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Defective Fas Ligand Expression and Activation-Induced Cell Death in the Absence of IL-2-Inducible T Cell Kinase

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The Tec family tyrosine kinase, IL-2-inducible T cell kinase (Itk), plays an important role in TCR signaling. Studies of T cells from Itk-deficient mice have demonstrated that Itk is critical for the activation of phospholipase-Cγ1, leading to calcium mobilization in response to TCR stimulation. This biochemical defect results in reduced IL-2 production by Itk-deficient T cells. To further characterize the downstream effects of the Itk deficiency, we crossed Itk<sup>−/−</sup> mice to a TCR-transgenic line and examined T cell responses to stimulation by peptide plus APC. These studies show that Itk is required for maximal activation of early growth responses 2 and 3 and Fas ligand transcription after TCR stimulation. These transcriptional defects lead to reduced activation-induced cell death of stimulated Itk<sup>−/−</sup> T cells, both in vitro and in vivo. Together these studies define an important role for Itk in TCR signaling, leading to cytokine gene expression and activation-induced cell death. The Journal of Immunology, 2002, 168: 2163–2172.

stimulation of the TCR induces a variety of different cellular responses, depending on the stage of development of the T cell and its Ag recognition history. In mature T lymphocytes, stimulation of the TCR can induce cytokine production, proliferation, anergy, or programmed cell death. In recent years, it has become increasingly clear that apoptotic cell death following a strong immune response is a major mechanism responsible for maintaining homeostasis in the immune system. For instance, after prolonged activation, T cells undergo a process termed activation-induced cell death (AICD), which is mediated by the induced expression of factors such as TNF and Fas ligand (FasL) (reviewed in Ref. 1). Activation of T cells via their TCR induces the synthesis of Fas (CD95), and its ligand, FasL (CD95L). The binding of Fas to FasL initiates a cascade of intracellular events in the Fas-expressing cell that ultimately results in the apoptotic death of that cell (reviewed in Ref. 2). The importance of this mechanism of AICD in the immune system is exemplified by the consequences of mutations in either the Fas or the FasL genes, which result in uncontrolled lymphoproliferation and autoimmunity in both humans and mice (3–5).

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3 Abbreviations used in this paper: AICD, activation-induced cell death; Fasl, Fas ligand; sFasl, soluble Fasl; MAP, mitogen-activated protein; MAPK, MAP kinase; SAPK, stress-activated protein kinase; ERK, extracellular signal-related kinase; JNK, c-Jun N-terminal kinase; xid, X-linked immunodeficiency; PLCγ1, phospholipase Cγ1; Egr, early growth response; SEB, staphylococcal enterotoxin B; PKC, protein kinase C; Mcc, moth cyochrome c; FLIP, Fas-associated death domain-like IL-1-converting enzyme-like inhibitory protein; EAE, experimental autoimmune encephalomyelitis; Itk, IL-2-inducible T cell kinase; Btk, Bruton’s tyrosine kinase; CHO, Chinese hamster ovary; Cy, CyChromae.
To further characterize the role of Itk in CD4+ T cell effector function, we crossed itk−/− mice to 5C.C7 TCR-transgenic mice (20), expressing a TCR specific for a moth cytochrome c peptide (MCC93-103) bound to the MHC class II molecule, IIE8 (21). These mice have provided a system for examining T cell signaling events and effector function in response to the natural receptor-ligand interaction. In this report, we show that in addition to calcium defects, itk−/− CD4+ T cells are defective in the activation of the ERK/MAPK and JNK pathways, the expression of early growth factors, and effector function in vivo, where T cells in the secondary responses of animals were incubated with anti-CD4-coated magnetic microbeads and purified. Puri fied CD4+ T cells were incubated in medium containing 5 µg/ml biotinylated anti-CD3ε for 45 s, followed by streptavidin (Life Technologies; 40 µg) cross-linking for 5 min. Ionomycin (1 µg) was added at 6 min. Data were analyzed by calculating the mean fluorescence ratio of iod-3 and fura-Red using FACScan software (BD Biosciences). 

**ERK and stress-activated protein kinase (SAPK)/JNK phosphorylation**

On day 8 post-primary stimulation with peptide and APCs, 5 × 10^6 5C.C7 itk−/− and 5C.C7 itk+/+ CD4+ T cells were incubated in 120 µl serum-free RPMI containing 25 µg/ml biotinylated anti-CD3ε for 10 min. Cells were quickly spun and resuspended in 120 µl serum-free RPMI containing 50 µg/ml streptavidin and incubated in a 37°C water bath for 0, 2, 5, or 10 min. As a positive control, cells were stimulated with PMA (2.5 ng/ml) and ionomycin (375 ng/ml) for 15 min at 37°C. Iod-3 and fura-Red staining was added to stop the reactions. Cells were then washed and for 15 min on ice in lysis buffer containing 25 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM Na3 VO4, and 10 mM of total RNA was reverse transcribed into cDNA using Superscript II and Random Hexamers (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. After DNase treatment (Promega, Madison, WI), 1 µg of total RNA was reverse transcribed into cDNA using a Bio-Rad iCycler using SYBR Green PCR Core

**Materials and Methods**

**Mice**

5C.C7 TCR transgenic mice (22) on the B10.BR (H-2k) background were crossed to the C57/BL6 background for at least eight generations, were used for in vivo experiments. All mice were between 6 and 12 wk of age and maintained in a specific-pathogen-free facility.

