR on *faim*$^{-/-}$ thymocytes leads to an elevated protein level of the orphan nuclear receptor Nur77, which plays a role in thymocyte apoptosis. Interestingly, in the absence of FAIM, there are reduced ubiquitination and degradation of the Nur77 protein. *Faim*$^{-/-}$ thymocytes also exhibit a defective TCR-induced activation of Akt whose activity we now show is required for Nur77 ubiquitination. Further analyses utilizing FAIM-deficient primary thymocytes and FAIM-overexpressing DO-11.10 T cells indicate that FAIM acts upstream of Akt during TCR signaling and influences the localization of Akt to lipid rafts, hence affecting its activation. Taken together, our study defined a TCR-induced FAIM/Akt/Nur77 signaling axis that is critical for modulating the apoptosis of developing thymocytes.

Apoptosis plays an important role in T cell biology as it eliminates excess antigen-specific T cells after an immune response to maintain lymphocyte homeostasis in periphery tissues (1, 2). This is mainly achieved via Fas-FasL interaction on T cells, which activates downstream death-inducing molecules and caspases that ultimately lead to the death of T lymphocytes (2–4).

Apoptosis also plays an important role in T cell maturation as it ensures that developing nonfunctional or autoreactive T cells are eliminated and prevented from seeding the peripheral immune system (1, 2). However, in this case, apoptosis is triggered by the engagement of the T cell receptor (TCR)[3] on thymocytes. Developing T cells progress through discrete stages of differentiation in the thymus. In the early stage, CD4$^-$CD8$^-$ precursors rearrange the TCR genes to generate a repertoire of CD4$^+$CD8$^+$ thymocytes with diverse specificities (5). These CD4/CD8 double-positive (DP) thymocytes subsequently undergo positive and negative selection before maturating into CD4$^+$ or CD8$^+$ single-positive thymocytes (6). During the selection process, DP thymocytes with TCR that fail to recognize the peptide-major histocompatibility complex on thymic epithelial cells would die of neglect, whereas those with TCR that bind the peptide-major histocompatibility complex with low affinity or avidity would mature into single-positive thymocytes. On the other hand, DP thymocytes that recognize the peptide-major histocompatibility complex on thymic antigen-presenting cells with high affinity or avidity are negatively selected and purged by apoptosis. Positive and negative selection of thymocytes are therefore important checkpoints in T lymphopoiesis as they ensure the establishment of a functional T cell repertoire capable of recognizing the peptide-major histocompatibility complex and are yet largely devoid of autoreactivity (7, 8).

Whether a developing T cell proceeds in differentiation or undergoes cell death depends very much on the quality and strength of its TCR signaling. TCR engagement is known to activate a number of intracellular signaling molecules such as tyrosine kinases, phosphatases, phospholipases, and adaptor proteins (9, 10). Some of these molecules are known to play a role in thymocyte differentiation. Mice lacking ζ-chain associated protein kinase (ZAP70) (11), phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (12), Grb2-related adaptor downstream of Shc (GADS) (13) and Grb2 (14), or the guanine nucleotide exchange factor Vav (15) have defective negative selection of thymocytes.

In addition to molecules specifically involved in TCR signaling, molecules implicated in canonical apoptosis pathways also play a role in TCR-mediated apoptosis of thymocytes. For example, caspase-3 is known to be activated by TCR signaling in thymocytes (16, 17). Deficiency in Bak and Bax, which are proapoptotic molecules of the Bcl-2 family and regulators of the intrinsic apoptotic pathway, also affects TCR-mediated apoptosis and interferes with negative selection of thymocytes (18). Another unique protein known to be involved in TCR-induced apoptosis of the thymocyte is the orphan steroid nuclear receptor Nur77 (19, 20), which regulates the transcription of apoptotic genes such as FasL, tumor necrosis factor-related apoptosis-inducing ligand, and NDG1 and

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[3] The abbreviations used are: TCR, T cell receptor; FAIM, Fas apoptosis inhibitory molecule; DP, double-positive; ERK, extracellular signal-regulated kinase; WT, wild-type; PBS, phosphate-buffered saline.

-2 (Nur77 downstream gene 1 and 2) (21). Indeed, overexpression of Nur77 in transgenic mice leads to massive apoptosis of thymocytes (22, 23).

However, the molecular mechanisms underlying TCR-mediated apoptosis of thymocytes are still not completely understood, and it is likely that there are other unappreciated players in this process. In this study, we examined the role of Fas apoptosis inhibitory molecule (FAIM) in TCR-mediated apoptosis of thymocytes. FAIM was cloned as a molecule that antagonized Fas-triggered apoptosis in B lymphocytes *in vitro* (24) and has also been shown to be involved in NF-κB and Ras-ERK activation in neurons (25). It is highly conserved and widely expressed, although it has no homology to other known proteins. In addition to being abundant in spleen, FAIM was also highly expressed in thymus (26), implying that FAIM could play a role in some aspects of thymocyte physiology.

We had previously inactivated *faim* in mice and found that FAIM regulates Fas-triggered apoptosis of lymphocytes and hepatocytes by modulating cellular FLICE-like inhibitory protein expression, hence affecting the binding of caspase-8 to Fas (27). Here, we examined the role of FAIM in TCR-triggered apoptosis of thymocytes and demonstrated that FAIM was up-regulated by TCR engagement and that it modulated TCR-induced apoptosis of thymocytes. In the absence of FAIM, TCR-induced activation of caspase-8, -9, and -3 was enhanced. FAIM deficiency also resulted in elevated levels of apoptotic molecules such as Nur77, Bak, and Bax, that had been shown to be involved in thymocyte apoptosis. Finally, we showed that FAIM acted upstream of Akt kinase during TCR signaling and influenced its localization to lipid rafts and hence activation. In turn, Akt influences the ubiquitination and possibly the degradation of Nur77. Thus, FAIM is a critical component in modulating TCR-induced apoptosis of thymocytes.

EXPERIMENTAL PROCEDURES

In Vivo and in Vitro TCR-mediated Apoptosis of Thymocytes—To study TCR-mediated apoptosis of thymocytes *in vivo*, mice were injected intraperitoneally with phosphate-buffered saline (PBS) or anti-CD3 antibody (145-2C11) at 0 and 24 h and sacrificed 24 h later. Thymocytes were collected for Western blot or flow cytometry analyses. To study TCR-mediated apoptosis *in vitro*, thymocytes from 6–8-week-old mice were added at 5×10^7 cells/ml to 6-well plates coated with anti-CD3 antibody or PBS. Anti-CD28 antibody (1 μg/ml) was added into culture medium. Cells were collected at indicated time points for Western blot or flow cytometry analyses.

