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Via an IL-2-Dependent Mechanism

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IL-4 Potentiates Activated T Cell Apoptosis Via an IL-2-Dependent Mechanism

Jian Zhang,† Tamás Bárdos,‡ Qing Shao,§ Jurg Tschopp,‖ Katalin Mikecz,‡‡ and Alison Finnegan†‡

Activation-induced cell death (AICD) of T cells is one of the major mechanisms of peripheral tolerance. The regulation of AICD by IL-4 is poorly understood. In this study, we report that AICD in IL-4-deficient T cells is significantly reduced compared with that in wild-type T cells. This impaired AICD correlates with the failure to induce degradation of cellular FLIP. IL-4-mediated enhancement of AICD and cellular FLIP degradation requires a Janus kinase/STAT-6 signaling pathway. Unexpectedly, these effects of IL-4 could be blocked by a neutralizing anti-IL-2 Ab, and addition of rIL-2 could completely restore the defective AICD in IL-4-deficient T cells. Furthermore, IL-4 regulates the T cell thresholds for IL-2 signaling during AICD. These data suggest that IL-4 promotes AICD via an IL-2-dependent mechanism. *The Journal of Immunology, 2003, 170: 3495–3503.*

 interleukin-4 is a cytokine produced by T cells, mast cells, and basophils that controls cell growth and regulates the immune system (1). IL-4 mediates its function through the binding to and stimulation of the IL-4R. The IL-4R consists of IL-4Rα, which binds IL-4 with high affinity, and the common γ-chain (γc) shared by IL-2, IL-7, IL-9, and IL-15 receptors (2–4). Binding of IL-4 to the IL-4R triggers phosphorylation of Janus kinase (JAK)-1 and JAK-3 (2, 3, 5, 6) leading to the activation of two major signaling pathways known as STAT-6, and insulin receptor substrates (IRS) which include IRS-1 and IRS-2 (2, 3). Tyrosine phosphorylated STAT-6 forms homodimers and translocates to the nucleus where they bind IL-4 responsive elements (2, 3). Recent studies using STAT-6-deficient (STAT-6−/−) mice have demonstrated that many IL-4-mediated effects on gene expression require the activation of STAT-6 (2, 3).

Activation-induced cell death (AICD) results from repeated stimulation through the TCR with Ags or anti-TCR Ab, and is dependent upon the interaction between Fas and Fas ligand (FasL) (7–12). Fas-mediated AICD is an important mechanism for maintaining tolerance to self-Ag (13). Ligation of Fas with FasL induces trimerization of the Fas receptor, and the adaptor protein, Fas-associated death domain protein (FADD), binds to the trimers Fas cytoplasmic region through the interaction of the respective death domains. Pro-caspase-8 (also called FLICE) is then recruited to FADD through binding of the death effector domains, which in turn induces self-activation of caspase-8. The Fas receptor, FADD, and pro-caspase-8 form a functional death-inducing signaling complex (DISC). The activated caspase-8 is released into the cytosol where it results in activation of a caspase cascade that initiates apoptosis (11, 14–17). Recently, a new family of viral FLIP has been described, which interferes with caspase-8 recruitment to the death effector domains of FADD (18). Subsequently, a cellular homolog of viral FLIP has been identified by several groups and variously named c-FLIP, Casper, inhibitor of FLICE, caspase homologue, FADD-like anti-apoptotic molecule, MORT1 associated CED-3 homolog-regulated inducer of toxicity, caspase-like apoptosis-regulatory protein, or usurpin (19–26). During Fas-mediated signaling, c-FLIP is recruited to the DISC and interferes with the recruitment of pro-caspase-8, thereby inhibiting Fas-induced apoptosis (19, 27–29).

Cytokines play an important role in the regulation of T cell apoptosis, but the specific role of IL-4 in the regulation of AICD is controversial. The defective AICD in IL-2Rα−/− T cells cannot be corrected by IL-4 (30). However, a high dose of IL-4 (100 ng/ml) has been shown to potentiate AICD in IL-2−/−, IL-2Rα−/−, and IL-2Rβ−/− T cells (31, 32). Therefore, whether and how IL-4 regulates AICD, especially in the presence of IL-2, remains to be further elucidated. In this study, we attempted to solve this discrepancy by comparing the levels of AICD in wild-type (Wt) T cells with those in IL-4−/− and STAT-6−/− T cells. We demonstrate that IL-4 regulates T cell susceptibility to AICD by an IL-2-dependent mechanism which possibly involves induction of c-FLIP degradation. Our observations indicate that IL-4 is not only a growth factor of T cells, but also an enhancer of AICD.