**Preparation and activation of CD4+ T cells**

Spleens and lymph nodes were removed from 6- to 12-wk-old 5C.C7 itk−/− and 5C.C7 itk+/+ littermates. After RBC lysis, single-cell suspensions were incubated with anti-CD4-coated magnetic microbeads and passed through LS columns according to the manufacturer’s protocol (Miltenyi Biotec, Auburn, CA). Purified CD4+ T cells were pooled and resuspended in RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS (HyClone, Logan, UT), 2 mM l-glutamine, 100 U penicillin, 100 µg/ml streptomycin, 10 mM HEPES, and 50 mM β-ME. This purification yielded routinely 90–97% CD4+ T cells (5C.C7 TCR is Va111). For in vitro activations, 1 × 10^6 CD4+ T cells were cultured in 24-well plates with 2 µM MCC93-103 peptide (DLLAYL KQAKTFF, Tufts Microtherapy, Bedford, MA) or 1 × 10^6 mitomycin C-treated (Calbiochem, La Jolla, CA) IE8 B7.1-expressing Chinese hamster ovary (CHO) cells (23). Cultures were all performed under Th1-skewing conditions (anti-IL-4 1 µg/ml and rIL-12 1 ng/ml; R&D, Minneapolis, MN). After 24 h, the cells were transferred into and expanded in fresh medium containing 5 ng/ml IL-2 (BD PharMingen, San Diego, CA). Addition of IL-2 immediately on primary stimulation had no effect on the secondary responses of 5C.C7 itk−/− or 5C.C7 itk+/+ cells. After stimulation, cells were maintained in medium supplemented with IL-2 until restimulation. Itk−/− and Itk+/+ T cells expanded comparably with these stimulation conditions, and similar numbers of cells were recovered from both types of cultures before secondary stimulation.

**Abs and flow cytometry**

Cells were stained with the indicated Abs in HBSS supplemented with 3% FCS for 30 min on ice. Cells were then washed and analyzed on a BD Biosciences (San Jose, CA) FACSCalibur. Data were analyzed using CellQuest software (BD Immunocytometry Systems, San Jose, CA). The Abs and flow cytometry reagents used were anti-CD4-CD4-Chrome (Cy), anti-Va111-FTTC, anti-Vβ8.1B-FTTC, anti-Vβ6-FTTC, anti-Fas-biotin, anti-CD69-PE, anti-CD44-FTTC, anti-CD25-PE, streptavidin-PE (BD PharMingen), and anti-Fasl-PE (eBioscience, San Diego, CA).

**In vitro proliferation assay**

For primary proliferation assays, 5 × 10^5 CD4+ Va111 T cells were stimulated with MCC93-103 peptide plus 5 × 10^5 mitomycin C-treated CHO cells expressing IE8 and B7.1 in a volume of 200 µl for 48 h. As a control, cells were stimulated with PMA (Sigma; 2.5 ng/ml) and ionomycin (Calbiochem; 375 ng/ml). [3H]Thymidine (NEN, Boston, MA) was added at 1 µCi/well and incubated for an additional 20 h, plates were harvested on a Tomtec Harvester 96 (Orange, CT), and [3H]thymidine incorporation was quantified on a Trilux microtiter counter (PerkinElmer, Wellesley, MA). Secondary proliferation assays were performed on day 14 after initial stimulation and the point at which the cells were no longer dividing. Previously activated cells were removed from culture, washed, and counted, and, as in the primary proliferation assay, 5 × 10^5 CD4+ Va111 T cells were stimulated. For the blocking of Fasl-mediated AICD in vitro, cells were stimulated in the same conditions as above with the addition of anti-Fasl (MFL3) or an isotopic control Ig (eBioscience) added at 5 µg/ml to each well at 0 and 20 h.

**Functional assay for Fasl-induced apoptosis**

The induction of Fasl-mediated cell death was determined by annexin V-FITC (BD PharMingen) and propidium iodide (Sigma) staining 15–20 h after 3 × 10^3 T cells were stimulated with 5 × 10^6 CHO (IE8 and B7.1 positive) cells and MCC93-103 peptide. Anti-Fasl or an isotopic control Ig (5 µg/ml) was added at 0 and again at 10 h poststimulation where indicated. Soluble Fasl (sFasL) was prebound for 30 min, followed by addition of a cross-linking enhancer Ig (Alexis, San Diego, CA) at 2 h poststimulation. Cells were immediately analyzed by flow cytometry. Specific apoptosis was determined by calculating the ratio of live cells in the treated wells to live cells in the wells incubated in the absence of MCC peptide.

**Intracellular IL-2 staining**

Day 8 post-primary stimulation with peptide and APCs, 5 × 10^6 5C.C7 itk−/− and 5C.C7 itk+/+ CD4+ T cells were incubated with 3 µg/ml fluo-3 and 5 µg/ml fura-Red (Molecular Probes, Eugene, OR) in RPMI containing 3% FCS for 45 min. Cells were washed twice and incubated in the dark at room temperature for 30 min. Cells (1 × 10^5) were placed in 1 ml of 37°C serum-free RPMI and analyzed on a BD Biosciences flow cytometer. Baseline calcium was measured, and cells were then stimulated with anti-CD3ε (145-2C11, 25 µg) (BD PharMingen) for 45 s, followed by streptavidin (Life Technologies; 40 µg) cross-linking for 5 min. Ionomycin (1 µg) was added at 6 min. Data were analyzed by calculating the mean fluorescence ratio of fluo-3 and fura-Red using FACScan software (BD Biosciences).
Reagents (PE Applied Biosystems, Foster City, CA). To quantify the amount of cDNA for an individual transcript, SYBR Green fluorescence was measured at the end of each cycle. The cycle threshold (Ct), the cycle at which exponential growth of the PCR product is first detected, was determined for known concentrations of plasmid DNA, and a standard curve was created. Template copy numbers were calculated for each sample by interpolating the Ct values on the standard curve using the iCycler software. All samples and standards were run in triplicate for any given experiment. The values of FasL, Egr3, and Egr2 were normalized to β-actin by dividing the average copy number of the respective transcript by the average copy number of β-actin in the respective sample. From 1 μg RNA, we consistently found there to be $-5 \times 10^6$ copies of β-actin in naive cells and $1.3 \times 10^6$ copies in restimulated cells.