Apoptosis Assay—Cells with DNA fragmentation was determined by propidium iodide staining as described (27). Briefly, thymocytes were treated with plate-bound anti-CD3 plus anti-CD28 antibodies for the indicated time and fixed in ethanol. After fixation, cells were washed and resuspended in staining buffer (20 μg/ml propidium iodide, 200 μg/ml RNase, and 0.1% Triton X-100 in PBS) for 1 h before fluorescence-activated cell sorter analysis. Results were analyzed with version 2.8 of the WinMDI software (Joseph Trotter, Scripps Research Institute).

Cell Culture, Transfections, Stimulations, and Western Blot Analyses—DO-11.10 cells were maintained in RPMI 1640 supplemented with 5% fetal calf serum, 100 international units/ml penicillin and 100 μg/ml streptomycin. A full-length cDNA fragment of murine *faim* was amplified by PCR and cloned into a pBluescript vector. After confirmation by sequencing analysis, *faim* cDNA was released and cloned into a pcDNA3.1 vector (Invitrogen), with a synthetic DNA fragment coding for the FLAG tag (DYKDDDDKH) being fused in-frame to the N terminus of *faim* cDNA. For certain cell stimulations, thymocytes were incubated with 10 μg/ml of biotinylated anti-TCRβ (H57-597) antibody at 4 °C for 30 min, followed by cross-linking with 25 μg/ml of streptavidin at 37 °C for various time points as indicated. DO-11.10 cells were treated with plate-bound anti-CD3 (10 μg/ml) and anti-CD28 (1 μg/ml) antibodies for the indicated time points. Whole cell extracts were prepared using lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% IGEPAL CA-630, 0.2 mM Na_3VO_4, and a protease inhibitor mixture (Roche Applied Science)). Protein concentration was measured by a colorimetric assay (Bio-Rad), and equal amount of proteins were loaded onto SDS gels. After transfer to polyvinylidene difluoride membranes, proteins were probed with primary antibodies (1 μg/ml) followed by horseradish peroxidase-conjugated secondary antibodies and were washed and visualized with chemiluminescent substrate (Pierce). Blots were reprobed with ERK2-specific antibody as loading control. Antibodies used were as follows: rabbit anti-ERK2 (C-14), mouse anti-caspase-8 p20 (D-8), rabbit anti-poly(ADP-ribose) polymerase (H250), rabbit anti-pT308 Akt, goat anti-linker for activation of T cells, mouse anti-ubiquitin (P4D1), and mouse anti-Akt1 (Santa Cruz Biotechnology); rabbit anti-caspase-9 (mouse-specific), rabbit anti-cleaved caspase-3 (Asp^{175}) (Cell Signaling); and mouse anti-Nur77, rabbit anti-Bak, and rabbit anti-Bax (BD Pharmingen); FAIM rabbit polyclonal antibody was raised in-house against full-length mouse FAIM.

Lipid Rafts Purification—Lipid rafts were prepared as described previously (28). Briefly, thymocytes (4×10^8) were lysed in 0.05% Triton X-100 in TNEV buffer (150 mM NaCl, 5 mM EDTA, and 25 mM Tris-HCl, pH 7.4), followed by addition of equal volume of 80% sucrose in lysis buffer and overlaid with 30 and 5% sucrose in the same buffer, respectively. Fractionation was performed in a SW60Ti rotor for 18 h at 4 °C and at 200,000 × *g*. Eleven fractions were collected, and lipid raft fractions (corresponding to 3rd, 4th, and 5th fractions) were solubilized in 10 mM *n*-octyl β-D-glucopyranoside (Sigma).

Statistical Analysis—All experiments were performed at least three times. Data are presented as mean ± S.E. Statistical comparison of the data were performed using Student's *t* test. Group difference with $p < 0.05$ was considered statistically significant.

RESULTS

FAIM Is Induced by TCR Stimulation and Inhibits TCR-mediated Apoptosis of Thymocytes—As FAIM is induced by antigen receptor stimulation in B cells (24, 27), we examined whether TCR cross-linking could up-regulate FAIM expression in thymocytes. WT thymocytes expressed a basal level of FAIM *ex vivo*, and we noticed an increase in FAIM protein levels in these cells following an 18-h treatment with a high dose (10 μg/ml) of anti-CD3 and anti-CD28 (1 μg/ml) antibodies (Fig. 1*A*). The expression level of FAIM was markedly higher in thy-