**Materials and Methods**

**Mice**

Wt and IL-4−/− BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The STAT-6−/− mice described previously (33) were backcrossed into a BALB/c background for 12 generations and then maintained as a homozygote colony in our animal facility. C57BL/6 mice were purchased from National Cancer Institute (Frederick, MD). c-FLIPL−/−
transgene (Tg) mice (C57BL/6 background) were described previously (34). All mice used in these experiments were 6–10 wk old.

**Reagents and cell culture**

The following reagents were purchased from BD PharMingen (San Diego, CA): anti-CD3 (145-2C11), anti-CD28 (37.51), PE-conjugated anti-Fasl (MFL-3), FITC-conjugated and purified anti-Fas (Jo2), PE-conjugated anti-CD3 (145-2C11), PE-conjugated anti-IL-2Rγ mAbs and FITC-conjugated annexin V. Anti-Bcl-2 (C-2) and Bcl-x<sub>d</sub> (t-19), anti-FADD (S-18), and anti-caspase-8 (T-16) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-c-FLIP<sub>L</sub> mAb (DAVE-2) was purchased from Alexis (San Diego, CA), Recombinant mouse (rm) IL-4, recombinant human (rh) IL-2, propidium iodide (PI), streptavidin, and anti-actin Ab (AC40) were purchased from Sigma-Aldrich (St. Louis, MO). A mouse Fas-Fc chimeric protein, rhLGFc, rmFasL which is generated from a DNA sequence encoding amino acid residues 132–279 of mouse FasL fused to the signal peptide of human CD3 and six histidine residues, T cell enrichment columns, anti-IL-4, anti-IL-2, anti-6X histidine, and fluorokine-conjugated rhIL-2 kit were obtained from R&D Systems (Minneapolis, MN). Lympholite-M was purchased from Cedarside Laboratories (Homby, Ontario, Canada). HRP-conjugated goat-anti-rabbit IgG, HRP-conjugated goat-anti-mouse IgG, and HRP-conjugated goat-anti-rat IgG Abs were purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD). Benzyloxy carbonyl (Cbz)-Val-Ala-Asp(Ome)-fluoromethylketone (zVAD-FMK) was purchased from Calbiochem (San Diego, CA). Sybr green I nucleic acid fluorescent dye was purchased from BioWhittaker Molecular Application (Rockland, ME). One step RT-PCR kit was obtained from Qiagen (Venica, CA).

**T cell isolation and activation**

Splenic T cells from naive WT, IL-4<sup>−/−</sup>, and STAT6<sup>−/−</sup> mice were isolated (purity ≥95% as determined by FACSscan analysis of CD3 cell surface expression) on T cell enrichment columns (R&D Systems). For in vitro activation, T cells (2 × 10<sup>6</sup>/ml) were stimulated with plate-bound anti-CD3 (2 μg/ml) and anti-CD28 (1 μg/ml) mAbs in 24-well plates, and then cultured for 72 h in RPMI 1640 supplemented with 10 mM HEPES, 0.1 mg/ml streptomycin, 100 U/ml penicillin, 0.05 mM 2-ME, 2 mM glutamine (all purchased from Life Technologies, Grand Island, NY) in the presence of 10 U/ml rhIL-2. Viable cells were separated from dead cells by Lympholite-M, and were then subjected for restimulation. Viability of cells was determined by PI staining.

**Proliferation assay**

Splenic T cells (2 × 10<sup>6</sup>/ml) from WT, IL-4<sup>−/−</sup>, and STAT6<sup>−/−</sup> mice were stimulated for 48 h at 37°C in round bottom 96-well plates precoated with anti-CD3 mAb (2 μg/ml) and anti-CD28 (1 μg/ml) mAbs. The cells were pulsed with 1 μCi [H]thymidine, and harvested 16 h later. The radioactivity was quantitated using a Wallac 1205 Betaplate beta liquid scintillation counter (Gaithersburg, MD).