The PCR were as follows: templates were initially denaturated at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 20 s, 25 s of primer annealing at 62°C, 62°C, 58°C, or 51°C for β-actin, FasL, Egr3, and Egr2, respectively, and lastly a 72°C extension for 25 s. Primers were: FasL sense 5'-TGAACGCCCTGTAGATCTCGG-3', antisense, 5'-GGTTAGGGCTTGGTGTGTC-3'; Egr3 sense, 5'-GCCCTTTGCTGTTGA-3', antisense, 5'-CCCCTTTTCCGACTTCTTT-3'; Egr2 sense, 5'-GGTACAAATTCCCAAAG-3', antisense, 5'-TCTCTTTCTCCAGGTATG-3'; β-actin sense, 5'-CGAGGCCCCAGACAAAGAGCGCAGG-3', antisense, 5'-CGTGTGGCCTTTAGGGTTCAGC-3'. Specific products were verified by melt-curve analysis and gel electrophoresis. For the generation of standard curves, plasmids containing cDNA clones of Egr3 (gift from J. Milbrandt, Washington University School of Medicine, St. Louis, MO), FasL (gift from A. Marshak-Rothstein, Boston University Medical Campus, Boston, MA), and β-actin (gift from R. Gerstein, University of Massachusetts Medical School, Worcester, MA) were used. A 190-bp fragment of Egr2 (135–324) was cloned into pGEM-T Easy (Promega) and used for the generation of a standard curve.

**SEB-induced deletion in vivo**

**itk**$^{-/-}$ and **itk**$^{-/-}$ littersmates were injected i.v. with 75 μg SEB (Toxin Technology, Sarasota, FL) on Day 0. Mice were tail bled on days -1, 3, 7, 11, and 15 into Alsever’s solution. After RBC lysis, cells were stained with anti-Vβ8.2-FITC or anti-Vβ6-FITC and anti-CD4-PE. Ten thousand live CD4$^+$ events were collected on a flow cytometer, and the percentage of CD4$^+$ cells expressing Vβ8 or Vβ6 was determined.

**Results**

**Naive itk**$^{-/-}$ CD4$^+$ T cells have defects in IL-2 production and proliferation in response to MHC/peptide stimulation

Numerous studies over the past few years have indicated that the recruitment and activation of specific signaling pathways in T lymphocytes are determined by the nature of the TCR-peptide-MHC interaction (reviewed in Ref. 24). Nonetheless, prior in vitro studies that have focused on elucidating the role of **itk** in T cell signaling and effector function have largely used Abs to T cell surface receptors, such as CD3 and/or CD28, to trigger TCR/costimulation signaling events. Moreover, although initial studies demonstrated that **itk**-deficient CD4$^+$ cells have functional defects in response to anti-CD3 stimulation (17, 18), **itk** has also been implicated as a negative regulator of CD28 costimulation (25). Therefore, we were interested in examining the role of **itk** in TCR-mediated signaling events in response to the physiological receptor-ligand interaction.

To accomplish this, we crossed **itk**$^{-/-}$ mice to mice transgenic for the 5C.C7 TCR, which is specific for a MCC peptide, MCC93–103, bound to H2Kd (21, 22).

To first determine the functional responses of TCR-transgenic **itk**$^{-/-}$ T cells, purified CD4$^+$ T cells from 5C.C7 **itk**$^{-/-}$ or 5C.C7 **itk**$^{-/-}$ mice were stimulated in vitro with MCC93–103 peptide plus APC (IE$^+$ and B7.1-expressing CHO cells). As shown in Fig. 1A, we found a modest (~2-fold) decrease in the Ag-induced proliferative responses of **itk**$^{-/-}$ CD4$^+$ T cells compared with control T cells at all peptide concentrations tested. In contrast, stimulation with a phorbol ester, PMA (P), plus a calcium ionophore, ionomycin (I), induced comparable levels of proliferation, confirming previous data indicating that these pharmacological agents bypass the **itk**$^{-/-}$ defect by directly activating the protein kinase C (PKC)/Ras and calcium pathways, respectively (18). Previous studies had also indicated a defect in anti-CD3 Ab-induced IL-2 secretion by **itk**$^{-/-}$ T cells. To re-examine this issue with primary TCR-transgenic T cells, we determined the extent of IL-2 production by 5C.C7 **itk**$^{-/-}$ or 5C.C7 **itk**$^{-/-}$ cells after stimulation with MCC93–103 peptide and APCs. For these studies, we used intracellular staining of permeabilized cells with an anti-CD4-Cy and anti-Vε11-FITC, fixed, permeabilized, and stained intracellularly with anti-IL-2-PE. Ten thousand CD4$^+$Vε11$^+$ events were collected on a flow cytometer. B, Example of raw data; C, summary of one representative experiment of three.
CD4 or anti-CD3 plus anti-CD28 Abs, showing decreased IL-2 production by naive itk−/− T cells (17, 18).