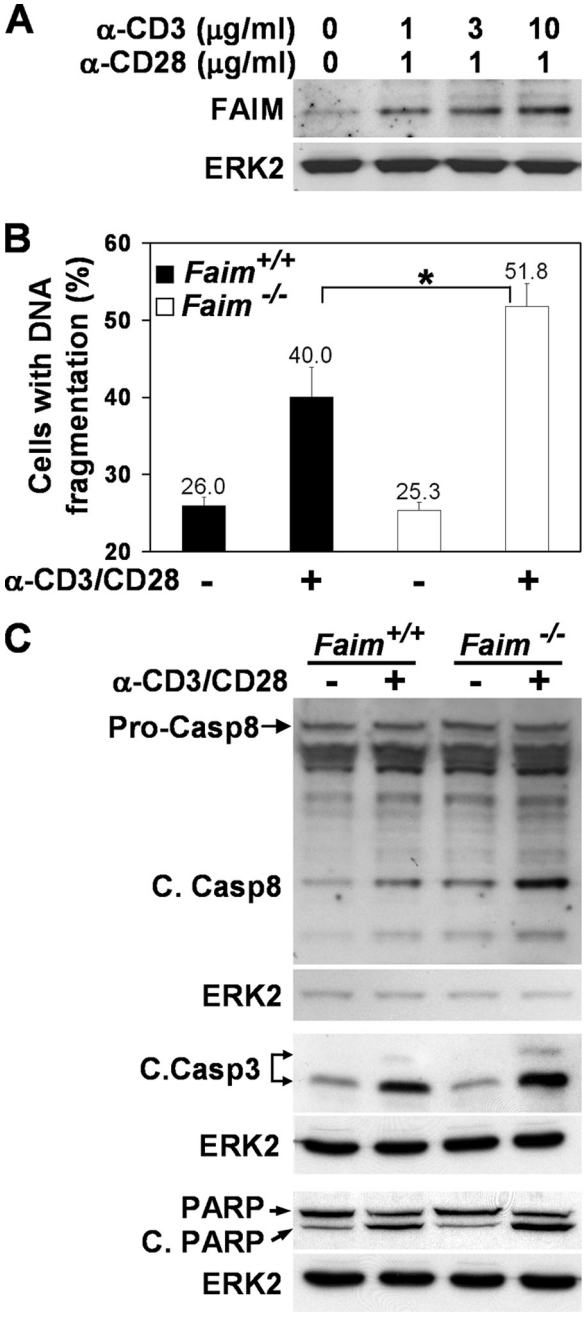

FIGURE 1. **Enhanced TCR-mediated apoptosis of *faim*$^{-/-}$ thymocytes.** *A*, TCR stimulation induces FAIM protein expression in thymocytes. WT thymocytes were treated with varying doses of anti-CD3/CD28 antibodies for 18 h, and cell lysates were probed for FAIM protein using specific antibodies. An anti-ERK2 blot was included as the loading control. Data shown are representatives of three independent experiments. *B*, graphical representation of the increase in TCR-induced apoptosis in WT and *faim*$^{-/-}$ thymocytes. Apoptosis was assessed after 18 h of stimulation with plate-bound anti-CD3 (10 μg/ml) and anti-CD28 (1 μg/ml) antibodies. Apoptosis was measured by propidium iodide staining of cellular DNA content and shown as a percentage of sub-G_1 to the whole population. *Numbers* indicate the mean percentage of cells with DNA fragmentation ± S.D. Data shown are representative of five independent experiments. *, $p = 0.024$ (two-sided Student's *t* test). *C*, Western blot analyses of caspase-8 and -3 activation and poly(ADP-ribose) polymerase cleavage in WT and *faim*$^{-/-}$ thymocytes after 18 h of anti-CD3/CD28 antibodies stimulation. Data shown are representative of six independent experiments. ERK2 blots were included as the loading control. *Casp8*, caspase-8; *PARP*, poly(ADP-ribose) polymerase.

mocytes receiving a stronger dose of TCR stimulation, suggesting that TCR signaling could directly regulate FAIM expression and that FAIM might play a role in TCR-mediated processes.

It is well documented that thymocytes are susceptible to apoptosis triggered by TCR engagement (29, 30) and that the activation of caspases are important in this process (16, 17). Hence, we determined whether FAIM was involved in thymocytes apoptosis triggered by TCR engagement by examining the extent of cell death in cultures of WT and *faim*$^{-/-}$ thymocytes treated with anti-CD3/CD28 antibodies. As shown in Fig. 1*B*, there was an increase in the percentage of cells with DNA fragmentation of WT thymocytes after 18 h of treatment with anti-CD3/CD28 antibodies as compared with untreated control (40% *versus* 26%). Interestingly, the increase in cells with DNA fragmentation was consistently higher in the anti-CD3/CD28 antibodies-treated *faim*$^{-/-}$ sample (51.8%). The increased apoptosis in mutant cells suggests that FAIM could play an inhibitory role in TCR-mediated apoptosis of thymocytes.

To further dissect the involvement of FAIM in TCR-mediated apoptosis of thymocytes, we next examined the activation of caspases in *faim*$^{-/-}$ cells (Fig. 1*C*). Compared with WT cells, *faim*$^{-/-}$ thymocytes exhibited enhanced activation of both caspase-8 and caspase-3, as indicated by the increased levels of the cleaved forms of these two caspases after 18 h of treatment with anti-CD3/CD28 antibodies. As a result of the higher level of caspase activation, the cleavage of poly(ADP-ribose) polymerase, which is a substrate of caspase-3, was also considerably enhanced in TCR-stimulated *faim*$^{-/-}$ thymocytes compared with the WT control. Thus, the data indicate that FAIM is involved in inhibiting TCR-mediated apoptosis and could modulate the activation of caspase-8 and caspase-3.

Augmented Depletion of CD4$^+$*CD8*$^+$ *Thymocytes in faim*$^{-/-}$ *Mice Injected with Anti-CD3 Antibody*—We further determined whether the lack of FAIM would affect thymocytes apoptosis *in vivo*. We employed a model that was frequently used to induce deletion of thymocytes *in vivo* by injecting WT and *faim*$^{-/-}$ mice with anti-CD3 antibody (31, 32). After injection of anti-CD3 antibody, we noticed a substantial reduction in the total number of thymocytes recovered from challenged WT and *faim*$^{-/-}$ mice compared with PBS-injected controls. More importantly, anti-CD3 antibody-injected *faim*$^{-/-}$ mice had significantly fewer thymocytes compared with similarly treated WT animals (*faim*$^{-/-}$, $13.4 \pm 1.4 \times 10^6$, $n = 7$ *versus* WT, $23.4 \pm 5.9 \times 10^6$, $n = 7$), whereas the thymic cellularity was comparable between *faim*$^{-/-}$ and WT mice injected with PBS (*faim*$^{-/-}$, $3.0 \pm 1.0 \times 10^8$, $n = 10$ *versus* WT, $2.9 \pm 1.1 \times 10^8$, $n = 10$). We further demonstrated that the injection of anti-CD3 antibody resulted in an ~2–3-fold reduction in the fraction of DP thymocytes in WT mice compared with PBS-injected WT controls (Fig. 2*A*). However, the same treatment led to a more drastic 5–15-fold decrease in the fraction of DP cells in *faim*$^{-/-}$ mice compared with PBS-injected *faim*$^{-/-}$ mice, and in some mice, this population of cells was almost obliterated. This severe reduction of DP thymocytes was also reflected by the drastic decrease in the absolute number of DP thymocytes in anti-CD3 antibody-challenged *faim*$^{-/-}$ compared with WT mice (Fig. 2*B*).