**Induction and analysis of AICD**

In vitro activated splenic T cells (2 × 10<sup>6</sup>) were restimulated with either plate-bound anti-CD3 for 8 h in the presence of 10 U/ml rhIL-2 or immobilized rmFasL for 6 h. To identify apoptotic cells specifically, a combination of FITC-conjugated annexin V and PI staining was used as described previously (35). Annexin V binds to phosphatidylserine exposed on the outer leaflet of the plasma membrane early in apoptosis, whereas PI, a marker of membrane permeability, is a late-stage event common to both apoptotic and necrotic cells. The annexin V/PI-stained samples were analyzed using a FACScan (BD Biosciences, Mountain View, CA). An isotype-matched control Ab served to define background values.

**Flow cytometric analysis**

WT and IL-4<sup>−/−</sup> T cells were activated with plate-bound anti-CD3 and anti-CD28 in the presence of 10 U/ml rhIL-2, and Fas expression was detected using flow cytometry. Preactivated splenic T cells from IL-4<sup>−/−</sup> mice were restimulated as indicated in the presence of rhIL-2 (10 or 2 U/ml). The cells were collected, washed twice with PBS containing 1% BSA and 0.05% sodium azide, and then incubated with FITC-conjugated anti-Fas or PE-conjugated anti-Fasl mAb (MFL-3, BD PharMingen) for 45 min at 4°C. After rinsing twice with PBS, cells were fixed in PBS and 1% paraformaldehyde, and the staining was assessed by a FACSscan flow cytometer with CellQuest software (BD Biosciences). An isotype-matched control Ab served to define background values.

To analyze IL-2R expression, purified IL-4<sup>−/−</sup> splenic T cells were stimulated for 72 h with plate-bound anti-CD3 and anti-CD28 mAbs in the presence or absence of 10 U/ml rhIL-2 with different concentrations of rmIL-4 (5–60 ng/ml), or in the presence of rhIL-2 (5–60 U/ml). By the end of the cultures, cells were harvested, and stained with biotin-labeled rhIL-2 followed by avidin-FITC. The expression of total IL-2R was determined by flow cytometry. To detect γc chain expression, IL-4<sup>−/−</sup> T cells were activated for 72 h with anti-CD3 and anti-CD28 in the presence of rhIL-2 (10 U/ml) with various amounts of rmIL-4 as described above, and γc chain expression was determined.

**Quantitative real-time RT-PCR assays of c-FLIP**

Total RNA was purified from cells by a guanidinium isothiocyanate/silica gel-based membrane RNAeasy method (Qiagen) according to the manufacturer’s instructions. The primers were designed using the Primer Express software package that accompanies the ABI PRISM 7900HT sequence detection System (Applied Biosystems, Foster City, CA) based upon the sequences of mouse c-FLIP<sub>L</sub> and c-FLIP<sub>S</sub> from GenBank Accession No.U97076 and Y141042. The forward primer of c-FLIP<sub>L</sub> was 5′-TGAGAAATGTTGACGGGAATGCCGTGAGATGT. The forward primer of c-FLIP<sub>S</sub> was 5′-GACGTCCTTCGAGTCTCACC and the reverse primer was 5′-TCTCCACGTTGCTCTTGC. The real-time RT-PCR was performed using the Qiagen one-step RT-PCR kit. The DNA-internalizing SYBR green I fluorescent dye was used for detection of the PCR product. To verify that equivalent amounts of RNA were added to each c-FLIP PCR, PCR amplification of the GAPDH housekeeping gene was performed in each sample. The forward primer of GAPDH was 5′-CCATGGGAAGACGTGGGC-3′ and the reverse primer was 5′-CAAGTTGTCAATGATCCAC-3′. RT-PCR was performed as follows: reverse transcription reaction, 50°C for 30 min and 95°C for 15 min; PCR amplification: 94°C for 30 s; 55°C for 30 s; 72°C for 30 s, repeated for 40 cycles (ABI PRISM 7900HT sequence detection System; Applied Biosystems).