**itk−/− CD4+ T cells proliferate more vigorously than control T cells on secondary stimulation**

Prior studies that have investigated the role of Itk in T cell signaling pathways have focused primarily on the responses of naïve T cells that lack Itk. Therefore, we were interested in determining how itk−/− cells respond on secondary stimulation. To accomplish this, SC.C7 itk+/+ and SC.C7 itk−/− CD4+ T cells were stimulated in vitro with 2 μM MCC93−101 peptide plus APCs and expanded in medium containing IL-2 until restimulation. In addition, to ensure a homogeneous population of activated T cells, these stimulations were performed in Th1-skewing conditions (IL-12 plus anti-IL-4 Ab). On day 14 after the initial stimulation, cells were restimulated with a range of concentrations of MCC93−101 peptide plus APCs, and T cell proliferation was assessed. Interestingly, itk−/− T cells proliferated far more vigorously than control (itk+/+) T cells on secondary stimulation, indicating that the control T cells had either failed to proliferate or had undergone AICD (Fig. 2A). To ensure that these previously activated itk−/− T cells still possessed defects in IL-2 production in a secondary response, we performed intracellular IL-2 staining. These assays indicated that previously activated itk−/− T cells consistently produced reduced levels of IL-2 over a wide range of peptide concentrations (Fig. 2B). These data support the notion that itk−/− CD4+ T cells retain a TCR signaling defect after secondary in vitro stimulation.

**FasL up-regulation is defective in itk−/− CD4+ T cells**

FasL transcription is regulated by a number of factors, including NF-κB, NF-AT, AP-1 (fos/jun), and Egr family members, all of which are activated in response to TCR stimulation (26–31). A previous study has demonstrated that itk−/− CD4+ T cells are impaired in their ability to efficiently translocate cytoplasmic NF-AT to the nucleus on TCR stimulation (19). Together with our observation that itk−/− T cells proliferate more vigorously on secondary stimulation compared with itk+/+ T cells, this finding suggested that itk−/− T cells may be impaired in the expression of FasL. As an initial effort to assess whether proliferative differences between control and itk−/− T cells were due to differences in Fas/Fasl-mediated AICD, we repeated the secondary in vitro proliferation assays in the presence of a neutralizing anti-FasL Ab (Fig. 2C). These experiments indicated that the presence of anti-Fasl Ab, but not an isotype control Ab, blocked AICD and restored the proliferative capacity of control (itk+/+) T cells. In contrast, the anti-Fasl Ab had no effect on the proliferative responses of itk−/− T cells. Interestingly, at high peptide concentrations (100 nM), both wild-type and itk−/− T cells undergo AICD in the presence of anti-Fasl Ab. This is likely due to the up-regulation of FasL in the itk−/− T cells in response to very strong TCR signaling (100 nM peptide vs 10−6 or 10−1 nM peptide), which may be more difficult to block with the concentrations of anti-Fasl Ab used. Nonetheless, at lower concentrations of peptide, it appears that itk−/− T cells fail to up-regulate FasL after stimulation.

As an additional measure of FasL up-regulation, previously activated T cells were restimulated with peptide and APCs for 9 h, stained for surface Fas and Fasl, and analyzed by flow cytometry. As shown in Fig. 3, we observed induced surface expression of Fasl on itk+/+ T cells at all peptide concentrations, with maximal levels at the highest peptide concentration tested (100 nM). In contrast, itk−/− T cells failed to detectably up-regulate Fasl, except perhaps at the highest peptide concentration where a slight shift in Fasl staining can be seen. Both itk+/+ and itk−/− T cells show no difference in the expression of Fas upon stimulation, indicating that differences in AICD between control and itk−/− T cells are not due to differences in surface expression of Fas.

To confirm that the decreased proliferative responses of itk+/+ T cells and the increased induction of FasL expression correlated with increased apoptosis, itk−/− and itk−/− T cells were stained with annexin V and propidium iodide after stimulation. As shown in Fig. 4, a substantial degree of apoptosis is induced in control T cells after peptide stimulation, whereas itk−/− T cells require stimulation with 10−2 to 10−9 higher concentrations of peptide to induce a comparable degree of apoptosis. The presence of a neutralizing anti-Fasl Ab was able to increase cell viability, on average, by 2.4-fold in stimulations of control T cells. In contrast, cell viability was only increased by 1.3-fold when anti-Fasl Ab was included in cultures of itk−/− T cells. This observation further supports the conclusion that greater levels of functional FasL are expressed on itk−/− compared with itk−/− previously activated CD4+ T cells. These data are also consistent with a previous finding that thymocytes from itk−/− mice are defective in activation-induced cell death in response to anti-CD3 Ab stimulation (32).

**FIGURE 2.** A and B, SC.C7 itk+/+ and SC.C7 itk−/− CD4+ T cells that were initially activated with 2 μM MCC93−101 were restimulated on day 14, and proliferative responses were measured. B, Cells were restimulated on day 12 for 6 h and analyzed for IL-2 production by intracellular staining. C, Proliferative responses of SC.C7 itk+/+ and SC.C7 itk−/− CD4+ T cells that were restimulated in the presence of anti-Fasl Ab or an isotype control Ig (5 μg/ml). Abs were added at time 0 and again at 20 h. P + I, PMA and ionomycin; Ctrl, control.

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One previous study has indicated that another Tec kinase family member, Btk, functions as an inhibitor of signaling through Fas in B cells (33). Therefore, to determine whether signaling through Fas is altered in \( itk^{-/-} \) T cells, cells were treated with a Fas agonist, sFasL. As shown in Fig. 4B, stimulation with sFasL led to comparable levels of apoptosis in both \( itk^{+/+} \) and \( itk^{-/-} \) T cells. These data indicate that signaling through Fas is unperturbed in \( itk^{-/-} \) T cells.