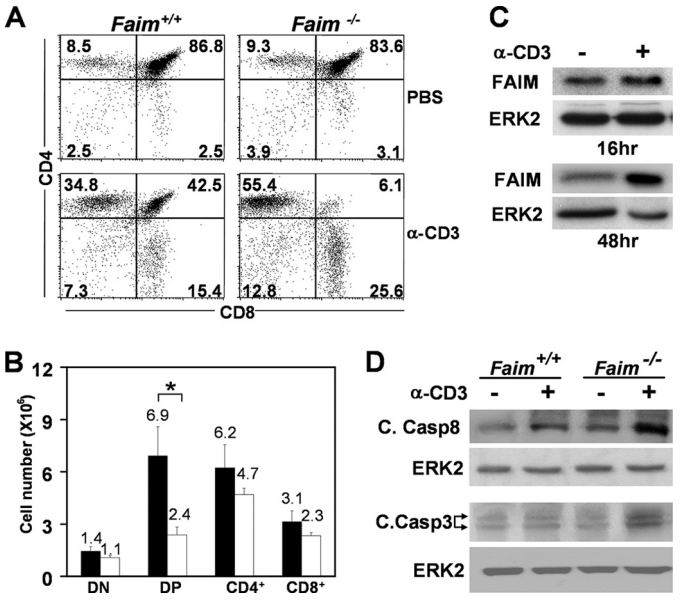

FIGURE 2. **Augmented depletion of CD4$^+$CD8$^+$ thymocytes in *faim*$^{-/-}$ mice after *in vivo* anti-CD3 antibody treatment.** *A*, flow cytometry analysis of thymocytes isolated from 8-week-old WT and *faim*$^{-/-}$ mice injected intraperitoneally with PBS or anti-CD3 antibody (25 μg). Percentages of cells in various thymic populations were shown. *B*, enumeration of cells in the various thymic fractions of *faim*$^{+/+}$ and *faim*$^{-/-}$ mice after intraperitoneal injection with anti-CD3 antibody. *, $p = 0.036$ (two-sided Student's *t* test). *DN*, double negative; *DP*, double positive. *C*, increased level of FAIM protein expression in WT thymocytes after anti-CD3 antibody injection *in vivo*. Cell lysates were prepared from thymocytes of mice injected 16 or 48 h earlier with anti-CD3 antibody and probed with anti-FAIM or anti-ERK2 antibody. *D*, WT and *faim*$^{-/-}$ mice were intraperitoneally injected with anti-CD3 antibody (25 μg) 16 h earlier, and whole cell lysates of thymocytes were probed for activated caspase-8 (*Casp8*) and -3 (*Casp3*).

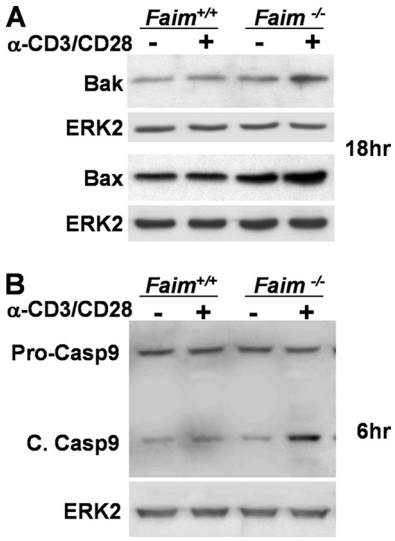

FIGURE 3. **FAIM deficiency leads to elevated levels of Bak and Bax proteins and increased activation of caspase-9 during TCR-mediated apoptosis of thymocytes.** *A*, increased protein expression of Bak and Bax in *faim*$^{-/-}$ thymocytes. WT and *faim*$^{-/-}$ thymocytes were stimulated with anti-CD3 (10 μg/ml) and anti-CD28 (1 μg/ml) antibodies for 18 h and analyzed for Bax and Bak induction via Western blot analysis. *B*, enhanced caspase-9 (*Casp9*) activation in *faim*$^{-/-}$ thymocytes. Thymocytes from WT and *faim*$^{-/-}$ mice were treated with anti-CD3/CD28 antibodies for 6 h, and cell lysates were probed for increase in the activated form of caspase-9. Anti-ERK2 blots were included as loading controls. Data are representative of four independent experiments.

Similar to the *in vitro* situation in Fig. 1, the *in vivo* injection of anti-CD3 antibody also led to elevated expression of FAIM protein in WT thymocytes, which was more prominent at 48 h compared with the 16-h time point (Fig. 2*C*). In addition, we also detected enhanced activation of caspase-8 and caspase-3 in *faim*$^{-/-}$ thymocytes compared with WT controls 16 h after the *in vivo* injection of anti-CD3 antibody, a time point at which thymocytes have not manifested massive apoptosis (Fig. 2*D*).

Increased Expression of Bak and Bax and Enhanced Activation of Caspase-9 in TCR-stimulated faim$^{-/-}$ *Thymocytes*—Given that FAIM could inhibit TCR-induced thymocyte apoptosis (Fig. 1), we sought to define the molecular pathways that FAIM might participate in by examining the effect of FAIM deficiency on several molecules shown previously to be involved in thymocyte apoptosis.

Among the molecules known to regulate thymocyte apoptosis are the proapoptotic Bak and Bax. Mice lacking Bak and Bax had altered thymic subsets, and mutant thymocytes were resistant to TCR-mediated apoptosis (18). To determine a possible relationship between FAIM and Bak and Bax, we examined whether the expression of these molecules was altered in TCR-stimulated *faim*$^{-/-}$ thymocytes. Western blot analyses revealed that the protein levels of Bak and Bax were increased in *faim*$^{-/-}$ compared with WT thymocytes following TCR stimulation (Fig. 3*A*), suggesting that the lack of FAIM leads to increased accumulation of the proapoptotic proteins Bak and Bax. The elevated protein levels of Bak and Bax were also consistent with the greater susceptibility of *faim*$^{-/-}$ thymocytes to anti-CD3 antibody-triggered apoptosis (Fig. 1) and with the finding that thymocytes lacking Bak and Bax were resistant to anti-CD3 antibody-induced apoptosis.

Bak and Bax are important regulators of the mitochondrial apoptosis pathway and because their expressions were increased in *faim*$^{-/-}$ thymocytes, we examined whether the activation of caspase-9 involved in the intrinsic pathway was also affected in mutant cells. As shown in Fig. 3*B*, the activation of caspase-9, as indicated by the presence of its cleaved form, was induced in WT thymocytes upon TCR stimulation. In addition, consistent with the higher accumulation of Bak and Bax in *faim*$^{-/-}$ thymocytes, the activation of caspase-9 was further enhanced in the mutant thymocytes treated with anti-CD3/CD28 antibodies. Thus, the data suggest that FAIM could be involved in modulating the levels of Bak and Bax during TCR-mediated apoptosis of thymocytes.

FAIM Modulates Nur77 Protein Levels during TCR-mediated Apoptosis of Thymocytes—Another important molecule implicated in thymocyte apoptosis is the orphan nuclear receptor Nur77, which was proapoptotic and induced by TCR engagement (19–21, 23). To determine whether FAIM is involved in the Nur77 signaling pathway, we first examined whether Nur77 expression and/or induction was altered in *faim*$^{-/-}$ thymocytes. Real-time reverse transcription-PCR analysis, as shown in Fig. 4*A*, revealed similar increases in Nur77 mRNA in WT and *faim*$^{-/-}$ thymocytes 6 h after TCR engagement, suggesting that FAIM does not affect the induction of Nur77 mRNA upon TCR stimulation.