The relative differences among the samples at different time points were determined using the ΔΔCT (CT: cycle threshold) method as outlined in the Applied Biosystems protocol for RT-PCR. A ΔCT value was calculated for each sample using the CT value for GAPDH to correct for small differences in the RT-PCR and the CT values for the input DNA samples to normalize the c-FLIP<sub>L</sub> or c-FLIP<sub>S</sub> results. A ΔΔCT value was then calculated by subtracting the ΔCT for the control (day 0) from each time point ΔCT within an experiment. The ΔΔCT values were converted to fold differences compared with the control (day 0 sample) by raising 2 to the ΔΔCT power (2<sup>−ΔΔCT</sup>).
**Results**

**IL-4-mediated potentiation of TCR-induced AICD is STAT-6-dependent**

To assess the effects of IL-4 on AICD of T cells, Wt T cells were activated for 72 h with plate-bound anti-CD3 and anti-CD28 mAbs with 10 U/ml rhIL-2 in the presence or absence of neutralizing anti-IL-4 mAb (30 μg/ml), and activated T cells were then restimulated for 8 h with plate-bound anti-CD3 with or without anti-IL-4 in the presence of 10 U/ml rhIL-2. The apoptotic cells were detected by staining of FITC-conjugated annexin V and PI. Apoptotic cells are scored as annexin V+PI−, whereas necrotic cells are annexin V−PI+. A significant number of Wt T cells were stimulated for 8 h with plate-bound anti-CD3 underwent early apoptosis which was annexin V-positive but PI-negative. As shown in Fig. 1A, specific TCR-mediated AICD was significantly abrogated in the presence of a neutralizing anti-IL-4, suggesting that IL-4 plays an important role in the induction of AICD. To further confirm this observation, TCR-mediated AICD was examined in IL-4−/− mice. Consistent with the above result, AICD in T cells lacking IL-4 was significantly impaired compared with that in Wt T cells (Fig. 1B). Furthermore, the defective AICD observed in IL-4−/− T cells was not due to a higher activation threshold because high concentrations of anti-CD3 stimulation (5–10 μg/ml) did not restore the impairment in AICD in IL-4−/− T cells. Taken together, the above observations suggest that IL-4 promotes AICD in T cells.

Upon IL-4R ligation, STAT-6 is tyrosine phosphorylated by JAK-1 and JAK-3, homodimerizes, and then translocates to the nucleus (1–3). We then hypothesized that IL-4-mediated regulation of AICD may be involved in the JAK/STAT-6 signaling pathway. To test this possibility, AICD levels in Wt, IL-4−/−, and STAT-6−/− T cells were compared. As shown in Fig. 1C, T cells from STAT-6−/− mice were also resistant to AICD, indicating that IL-4 regulates AICD via the JAK/STAT-6 signaling pathway. To further confirm this finding, IL-4−/− and STAT-6−/− T cells were activated and then restimulated with anti-CD3 in the presence of rmIL-4. As expected, rmIL-4 did restore the defective AICD in IL-4−/− T cells but not in STAT-6−/− T cells (Fig. 1C). IL-4-mediated restoration of AICD in IL-4−/− T cells is regulated by Fas-FasL interaction because a mouse Fas-Fc chimeric protein significantly abrogated this enhancement (Fig. 1D). To further confirm whether defective Fas-mediated signaling existed in IL-4−/− T cells, Wt and IL-4−/− T cells were preactivated as described above, restimulated for 6 h at 37°C with cross-linked rmFasL, and the apoptotic cells were determined. Consistent with Fig. 1C, FasL-induced T cell death in IL-4−/− mice was significantly impaired (Fig. 1E). It has also been shown that cytokines stimulating cell division will allow cells to become sensitive to AICD (31, 36). Thus, it is critical to know the cell cycle status of IL-4−/− and STAT-6−/− T cells under these conditions. To this end, a thymidine uptake assay was performed. As shown in Fig. 1F, the rates of IL-4−/− and STAT-6−/− T cell proliferation were comparable or even higher than those of Wt T cells. This finding suggests that there may be a special role of IL-4 signaling in priming T cells for AICD. We should note that the induction of AICD in Wt T cells does not require exogenous IL-2 (Fig. 1G) (37), whereas AICD of IL-4−/− T cells requires medium levels of IL-2 plus exogenous IL-4 (Fig. 1C).