Although IL-2 is commonly recognized as a growth-promoting cytokine that triggers survival and proliferative signals upon binding its receptor, IL-2 can also potentiate AICD by inducing maximal FasL expression (34, 35). This is thought to occur through the action of IL-2R-mediated transcription factors such as SP-1 (36). Furthermore, IL-2R signals have also been shown to down-regulate Fas-associated death domain-like IL-1-converting enzyme-like inhibitory protein (FLIP), an anti-apoptotic molecule (37). Thus, signals through the IL-2R can cooperate with TCR signals to provide a feedback mechanism that renders activated T cells more susceptible to apoptotic death. In light of these data, we were interested to determine whether the reduced ability of \( itk^{-/-} \) T cells to undergo AICD was due, in part, to decreased levels of IL-2 production (Fig. 2B). To address this issue, exogenous IL-2 was added to cultures during restimulation. We found that addition of exogenous IL-2 did not enhance FasL-induced cell death or FasL surface expression on \( itk^{-/-} \) T cells (data not shown), indicating that the defect in FasL expression is not secondary to the decreased levels of IL-2 production seen in \( itk^{-/-} \) CD4\(^{+}\) T cells. Furthermore, because both \( itk^{+/+} \) and \( itk^{-/-} \) T cells are cultured in an excess of exogenous IL-2 during the primary stimulation, differences in IL-2R signaling are unlikely to account for differential expression of FasL or responsiveness to AICD during the subsequent in vitro stimulations.

Calcium, ERK, and JNK pathways are defective in previously activated \( itk^{-/-} \) CD4\(^{+}\) T cells

Stimulation of the TCR leads to the activation of signaling pathways that ultimately result in the generation of active transcription factors leading to new gene expression (38). Previous biochemical studies have demonstrated that Itk plays a role in the phosphorylation and activation of PLCy1 following stimulation of the TCR (17, 39). Activated PLCy1 then converts the membrane phospholipid, phosphatidylinositol 4,5-bisphosphate, into inositol 1,4,5-trisphosphate, an activator of calcium release channels in the endoplasmic reticulum, and 1,2-diacylglycerol, an activator of the

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**FIGURE 3.** Previously activated 5C.C7 \( itk^{+/+} \) and 5C.C7 \( itk^{-/-} \) CD4\(^{+}\) T cells were restimulated on day 14 with APCs and the indicated concentrations of MCC\(_{93-103}\) peptide. After 9 h, cells were stained for CD4, V\(_{e11}\), Fas, and FasL and analyzed by flow cytometry. Ten thousand CD4\(^{+}\)V\(_{e11}^{+}\) events were collected. Dotted line, Nonstimulated cells; bold line, staining of stimulated cells. These data are representative of three independent experiments.

**FIGURE 4.** A and B, Previously activated 5C.C7 \( itk^{+/+} \) and 5C.C7 \( itk^{-/-} \) CD4\(^{+}\) T cells were stimulated for 16 h with \( (w) \) MCC\(_{93-103}\) peptide and APCs, in the presence of anti-FasL or an isotype control Ig. Following stimulation, cells were stained with annexin V and propidium iodide (PI) and analyzed by flow cytometry. A, Example of dot plots of annexin V vs propidium iodide fluorescence; numbers in the lower left quadrant, percentage of live cells present at the time of analysis. B, Summary of data from all stimulation conditions. As a control (Ctrl), cells were stimulated with sFasL alone to ensure comparable levels of susceptibility with that of Fas-mediated apoptosis of the \( itk^{+/+} \) and \( itk^{-/-} \) T cells. In each case, the percentage of live cells was determined by calculating the ratio of live cells in the treated wells to live cells in the wells incubated in the absence of MCC peptide.
Ras and PKC pathways. A sustained increase in intracellular calcium concentrations after TCR stimulation leads to the calcineurin-dependent dephosphorylation of cytoplasmic NF-AT, resulting in NF-AT translocation to the nucleus (40–42). To confirm that previously activated itk−/− CD4+ T cells retain the biochemical defects characterized in primary resting itk−/− CD4+ T cells (17), intracellular calcium mobilization was measured upon re-stimulation of 5C.C7 itk−/− T cells initially stimulated with 2 μM MCC93–103 plus APCs and cultured for 10 days in exogenous IL-2. As demonstrated in Fig. 5A, itk−/− T cells show a significant defect in calcium mobilization compared with itk+/+ T cells after stimulation through the TCR. These data indicate that previously activated itk−/− T cells exhibit a comparable deficit in signaling compared with freshly isolated ex vivo itk−/− T cells.

Recently, several reports focusing on Itk biochemistry have demonstrated that Itk is recruited to the linker of activated T cells/SH2 domain-containing 76-kDa leukocyte protein (SLP-76) complex in response to TCR signaling, thereby providing a scaffold for Itk to activate PLCγ1, potentially by direct phosphorylation. These data place Itk intermediate between proximal TCR signaling events and downstream events such as the activation of the Ras pathway (Refs. 43–46; reviewed in Ref. 16). Activated Ras is known to activate the ERK/MAPK pathway, which subsequently leads to the transcription of fos proteins, and ultimately to the formation and activation of AP-1 complexes (reviewed in Ref. 47). In addition, the ERK proteins, ERK1 and ERK2, have recently been shown to play a role in AICD by inducing FasL transcription (9). Consistent with these findings, the FasL promoter was also found to possess target sites for AP-1 transcription complexes (30).

In light of these data, we were interested in determining whether Ras and PKC pathways. A sustained increase in intracellular calcium concentrations after TCR stimulation leads to the calcineurin-dependent dephosphorylation of cytoplasmic NF-AT, resulting in NF-AT translocation to the nucleus (40–42). To confirm that previously activated itk−/− CD4+ T cells retain the biochemical defects characterized in primary resting itk−/− CD4+ T cells (17), intracellular calcium mobilization was measured upon re-stimulation of 5C.C7 itk−/− T cells initially stimulated with 2 μM MCC93–103 plus APCs and cultured for 10 days in exogenous IL-2. As demonstrated in Fig. 5A, itk−/− T cells show a significant defect in calcium mobilization compared with itk+/+ T cells after stimulation through the TCR. These data indicate that previously activated itk−/− T cells exhibit a comparable deficit in signaling compared with freshly isolated ex vivo itk−/− T cells.