We next examined the protein level of Nur77 in WT and *faim*$^{-/-}$ thymocytes after TCR activation (Fig. 4*B*). Western

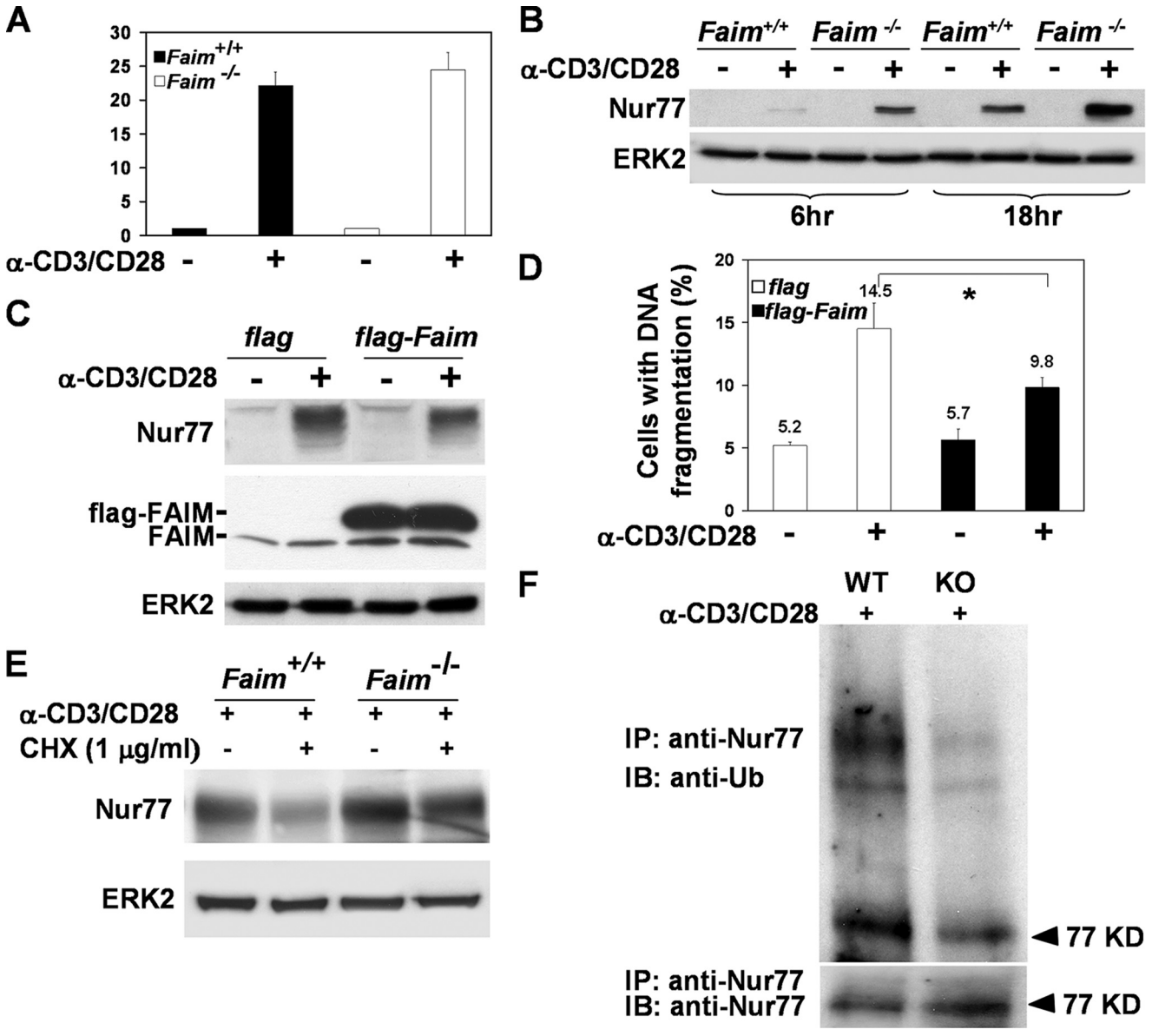

FIGURE 4. **FAIM regulates Nur77 protein expression in TCR-stimulated thymocytes.** *A*, shown is a real time reverse transcription-PCR analysis of Nur77 mRNA expression in WT and *faim*$^{-/-}$ thymocytes stimulated with anti-CD3/CD28 antibodies for 6 h. TaqMan assays for Nur77 and glyceraldehyde-3-phosphate dehydrogenase were purchased from Applied Biosystems. Data are representative of three independent experiments. *B*, Nur77 protein levels in *faim*$^{-/-}$ thymocytes were increased after TCR stimulation. Western blot analysis of Nur77 protein expression in *faim*$^{+/+}$ and *faim*$^{-/-}$ thymocytes at 6- and 18-h time points after stimulation with anti-CD3 (10 μg/ml) and anti-CD28 (1 μg/ml) antibodies. An ERK2 blot was included as loading control. Data are representative of five independent experiments. *C*, shown is a reduced induction of Nur77 protein expression in DO-11.10 cells overexpressing FAIM. Cells were transfected with pcDNA3.1-FLAG (*flag*) or pcDNA3.1-FLAG-FAIM (*flag-FAIM*) plasmid and subsequently stimulated with anti-CD3/CD28 antibodies for 18 h and analyzed via Western blot for Nur77 protein levels. The FAIM and ERK2 blots were included as control for transfection and loading, respectively. Data are representative of three independent experiments. *D*, overexpression of FAIM reduces TCR-mediated apoptosis in DO-11.10 cells. Graphical representation of the percentage of cells with DNA fragmentation of TCR-stimulated DO-11.10 cells transfected with pcDNA3.1-FLAG (*flag*) or pcDNA3.1-FLAG-FAIM (*flag-FAIM*) plasmid. Calculation of apoptosis is as defined in Fig. 1. *Numbers* indicate mean percentage of cells with DNA fragmentation ± S.E. ($n = 4$); *, $p = 0.010$ (two-sided Student's *t* test). *E*, FAIM promotes Nur77 degradation after TCR stimulation. Western blot analysis of Nur77 protein expression in *faim*$^{+/+}$ and *faim*$^{-/-}$ thymocytes stimulated with anti-CD3 (10 μg/ml) and anti-CD28 (1 μg/ml) antibodies for 18 h. The protein synthesis inhibitor cycloheximide (*CHX*) (1 μg/ml) was added in the last hour of stimulation. An ERK2 blot was included as loading control. Data are representative of three independent experiments. *F*, FAIM affects Nur77 ubiquitination in TCR-stimulated thymocytes. *faim*$^{+/+}$ and *faim*$^{-/-}$ thymocytes were stimulated with anti-CD3 (10 μg/ml) and anti-CD28 (1 μg/ml) antibodies for 18 h. Nur77 protein was immunoprecipitated (*IP*) with an anti-Nur77 monoclonal antibody and subsequently probed for ubiquitination. Data shown are representative of four independent experiments. *KO*, knock-out; *IB*, immunoblot.

blot analysis indicated that the protein level of Nur77 was slightly increased in WT thymocytes 6 h after activation by anti-CD3/CD28 antibodies, and the induction became more prominent after 18 h of treatment. In comparison, Nur77 protein was already prominently detected in *faim*$^{-/-}$ thymocytes at the 6-h time point following TCR engagement and far exceeded the level found in WT cells by the 18-h time point. Thus, the data suggested that FAIM likely acted upstream of Nur77 and that the presence of FAIM could lead to the suppression of Nur77 protein expression level during TCR stimulation of thymocytes.