**IL-4 up-regulates FasL expression but does not affect Bcl-2 expression**

AICD is mediated by Fas-FasL interaction (9, 10, 37). The expression of Fas or FasL on T cells might affect the susceptibility of T cells to AICD. However, we could not detect any difference in Fas expression between Wt and IL-4−/− T cells after primary CD3/CD28 stimulation (data not shown). It has been shown that FasL expression requires CD3 restimulation (35) and IL-2 can up-regulate FasL expression during primary stimulation (37). We then detected FasL expression after CD3 restimulation in the presence of 10 U/ml rhIL-2. There was no difference of FasL expression observed in Wt and IL-4−/− T cells (Fig. 2, upper panel). This is possibly due to the presence of 10 U/ml rhIL-2 which may be sufficient for induction of FasL expression in IL-4−/− T cells. To test whether this was the case, activated Wt and IL-4−/− T cells were restimulated for 24 h with anti-CD3 in the presence of 2 U/ml rhIL-2, and FasL expression was determined by flow cytometry. IL-4−/− T cells expressed significantly low levels of FasL at the cell surface compared with Wt T cells (Fig. 2A, lower panel).
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These observations suggest that IL-4 can up-regulate FasL expression induced by IL-2, but the defective AICD in IL-4−/− T cells cannot be ascribed to the defective expression of FasL, because equivalent levels of FasL expression were observed at the concentration (10 U/ml) of rhIL-2 used in Wt and IL-4−/− T cells.

IL-4 has been shown to inhibit death in resting T cells and suppresses superantigen-induced T cell death by preventing degradation of Bcl-2 and Bcl-xL (38, 39). Therefore, it was important to determine whether decreased expression of Bcl-2 and Bcl-xL was involved in IL-4-mediated promotion of T cell AICD. Splenic T cells from Wt and IL-4−/− mice were activated in wells coated with anti-CD3 and anti-CD28 for 0, 24, 48, and 72 h in the presence of 10 U/ml rhIL-2. The cells were harvested at each time point, lysed in 1% Triton X-100, and blotted with anti-Bcl-2 and anti-Bcl-xL, respectively. The loading control was performed using anti-actin immunoblotting. The data are from one of three independent experiments.

Enhancement of AICD by IL-4 correlates with increased c-FLIP degradation

It has been shown that c-FLIP associates with FADD following Fas ligation, and functions as an inhibitor of Fas-mediated apoptosis through its interference with the recruitment of caspase-8 to FADD (19, 27–29, 37, 40–42). Therefore, c-FLIP may determine the susceptibility of T cells to AICD. To analyze the effect of IL-4 on c-FLIP expression during TCR-mediated AICD, Wt and IL-4−/− T cells were activated with a combination of plate-bound anti-CD3 and anti-CD28 mAbs for 0, 24, 48, and 72 h in the presence of 10 U/ml rhIL-2. Aliquots of T cells were harvested at each time point, and RNA was extracted and assayed for c-FLIP transcripts by real-time RT-PCR. No decreases but slight increases in both c-FLIPL and c-FLIPS mRNA expression was observed after CD3/CD28 stimulation (Fig. 3A). These findings suggest that IL-4 may not control c-FLIP expression at the transcriptional level.

To investigate how IL-4 regulates c-FLIP expression in the presence of IL-2, splenic T cells from Wt and IL-4−/− mice were stimulated for 0–72 h with plate-bound anti-CD3 and anti-CD28 mAb in the presence of 10 U/ml rhIL-2. The activated T cells were collected at each time point, and c-FLIP expression was assessed by immunoblotting with anti-c-FLIPL and anti-c-FLIPS mAbs. As shown in Fig. 3B, in Wt T cells a degradation of both c-FLIPL and c-FLIPS occurred at 48 h, being evident at 72 h after stimulation, whereas in IL-4−/− T cells c-FLIP degradation was significantly reduced (Fig. 3B). To further determine whether c-FLIP degradation was induced by caspase activation, Wt T cells were pretreated for 15 min with zVAD-FMK, a pan-caspase inhibitor, and then stimulated for 24 and 72 h with anti-CD3 and anti-CD28 in the presence of 10 U/ml rhIL-2. As shown in Fig. 3C, the degradation of c-FLIPL and c-FLIPS was significantly inhibited in zVAD-treated Wt T cells. These results clearly indicate that IL-4 does not regulate c-FLIP expression at the transcriptional levels but rather favors c-FLIP degradation. Furthermore, the presence of rmIL-4 favored c-FLIP degradation in IL-4−/− T cells (Fig. 3B). These observations suggest that IL-4 directly or indirectly controls c-FLIP expression at the posttranslational level, and might mediate the susceptibility of T cells to AICD. In support of this hypothesis, addition of exogenous IL-4 restored AICD in IL-4−/− T cells to a comparable level with that of Wt T cells (Fig. 1C).