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Activated Ras also plays a role in the activation of the MAPK kinase kinase-1/JNK pathway, which is essential for transcriptional activation of the FasL promoter via the activation of c-Jun (8, 49). To further assess the role of Itk in the activation of the Ras pathway and its downstream effectors, we examined the level of JNK/SAPK phosphorylation in itk−/− cells upon TCR stimulation. As demonstrated in Fig. 5B, the activation of the JNK/SAPK pathway is also impaired in itk−/− CD4+ T cells. Similar to ERK phosphorylation, treatment with PMA and ionomycin induced comparable levels of SAPK/JNK phosphorylation in itk+/+ and itk−/− cells. Collectively, these biochemical data strongly suggest that itk−/− CD4+ T cells are unlikely to accumulate normal levels of active c-Fos and c-Jun and thus are likely to have reduced levels of AP-1 complexes after TCR stimulation.

**Reduced Egr2, Egr3, and FasL transcription after TCR stimulation of itk−/− T cells**

Several transcription factors such NFAT, NF-κB, Egr2, as well as Egr3, have been implicated in the TCR-mediated activation of the FasL promoter. In fact, the FasL promoter contains consensus sequences for NF-AT, NF-κB, and Egr, as well as the AP-1 factors, c-Fos, and c-Jun (27, 29, 50, 51). The Egr2 and Egr3 genes are normally expressed at low basal levels in resting T cells, and are transcriptionally induced following TCR stimulation (52). Furthermore, the Egr2 and Egr3 promoters themselves are targets of NF-AT proteins and in turn function as strong trans activators of the FasL promoter (31). Consistent with these findings, the over-expression of either Egr2 or Egr3 in T cell hybridomas or HeLa cells induces FasL transcription (27, 50). Additional evidence also indicates that the induction of Egr2 and Egr3 transcription, and consequently FasL expression, is inhibited by the calcineurin inhibitor, cyclosporin A (27, 50). Despite these compelling data, there have been conflicting results regarding which factor, Egr2 or Egr3, is more critical for FasL transcription. Nonetheless, the bulk of the evidence indicate that NF-AT and Egr factors act synergistically in the activation of the FasL promoter.

Recent studies have demonstrated that itk−/− CD4+ T cells are defective in the nuclear translocation of NF-AT upon TCR stimulation (19); furthermore, dominant-negative Itk can inhibit TCR-induced NF-AT-dependent transcription (43). Given these observations and the fact that the Egr2 and Egr3 promoters are regulated by NF-AT, we reasoned that Egr2 and Egr3 transcription might be defective in itk−/− T cells, resulting in impaired FasL transcription. To test this idea, we used real-time quantitative PCR analysis to determine the mRNA levels of Egr2, Egr3, FasL, and β-actin in resting and stimulated T cells.

**FIGURE 5.** A. 5C.C7 itk+/+ and 5C.C7 itk−/− CD4+ T cells were loaded with fluo-3 and fura-Red on day 10 post-initial stimulation with 2 μM MCC93–103 peptide. Intracellular calcium was then measured in response to anti-CD3 Ab cross-linking, followed by ionomycin (Iono.) stimulation. Data are displayed as the ratio of fluo-3 to fura-Red fluorescence. B. Previously activated 5C.C7 itk+/+ and 5C.C7 itk−/− CD4+ T cells were restimulated on day 14 by anti-CD3 Ab cross-linking for 0, 2, 5, or 10 min. As a control, cells were treated with PMA and ionomycin (P+1) for 15 min. Total lysates were immunoblotted with an anti-phospho-ERK (p-ERK1 and 2) or an anti-phospho-SAPK/JNK (p-SAPK/JNK) Ab. The membrane was stripped and reprobed for ERK or SAPK/JNK protein, respectively.
Since Fas-dependent death can occur at low levels in naive CD4+ T cells upon strong stimulation of the TCR within the first 18 h (53), we first sought to determine the expression levels of the Egr2, Egr3, and FasL transcripts in naive 5C.C7 itk+/+ or itk−/−CD4+ T cells that were stimulated with anti-CD3 Ab for 0, 6, 18, or 48 h. For these experiments, CD4+CD44low T cells were sorted from mice of each genotype, to prevent ambiguities caused by the presence of activated/memory T cells in the unsorted populations. For the analysis of previously activated T cells, 5C.C7 itk+/+ and 5C.C7 itk−/−CD4+ T cells were stimulated with 2 μM MCC957,103 peptide plus APCs in Th1-skewing conditions and then expanded in IL-2. These cells were then restimulated on day 14 with anti-CD3 Ab for 0, 1, 2, 4, and 6 h.

Fig. 6A shows an example of raw data obtained from this analysis. The amount of PCR product present, as measured by fluorescence intensity, is indicated for each PCR cycle. As can be seen, the β-actin curves for both samples (itk+/+ and itk−/−) are virtually superimposable, indicating nearly identical amounts of cDNA in these samples. In contrast, the FasL curves do not superimpose, indicating a difference in the copy number of FasL transcripts between the stimulated itk+/+ and itk−/− T cells. By interpolation of these data to a standard curve, absolute values for FasL copy numbers can be obtained for each sample. To normalize for the amount of cDNA present in each sample, a ratio of the average copy numbers of Egr2, Egr3, and FasL to β-actin copy numbers was calculated for each data point. The data from a representative experiment of each type are shown in Fig. 6B. This analysis demonstrated decreased levels of Egr2, Egr3, and FasL transcripts in primary ex vivo itk−/−/CD4+ T cells after stimulation, although the magnitude of this reduction is modest. A more striking deficit was seen in the analysis of previously activated itk−/− T cells, where we observed, on average, a 5- and 6-fold decrease in Egr2 and Egr3 levels, respectively, and a 3-fold decrease in levels of FasL transcripts compared with the levels in the itk+/+ T cells at the peak of the response. Interestingly, these data also clearly demonstrate the dramatic enhancement in TCR-mediated Egr3 and FasL transcription in previously activated compared with naive CD4+ T cells. For example, the peak of Egr3 expression in previously activated itk−/− T cells is nearly 40-fold higher than in naive T cells, as is the case for FasL as well. In contrast, peak Egr2 levels only increase by ∼2-fold in previously activated compared with naive CD4+ T cells. These data are consistent with a more important role in FasL transcription for Egr3 than for Egr2. Together these data clearly indicate that signaling through Itk plays an important role in the TCR-induced up-regulation of Egr factors and that impaired expression of Egr2 and Egr3 in itk−/− T cells correlates with impaired FasL expression.