To determine whether FAIM acted upstream of Nur77, we overexpressed FAIM in T cell hybridoma DO-11.10. As shown in Fig. 4*C*, DO-11.10 cells that overexpressed FAIM have

reduced levels of Nur77 protein compared with the vector-transfected control upon TCR stimulation, and consistent with the findings so far, FAIM-overexpressing DO-11.10 cells were also more resistant to TCR-mediated apoptosis (14.5% *versus* 9.8%, vector-transfected control *versus* FAIM-overexpressing cells, respectively) (Fig. 4*D*). Taken together, the data indicate that FAIM functions upstream of Nur77 during thymocyte apoptosis and that FAIM signaling could modulate the level of Nur77 protein expression and thus the level of thymocyte apoptosis upon TCR stimulation.

The significantly elevated protein but not mRNA level of Nur77 in TCR-stimulated *faim*$^{-/-}$ thymocytes compared with their WT counterparts indicated that FAIM could affect Nur77 expression at the post-transcriptional level. We next examined whether the turnover of Nur77 protein was affected in the absence of FAIM. Due to the inducible nature of Nur77 expression in thymocytes, we first treated *faim*$^{-/-}$ and WT thymocytes by TCR cross-linking for 18 h and subsequently used cycloheximide to inhibit the *de novo* protein synthesis for an additional hour to detect the protein level of Nur77. As shown in Fig. 4*E*, treatment with cycloheximide resulted in a drastic reduction of Nur77 protein in WT thymocytes, suggesting that Nur77 undergoes rapid protein degradation. In contrast, the decrease of Nur77 protein level after the same treatment was much less in mutant cells, which indicated that the elevated Nur77 protein level in *faim*$^{-/-}$ thymocytes could be partially ascribed to its reduced protein degradation.

Protein ubiquitination is known to be a major mechanism responsible for protein degradation (33). The defective protein degradation of Nur77 in *faim*$^{-/-}$ thymocytes led us to examine whether the ubiquitination of Nur77 occurred in WT thymocytes and whether the process was affected in *faim*$^{-/-}$ thymocytes. As shown in Fig. 4*F*, Nur77 exhibited a significant degree of ubiquitination in TCR-stimulated WT thymocytes, whereas the ubiquitination of Nur77 was much less prominent in mutant cells. Taken together, these data suggest that FAIM could be involved in the pathway that regulates Nur77 ubiquitination and degradation.

FAIM Acts Upstream of Akt and Affects Its Activation—To gain more insight to the role of FAIM in thymocyte apoptosis, we also examined the activation of Akt, which has been shown to promote the survival of thymocytes (34). Akt is known to interact with Nur77 (35) and negatively regulates its transcriptional activity in T cells and thereby antagonizes apoptosis (36). As our results depicted in Fig. 4 indicated that FAIM could regulate Nur77 protein level, we were interested in examining the relationship between FAIM and Akt. It is possible that FAIM could act either independently or downstream of Akt and not affect it in any manner, or it can act upstream of the kinase and directly influence its activation.

As shown in Fig. 5*A*, TCR stimulation of WT thymocytes led to the activation of Akt, as evidenced by the phosphorylation of Thr^{308} at the 3- and 10-min time points. Interestingly, in the absence of FAIM, there was reduced activation of Akt at the 3-min time point following TCR stimulation, and Akt activation was returned to basal level at the 10-min time point. This suggests that the absence of FAIM could affect the activation of Akt, and, furthermore, FAIM functions upstream of Akt.