Following ligation of the FasR with Fasl, Fas, FADD, and procaspase-8 form a DISC, c-FLIP recruited to the DISC determines the susceptibility of T cells to Fas-mediated apoptosis (19, 27–29, 37, 40–42). Therefore, in IL-4−/− T cells enhanced recruitment of c-FLIP to the DISC may preclude procaspase-8 recruitment to the DISC thereby preventing apoptosis. To test this possibility, splenic T cells from Wt and IL-4−/− mice were activated for 72 h with plate-bound anti-CD3 and anti-CD28 in the presence of rhIL-2, and activated T cells were incubated for 30 min on ice with rmFasl, followed by cross-linking at 37°C for 1, 5, and 15 min with anti-6x histidine mAb or were left uncross-linked (35). The cells were lysed, immunoprecipitated with anti-Fas, and blotted with anti-caspase-8, anti-c-FLIPL, anti-FADD and anti-Fas, respectively. As shown in Fig. 3D, following ligation of Fas with Fasl, high amounts of c-FLIPL and c-FLIPS were recruited to the DISC in IL-4−/− T cells. This recruitment was significantly impaired in Wt T cells, and the only c-FLIP that could be detected in the DISC was p43 intermediates of c-FLIPL. Although pro-caspase-8 was detected at the DISC in IL-4−/− T cells, the amounts of procaspase-8 and active p20 fragments were significantly reduced (Fig. 3D). In contrast, procaspase-8 and its p43 intermediates and active p20 fragments were present in Wt T cells after Fasl stimulation (Fig. 3D). Our data suggest that c-FLIP prevents procaspase-8 recruitment to the DISC.

To further confirm the role of c-FLIP in Fas-mediated T cell death, c-FLIPL Tg mice were generated to show increased expression of c-FLIPL in T cells under the control of the T cell-specific human CD2 enhancer element (34). T cells from Wt C57BL/6 and c-FLIPL Tg mice were preactivated for 72 h with plate-bound anti-CD3 and anti-CD28 in the presence of 10 U/ml rhIL-2. Activated T cells were then stimulated with immobilized rmFasl, for 6 h, and apoptotic cells were determined. c-FLIPL Tg T cells were significantly resistant to Fas-mediated cell death compared with Wt T cells (Fig. 3E). These results indicate that c-FLIP is indeed involved in Fas-mediated cell death in T cells. Note that a small but significant number of T cells from c-FLIPL Tg mice still underwent apoptosis, suggesting that c-FLIPS may also play a role in the regulation of T cell susceptibility to Fas-mediated cell death in T cells.
FIGURE 3. Resistance of IL-4−/− T cells to AICD correlates with the failure of inducing c-FLIP degradation. A. Wt and IL-4−/− splenic T cells were stimulated for 0–72 h with plate-bound anti-CD3 and anti-CD28 mAb in the presence of 10 U/ml rhIL-2. RNA was isolated from all samples, and aliquots from each time point were subjected to c-FLIP L and c-FLIP S mRNA analysis by real-time RT-PCR using a one-step PCR kit from Qiagen. For comparison of RT-PCR results for each time point from Wt and IL-4−/− T cells, a relative quantitation using the comparative CT method was applied. B. Wt and IL-4−/− T cells, either treated with 60 ng/ml rmIL-4 or left untreated, were stimulated as in Fig. 1A. Cells were collected at 0, 24, 48, and 72 h, and lysed in 1% Triton X-100 lysis buffer. The cell lysates were blotted with anti-c-FLIP L/S mAb. The membranes were stripped and reprobed with anti-actin mAb. C. Wt T cells were activated for 24 or 72 h in the presence or absence of a pan-caspase inhibitor zVAD (50 μM). The cell lysates were blotted with anti-c-FLIP L/S. D. Splenic T cells from Wt and IL-4−/− mice were activated for 72 h as described in Fig. 1A. Activated T cells were then incubated for 30 min with recombinant mouse FasL, followed by cross-linking for 0, 1, 5, and 15 min with anti-6X histidine, and lysed in 1% Brij 97 lysis buffer. The DISC was immunoprecipitated with anti-Fas, and determined by immunoblotting with anti-c-FLIP L/S, anti-caspase-8, anti-FADD, and anti-Fas Abs, respectively. E. Splenic T cells from C57BL/6 and c-FLIP L Tg mice were preactivated in Fig. 1A. Activated T cells were ligated for 6 h with cross-linked smFasL, and apoptotic cells were determined. The transgenic expression of c-FLIP L was determined using anti-Flag mAb. Similar results were obtained in three independent experiments.