**FIGURE 6.** Purified naive (CD44low) 5C.C7 itk+/+ or 5C.C7 itk−/− CD4+ T cells (2 × 10^6) were stimulated with anti-CD3 Ab for 0, 6, 18, and 48 h (primary). For secondary cells, 2 × 10^5 5C.C7 itk+/+ and 5C.C7 itk−/− CD4+ T cells were initially stimulated with 2 μM MCC peptide plus APCs on day 0 and restimulated with anti-CD3 Ab on day 14 for 0, 1, 2, 4, and 6 h. Following stimulation, RNA was isolated, and 1 μg was reverse transcribed into cDNA and subjected to real-time quantitative PCR analysis for β-actin, FasL, Egr2, and Egr3. A, Example of raw data obtained from real-time quantitative PCR analysis. Dashed line, Cycle threshold value at which individual samples were compared. B, Egr2, Egr3, and FasL transcript levels were determined for naive (primary) and secondary cells. Each sample was run in triplicate, and the average template copy number was determined by interpolating the cycle threshold (Ct) value on a standard curve. These values were normalized to β-actin transcript values determined for each respective data point. The y-axis represents the ratio of Egr2, Egr3, or FasL to β-actin in the sample. Error bars, SD of the Egr2, Egr3, or FasL values obtained from triplicate reactions. These data are representative of three independent experiments. RFU, Relative fluorescence unit.

**CD4+ T cells in itk−/− mice are defective in AICD in vivo**

It has been well documented that mice deficient in the expression of Fas (lpr) or FasL (gld) possess profound defects in the peripheral deletion of activated lymphocytes and develop severe autoimmune disorders as a result of a failure to maintain peripheral T cell tolerance (reviewed in Ref. 2). Furthermore, CD4+ T cells...
from mice bearing the lpr or gld mutations are resistant to TCR-mediated apoptosis upon anti-CD3 or superantigen stimulation (54–57). When injected into mice, SEB, a bacterial superantigen, selectively activates Vβ8+ T cells. This leads first to the expansion of Vβ8+ T cells, followed by a steep decline in the percentage of these cells as a result of Fas-mediated cell death (58, 59). Thus, we were interested in determining whether Itk-deficient T cells would possess defects in AICD in vivo, as a result of the defective FasL expression we observed in vitro. To assess this, we injected itk+/− and itk−/− mice with SEB and examined peripheral blood T cells on days 3, 7, 11, and 15 postinjection. Fig. 7 shows the percentage of CD4+ cells bearing Vβ8, or Vβ6 as a control, over the course of the response to SEB. Interestingly, itk+/− and itk−/− mice initially responded similarly to SEB, as seen by the equivalent increases in Vβ8+CD4+ T cells on day 3 after injection. Following the expansion phase of the response, itk−/−/Vβ8+ T cells under went deletion, as has previously been reported. In contrast, itk−/−/Vβ8+ T cells survived to a much greater degree than control T cells, indicative of reduced AICD. Fig. 7B demonstrates that both itk+/− and itk−/− mice had comparable percentages of the control Vβ6+CD4+ T cells, which are not reactive to SEB. Overall, these data suggest that itk−/−CD4+ T cells are inefficient at undergoing AICD, most likely as a result of reduced FasL expression. Consistent with this conclusion, we routinely observe a 2-fold increase in the proportion of CD4+ T cells with an activated/memory phenotype in itk−/− compared with itk+/− control mice (data not shown). However, we cannot rule out the possibility that the levels of IL-2 in vivo may be decreased in SEB-injected itk−/− mice, thereby rendering itk−/−CD4+ T cells less susceptible to FasL-mediated death.

Discussion

Aberrant regulation of the Fas/FasL system has detrimental effects on the health of an organism. Mutations in the Fas or FasL genes result in autoimmunity and lymphadenopathy in mice and autoimmune lymphoproliferative syndrome in humans (3–5). Conversely, increased expression of FasL in HIV-1-infected T cells has been found to be a factor in the T cell depletion that ultimately causes AIDS (60, 61). Currently, the signaling requirements for proper FasL expression are not completely understood. Therefore, investigation of the molecular mechanisms regulating this pathway is warranted. In our efforts to elucidate the role of a Tec family kinase, Itk, in CD4+ T cell effector function, we discovered that Itk plays a crucial role in the signaling pathway that induces FasL expression.

Previous studies by several groups have established that Itk is important for T cell effector functions, including cytokine production as well as the development of protective immunity to pathogen infections (17–19, 48). The data presented in this article address the role of Itk in T cell homeostasis, as opposed to effector function, and demonstrate that Itk is required for efficient apoptosis induced by TCR stimulation. These experiments further strengthen the notion that Itk is a crucial component of the TCR signaling cascade required for the transcription of genes important for proper immune function. The physiological relevance of these findings is reflected in the altered response of itk−/− mice to the superantigen, SEB.