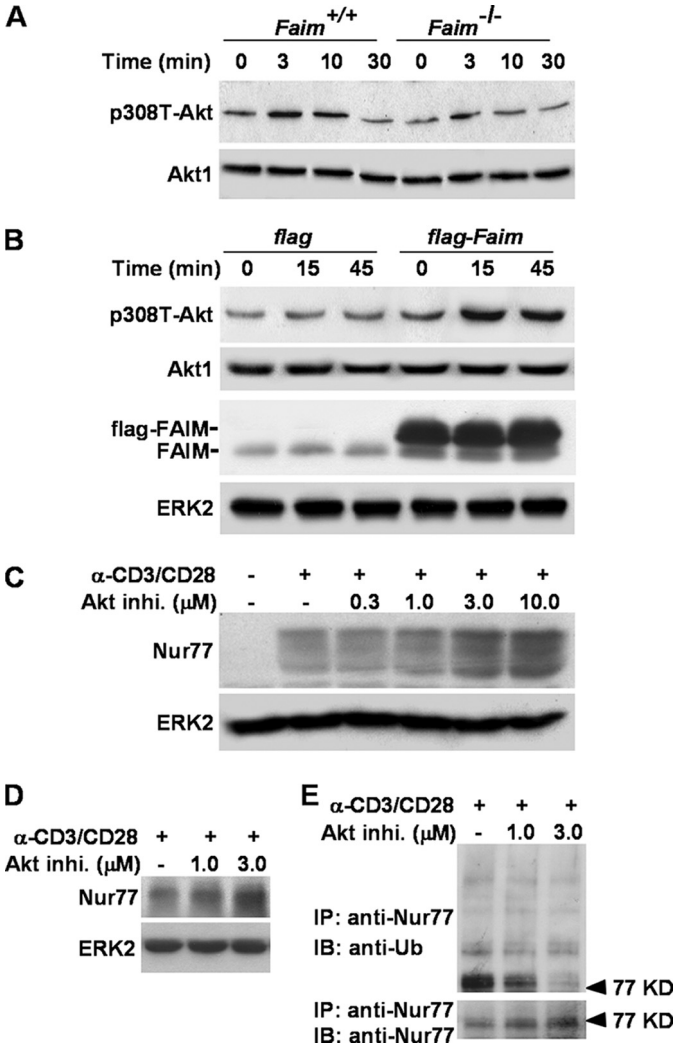

FIGURE 5. **Akt activation is regulated by FAIM and affects Nur77 protein levels.** *A*, reduced Akt activation in *faim*$^{-/-}$ thymocytes. Western blot analysis of Akt threonine 308 phosphorylation in WT and *faim*$^{-/-}$ thymocytes stimulated via the TCR. Data shown are representative of three independent experiments. *B*, overexpression of FAIM leads to the sustained activation of Akt during TCR engagement. DO-11.10 cells were transfected with pcDNA3.1-FLAG (*flag*) or pcDNA3.1-FLAG-FAIM (*flag-FAIM*) plasmid and examined for Akt phosphorylation after anti-CD3/CD28 stimulation by Western blot analysis. The anti-Akt1, anti-FAIM, anti-FLAG, and anti-ERK2 blots served as controls for transfections and loading of cell lysates, respectively. Data shown are representative of three independent experiments. *C* and *D*, Akt controls the protein level of Nur77. DO-11.10 cells (*C*) or WT thymocytes (*D*) were stimulated with anti-CD3/CD28 antibodies for 18 h in the absence or presence of varying concentrations of the Akt inhibitor (1,3-dihydro-1-(1-((4-(6-phenyl-1*H*-imidazo[4,5-g]quinoxalin-7-yl)phenyl)methyl)-4-piperidinyl)-2*H*-benzimidazol-2-one trifluoroacetate salt) and examined for the protein levels of Nur77 in Western blot analysis. Anti-ERK2 blot was included as loading controls. Data shown are representative of three independent experiments. *E*, Akt regulates Nur77 ubiquitination in TCR-stimulated thymocytes. WT thymocytes were stimulated with anti-CD3 (10 μg/ml) and anti-CD28 (1 μg/ml) antibodies for 18 h with varying concentrations of the Akt inhibitor (*Akt inhi.*). Nur77 protein was immunoprecipitated (*IP*) with an anti-Nur77 monoclonal antibody and probed for Nur77 ubiquitination. Data shown are representative of three independent experiments. *IB*, immunoblot.

Again, we examined the activation of Akt in DO-11.10 cells overexpressing FAIM. Indeed, the engagement of the TCR led to a more robust and sustained induction of Akt phosphorylation in DO-11.10 cells overexpressing FAIM compared with the vector-transfected control (Fig. 5*B*). Hence, FAIM likely acts upstream of Akt and directly influences its activation.

Because FAIM acted upstream of Akt (Fig. 5, *A* and *B*) and Nur77 (Fig. 4) in signaling thymocyte apoptosis, we explored the relative positioning of Akt and Nur77 in this pathway. It is possible that Akt and Nur77 represent two independent signaling branches downstream of FAIM and do not influence each other, or they function in a linear signaling pathway with one upstream of the other. To examine these possibilities, we treated DO-11.10 cells with an Akt inhibitor before stimulating them with anti-CD3/CD28 antibodies. As seen in Fig. 5*C*, TCR stimulation of DO-11.10 cells led to the induction of Nur77 protein expression. Interestingly, the expression of Nur77 protein was further enhanced in the presence of increasing concentrations of the Akt inhibitor (Fig. 5*C*). In TCR-stimulated WT thymocytes, we also found that the inhibition of Akt activity resulted in an accumulation of Nur77 protein (Fig. 5*D*). These data suggest that Akt acts upstream of Nur77 and signals to down-modulate Nur77 protein level during TCR signaling. In accordance with the elevated level of Nur77 protein and consistent with our data in Fig. 4*F*, the inhibition of Akt also led to a decreased ubiquitination of Nur77 in TCR-stimulated WT thymocytes (Fig. 5*E*). Taken together, the data support a model in which TCR engagement activates a FAIM/Akt/Nur77 signaling axis that modulates the apoptosis of thymocytes.

FAIM Modulates the Localization of Akt to Lipid Rafts—Next, we examined the mechanism underlying the regulation of Akt activation by FAIM. It is known that Akt translocates to lipid rafts upon TCR stimulation of T cells (37), and it is activated upon recruitment to cell membranes (38). Because TCR-induced Akt activation was compromised in *faim*$^{-/-}$ thymocytes, we examined whether the defect was due to perturbed translocation of Akt to lipid rafts in the absence of FAIM. Indeed, we were able to recapitulate previous findings (38) and showed that TCR stimulation led to increased localization of Akt to lipid rafts (fraction 4, as indicated by the presence of the lipid raft-located protein linker for activation of T cells) of WT thymocytes (Fig. 6*A*). Interestingly, there was also an increase in the amount of FAIM in the lipid raft fraction of TCR-stimulated WT thymocytes (Fig. 6*A*). This suggests that FAIM and Akt co-localize to lipid rafts in thymocytes following TCR engagement.

We further examined TCR-mediated localization of Akt to lipid rafts in the absence of FAIM. Compared with the situation in WT thymocytes, the amount of lipid raft-localized Akt was reduced in TCR-stimulated *faim*$^{-/-}$ thymocytes (Fig. 6*B*), indicating that FAIM could modulate the localization of Akt to lipid rafts. This was reaffirmed by examining the amount of Akt in lipid raft fractions in TCR-stimulated DO-11.10 cells overexpressing FAIM. As shown in Fig. 6*C*, the amount of Akt in lipid raft fraction was significantly increased in anti-CD3/CD28 antibodies-treated DO-11.10 cells upon overexpression of FAIM. Taken together, the results suggest that FAIM acts to facilitate the localization of Akt to lipid rafts during TCR stimulation and thereby influences its activation and survival signaling.