IL-4 regulates the sensitivity of T cells to AICD via an IL-2-dependent mechanism

Previous studies suggested that IL-2 predisposes T cells to Fas-mediated AICD (37, 43). IL-2 regulates the susceptibility of T cells to Fas-mediated AICD via the down-regulation of c-FLIP expression (37, 43). To test whether degradation of c-FLIP in Wt T cells correlated with increased IL-2 and IL-4 production, the kinetics of IL-2 and IL-4 production by Wt T cells stimulated with anti-CD3 and anti-CD28 were determined. As shown in the upper and middle panels of Fig. 4A, time-dependent increase in IL-2 and IL-4 production and IL-2R expression was observed in Wt T cells, being highest at 72 h following stimulation which correlates with significant degradation of c-FLIP (Fig. 3B). Because it has been shown that IL-2 is a critical cytokine for induction of AICD, it is possible that IL-4 may promote AICD via the increased secretion of IL-2. To test this possibility, IL-2 production during the primary stimulation with anti-CD3 and anti-CD28 mAbs was determined by ELISA. IL-2 production in activated Wt and IL-4−/− T cells was equivalent (Fig. 4A, lower panel). Our data indicate that the production of IL-2 by CD3/CD28-triggered T cells was significantly higher at 48 and 72 h than 24 h. However, an equivalent amount of IL-2 production by IL-4−/− T cells in response to CD3/CD28 stimulation cannot completely rule out the possibility that, in the absence of IL-4, the sensitivity of T cells to IL-2 may be decreased. If this is the case, exogenous IL-2 should restore the defective AICD in IL-4−/− T cells. Indeed, the addition of rhIL-2 restored defective AICD observed in IL-4−/− T cells in a dose-dependent manner (Fig. 4B), suggesting that the role of IL-2 in AICD may be mediated by IL-2. In support of this observation, IL-4-mediated restoration of defective AICD in IL-4−/− T cells was abrogated by a neutralizing anti-IL-2 mAb (Fig. 4B). Most importantly, IL-2R expression in IL-4−/− T cells was significantly reduced after anti-CD3 and anti-CD28 stimulation (Fig. 4C, left panel). Taken together, our observations indicate that IL-4 may regulate the sensitivity of T cells to IL-2 during the induction of AICD possibly via the up-regulation of IL-2R expression.

To verify this result, IL-4−/− T cells were stimulated for 72 h with various concentrations of IL-4 (5–60 ng/ml) with rhIL-2 or without rhIL-2 (10 U/ml), or in the presence of rhIL-2 (5–60 U/ml) alone. The expression of IL-2R was determined by first incubation
with biotinylated rhIL-2 followed by avidin-FITC. Using this approach, we should be able to directly demonstrate whether the binding capacity of IL-2 to IL-2R was up-regulated by IL-4 during induction of AICD. IL-4 itself only had a marginal effect on IL-2R binding capacity of IL-2 to IL-2R was up-regulated by IL-4 during AICD in IL-4

The requirements of IL-2 signaling thresholds for induction of FasL expression and c-FLIP degradation are different

We have shown that at low concentrations of rhIL-2 (2 U/ml), the optimal FasL induction requires IL-4 (Fig. 2). At medium concentrations of rhIL-2 (10 U/ml), induction of AICD requires IL-4/STAT-6 signaling, whereas at high concentrations of rhIL-2 (60 U/ml), IL-4 is not required for induction of AICD (Fig. 4). Thus, these data suggest that FasL expression and induction of AICD require different thresholds for IL-2 signaling. IL-4, by some unknown mechanism, can lower this threshold. To test this hypothesis, T cells from IL-4-/− and STAT-6-/− mice were activated with plate-bound anti-CD3 and anti-CD28 mAbs in the presence of rmIL-4 with 2 or 10 U/ml rhIL-2 or in the presence of 60 U/ml rhIL-2 alone, and cells were collected at 24 and 72 h, respectively. As shown in the left panel of Fig. 5A, the addition of rmIL-4 or rhIL-2 to IL-4-/− T cells favored the degradation of c-FLIP in these cells, whereas the addition of rmIL-4 did not have any effect upon c-FLIP expression in STAT-6-/− T cells. Of note, the addition of 2 U/ml rhIL-2 did not induce any c-FLIP degradation in WT T cells (Fig. 5A, right panel), but 10 U/ml rhIL-2 induced c-FLIP degradation (Fig. 3, B and C). Similarly, the addition of rhIL-2 also induced degradation of both c-FLIPL and c-FLIPΔN in STAT-6-/− T cells (Fig. 5A, left panel). Our data support the idea that the induction of FasL expression and c-FLIP degradation requires different thresholds for IL-2 signaling. To confirm this hypothesis, tyrosine phosphorylation of STAT-5 in IL-4-/− T cells, under the stimulation conditions described above, was detected. Consistent with the above data, certain levels of IL-2 are critical for IL-4-mediated STAT-5 phosphorylation. Low levels of tyrosine phosphorylation were observed in 2 U/ml rhIL-2 and 60