These results also support the notion that Itk may be involved in setting the threshold for TCR signaling (32). In the absence of Itk, the efficiency of TCR signaling is reduced, as assessed by a variety of biochemical and functional readouts, including reduced PLCγ1 activation, calcium mobilization, MAPK activation, and cytokine production. This reduced signaling lowers the overall effector response of the cell. Given that the ability of CD4+ T cells to up-regulate FasL is dependent on the integration of TCR signals, itk−/− T cells may require more receptor stimulation to attain a threshold of signals great enough to induce FasL up-regulation. This is consistent with our observation that, at high peptide concentrations, itk−/− T cells do up-regulate low levels of FasL and can undergo AICD.

These experiments led to the surprising finding that, during the initial phase of the response, itk−/− T cells expand comparably with itk+/− T cells in response to SEB injection in vivo. Given that previous studies have documented both reduced IL-2 production and proliferation of itk−/− T cells in vitro, these findings were somewhat unexpected. One interesting explanation for this discrepancy is the possibility that SEB produces such a strong activation signal in naïve T cells that FasL expression may be induced early during the activation process. Thus, the net expansion of Vβ8+ T cells in the control mice may reflect the combined effects of proliferation being offset by some apoptosis. Consistent with this possibility, upon infection of lpr/lpr mice with lymphocytic choriomeningitis virus, there is a notable increase in the rate of expansion of antiviral CTLs during the initial phase of the response compared with what is typically observed in wild-type mice (62). Thus, T cells in itk−/− mice may proliferate more poorly but may also undergo less apoptosis, yielding the same net outcome of Vβ8+ T cell numbers at the peak of the response as are found in itk−/− mice.

Although our data directly demonstrate reduced transcription of FasL in stimulated itk−/− T cells in vitro, the interpretation of the in vivo experiments is clearly more complex. Activation-induced cell death can involve the action of other molecules in addition to Fas/FasL, such as TNF-α and Bcl-2 family members. We have not fully ruled out the possibilities that itk−/− T cells are defective in the expression of one or more of these additional molecules and that such differences might also contribute to the decreased AICD.
of Itk−/− cells in vitro and in vivo. Studies addressing this possibility are currently underway. In addition, it is also possible that Itk−/− T cells express greater amounts of FLIP, an inhibitor of the Fas pathway. Because IL-2R signaling inhibits FLIP transcription (37), it is possible that reduced production of IL-2 by Itk−/− T cells in vivo may result in increased FLIP, thereby rendering Itk−/− T cells less susceptible to Fas-mediated apoptosis. Although differences in FLIP expression in vivo between control and Itk−/− T cells will be interesting to examine in the future, this concern is unlikely to be relevant to our in vitro studies, because stimulated T cells were cultured in an excess of exogenous IL-2. Another concern is the observation by Bonfoco et al. (63) that nonlymphoid FasL is essential for the deletion of SEB-reactive T cells. These investigators also demonstrated that T cell activation was necessary for the induction of nonlymphoid FasL transcription. Therefore, we cannot fully rule out the possibility that Itk−/− mice may be defective in the expression of cytokine(s) or effector molecule(s) necessary to induce the up-regulation of nonlymphoid FasL. Again, this possibility applies only to the in vivo studies and is not relevant to our in vitro studies using purified T cells.

Finally, we also considered the possibility that reduced AICD in vivo by Itk−/− /CD4+ T cells may reflect reduced activation and/or preferential differentiation of Itk−/− T cells into Th2 effectors that express FasL poorly (64, 65). These possibilities were tested by examining Vβ8+CD4+ T cells at the peak of the response (day 3) after SEB injection. When examined by flow cytometry for a panel of activation markers, both Itk+/+ and Itk−/− T cells showed comparable percentages of activated T cells. Furthermore, in vitro stimulation of these cells followed by intracellular staining for IL-2, IFN-γ, and IL-4 indicated no increased proportion of IL-4-producing cells among Itk−/− T cells compared with controls (data not shown). Thus, we found it unlikely that the reduced AICD we observe in vivo in Itk−/− mice is due to a skewed differentiation of Itk−/− T cells into Th2 effectors.

Similar to the FasL gene, the Fas gene is transcriptionally regulated by factors such as c-Fos and c-Jun. A report by Li et al. demonstrated that, although PKC and JNK are involved in the activation of the Fas gene upon TCR stimulation, phosphatidylinositol 3-kinase, calcineurin, and ERK kinases play no role in the signaling pathway leading to Fas transcription (66, 67). These findings suggest that the TCR signaling pathways leading to Fas and FasL transcription are distinct. Our data support the notion that Itk does not play a role in the TCR-mediated up-regulation of Fas. Another recent study indicated that Btk, a Tec kinase family member, acts as an inhibitor of the Fas-signaling pathway in B cells (33). Based on our observations that Itk−/− T cells are equally susceptible to apoptosis after treatment with sFasL, the possibility that Itk plays a comparable role in the Fas-signaling pathway in T cells seems unlikely.

On the basis of the data presented here, we speculate that Itk−/− mice may have altered susceptibility to autoimmune disease. For instance, experimental autoimmune encephalomyelitis (EAE), which can be induced in mice upon adoptive transfer of myelin-specific Th1 cells, is a result of Th1 CD4+ T cells initiating tissue damage to the CNS (68, 69). Interestingly, mice possessing the lpr or gld mutations are resistant to the induction of EAE (70). These findings suggest that FasL-expressing T cells may mediate apoptosis within a target tissue, thereby contributing to the pathology of this disease. Given these observations, it is possible that Itk−/− mice may also show decreased susceptibility to EAE. Alternatively, because the Fas/FasL pathway has been shown to be crucial for the removal of autoreactive T cells in the periphery (71), it is also possible that Itk−/− mice may be more susceptible to other types of autoimmune diseases. For instance, FasL-expressing CD4+ T cells have been shown to be important in the deletion of autoreactive B cells (72), raising the intriguing possibility that Itk−/− mice might have increased susceptibility to autoantibody-mediated autoimmune diseases.

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