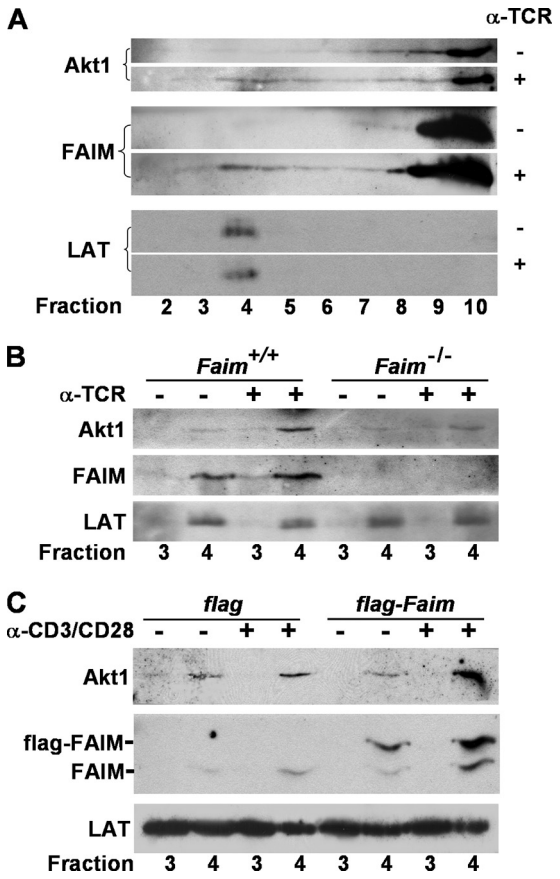

FIGURE 6. **FAIM facilitates the recruitment of Akt into lipid rafts.** *A*, FAIM and Akt localize to lipid rafts upon TCR stimulation. WT thymocytes were nontreated (−) or stimulated (+) via the TCR for 30 min and fractionated and examined for the presence of Akt1 and FAIM proteins. Fraction 4 represents the lipid raft fraction as indicated by the presence of lipid raft-located linker for activation of T cells. Data are representative of three independent fractionation experiments. *B*, shown are reduced Akt in lipid rafts of *faim*$^{-/-}$ thymocytes upon TCR stimulation. Cells were treated and processed as above to examine the presence of Akt1 and FAIM in lipid rafts. Only fractions 3 and 4, which represented the lipid raft fractions, are shown. Data are representative of three independent experiments. An anti-linker for activation of T cells (*LAT*) blot was included to indicate the lipid raft fractions. *C*, shown is an increased amount of Akt in lipid rafts of FAIM-overexpressing DO-11.10 cells upon TCR stimulation. Cells were transfected with pcDNA3.1-FLAG (*flag*) or pcDNA3.1-FLAG-FAIM (*flag-FAIM*) plasmid and stimulated with anti-CD3/CD28 antibodies for 30 min and subsequently fractionated and examined as above for the presence of Akt1, FLAG-FAIM, and endogenous FAIM in lipid raft factions. Only fractions 3 and 4 representing the raft fractions are shown. Data shown are representative of three independent experiments.

DISCUSSION

FAIM was first identified as an evolutionarily conserved protein that antagonizes Fas-mediated apoptosis of mature B cells *in vitro* (24). Subsequently, it was also shown to play a role in NF-κB activation during neurite outgrowth (25). We also recently demonstrated that it played a role in Fas-triggered apoptosis of lymphocytes and hepatocytes (27). In this report, we further demonstrate a role for FAIM in TCR-mediated apoptosis of thymocytes using cells with loss and gain of function of FAIM. Our mechanistic data further suggest that FAIM could act by regulating Akt activation and Nur77 expression to modulate TCR-mediated apoptosis of thymocytes.

The experimental support for a role for FAIM in TCR signaling first arose from our observation that TCR stimulation could induce high level of FAIM protein expression in WT thymo-

cytes (Figs. 1*A* and 2*C*). Next, engagement of the TCR in the absence of FAIM led to enhanced activation of caspase-8 and -9 and increased apoptosis of thymocytes. Thus, FAIM could function to reduce the susceptibility of developing thymocytes to apoptotic signals. It is well established that TCR signaling regulates the process of positive and negative selection of thymocytes during T cell development (5, 39).

The signaling proteins and pathways that promote or modulate TCR-mediated apoptosis of thymocytes are not well characterized. Among the better known proteins involved in thymocytes apoptosis is the orphan nuclear receptor Nur77 (22, 23). Nur77 is up-regulated upon TCR stimulation and is involved in TCR-induced apoptosis (19, 20). This pattern is similar to what we had found with FAIM (Fig. 4). We have therefore examined Nur77 in the context of a FAIM deficiency and interestingly, have found that *faim*$^{-/-}$ thymocytes have elevated levels of Nur77 protein expression upon TCR stimulation. This suggests that FAIM is involved in the Nur77 pathway and probably functions upstream of this protein in apoptotic signaling. It is tempting to hypothesize that FAIM could regulate Nur77 protein at a post-transcriptional level as TCR stimulation led to equivalent increase in Nur77 transcripts in WT and *faim*$^{-/-}$ thymocytes, but the latter has enhanced levels of Nur77 protein. In support of this hypothesis, we had directly examined the protein degradation of Nur77 and found that Nur77 protein degradation was reduced in *faim*$^{-/-}$ thymocytes compared with WT cells. Nur77 could convert antiapoptotic Bcl-2 family members to proapoptotic molecules and trigger the mitochondrial pathway in apoptosis (40, 41). Nur77 could also function as a transcription factor to induce the expression of apoptosis-inducing genes (21). Thus, the higher level of Nur77 protein expression in *faim*$^{-/-}$ thymocytes correlates well with the enhanced susceptibility of these cells to TCR-mediated apoptosis.

Consistent with the participation of FAIM in the Nur77 pathway of thymocyte apoptosis (this study) and with the role of Nur77 in effecting the mitochondrial pathway of apoptosis (40), we were also able to demonstrate that *faim*$^{-/-}$ thymocytes have augmented activation of caspase-9 (Fig. 2). Moreover, we found enhanced Bak and Bax expression in TCR-stimulated *faim*$^{-/-}$ thymocytes. Bak and Bax have recently been shown to play a critical role in thymocyte negative selection, and in their absence, thymocytes were resistant to apoptosis (18). Thus, taken together, these data suggested that FAIM may play an important role in modulating the cell death pathway during TCR-mediated negative selection of thymocytes.

Another molecule that has been implicated in thymocyte apoptosis and negative selection is the Akt kinase (34). Interestingly, we found that the activation of Akt was compromised in the absence of FAIM (Fig. 5), suggesting that FAIM also participated in the Akt survival signaling pathway. Furthermore, through the use of chemical inhibitors, we showed that the activity of Akt could directly affect the protein level of Nur77 during TCR stimulation by at least partially, regulating its ubiquitination (Fig. 5, *C–E*). Thus, our data propose a signaling cascade between FAIM, Akt, and Nur77, in which FAIM affects the activation of Akt, and, in turn, Akt controls Nur77 protein degradation by regulating its ubiquitination.

To understand the underlying mechanism of how FAIM regulates the activation of Akt, we further demonstrated that FAIM could facilitate the recruitment or retention of Akt in the lipid rafts, which is known to be essential for its activation. Thus, in the absence of FAIM, less Akt is located in the lipid raft of thymocytes upon TCR stimulation (Fig. 6*B*), leading to reduced Akt activation and resulting in decreased ubiquitination of Nur77 and elevated Nur77 protein levels and consequently, the enhanced susceptibility of thymocytes to TCR-mediated apoptosis. Conversely, overexpression of FAIM leads to increased amount of Akt in lipid rafts and consequently more enhanced activation of Akt upon TCR engagement (Fig. 6, *B* and *C*). The augmented Akt activation leads to increased ubiquitination of Nur77 and reduced Nur77 protein level and decreased apoptosis (Fig. 4). Thus, our study suggests that FAIM plays an important role in TCR-triggered apoptosis by modulating the level of Akt in lipid rafts and affecting its activation. However, we have been unsuccessful in detecting physical interaction between FAIM and Akt through co-immunoprecipitation experiments (data not shown). It is tempting to speculate that FAIM could indirectly facilitate the translocation or retention of Akt in lipid rafts through a third party. Experiments are ongoing to identify possible molecules that interact with FAIM. Nevertheless, we have identified a new player, FAIM, that attenuates rather than promotes TCR-triggered apoptosis, and we have also defined a TCR-induced FAIM/Akt/Nur77 signaling axis that may regulate thymocyte negative selection.

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