FIGURE 4. IL-4 regulates IL-2R expression during AICD in IL-4-/− T cells. A, Top panel, WT T cells were stimulated for 24, 48, and 72 h with plate-bound anti-CD3 and anti-CD28 mAbs; IL-2 and IL-4 and IL-4 production in the culture supernatants was detected by ELISA; IL-2R expression was detected by staining T cells with biotinylated rhIL-2 followed by avidin-FITC; and IL-2R expression was determined using flow cytometry (middle panel). Bottom panel, WT and IL-4-/− T cells were activated for 72 h with plate-bound anti-CD3 and anti-CD28 mAbs. IL-2 content in the supernatants was determined. B, Splenic T cells from WT, IL-4-/−, and STAT-6-/− mice were activated and restimulated as Fig. 1A in the presence of 10 U/ml rhIL-2. Some of the IL-4-/− T cells were treated with rmIL-4 (60 ng/ml), rhIL-2 (10–60 U/ml), or rmIL-4 (60 ng/ml) plus anti-IL-2 (50 μg/ml). STAT-6-/− T cells were treated with rhIL-2 (10–60 U/ml). Apoptotic cells were scored as in Fig. 1A. C. Left panel, WT and IL-4-/− T cells were stimulated as in Fig. 1A, and IL-2R expression was determined. Middle panel, IL-4-/− T cells were cultured for 72 h in the presence or absence of 10 U/ml rhIL-2 with different concentrations of rmIL-4 (5–60 ng/ml) (IL-2/IL-4 or IL-4), or in the presence of different concentrations of rhIL-2 (5–60 U/ml) with or without rmIL-4 (60 ng/ml) (IL-2/IL-4 or IL-2). Total IL-2R expression was determined. Right panel, IL-4-/− T cells were activated for 72 h with 10 U/ml rhIL-2 with different concentrations of rmIL-4 (5–60 ng/ml), and γc chain expression was determined by flow cytometry. The data represent one of three independent experiments.
anti-c-FLIPL/S mAb. The equal protein loading was confirmed by anti-actin blotting. B, Spleenic T cells from IL-4−/− mice were activated for 15 and 30 min at 37°C with rmIL-4 (60 ng/ml), IL-2 (2 U/ml) and rmIL-4 (60 ng/ml), rmIL-4 (60 ng/ml), rhIL-2 (10 U/ml) plus rmIL-4 (60 ng/ml), and rhIL-2 (60 U/ml). The cells were lysed in 1% Brij 97 lysis buffer, and immunoprecipitated with anti-STAT-5 Ab. The STAT-5 immunoprecipitates were blotted with anti-phosphotyrosine (pTyr) mAb. The membranes were stripped and reprobed with anti-STAT-5 Ab. Data are shown from one of three independent experiments.

Discussion
AICD is one of the major mechanisms for maintaining peripheral tolerance in T cells, and is dependent upon the interaction between Fas and FasL (7, 9, 37, 45). In this study, we demonstrate that AICD in IL-4−/− T cells is impaired, and this impaired AICD cannot be ascribed to the defective expression of FasL or Bcl-2 and Bcl-xL, but rather correlates with the failure of down-regulation of c-FLIP expression. IL-4 favors T cells undergoing AICD by inducing degradation of c-FLIP, and this effect requires signaling through STAT-6. Most importantly, our data indicate that IL-4 regulates the susceptibility of T cells to AICD by influencing T cell sensitivity to IL-2.

Although several studies suggest that cytokines play an important role in T cell apoptosis, the roles of IL-4 in AICD remain to be further elucidated. We found that AICD was defective in IL-4−/− T cells. The addition of rmIL-4 was able to restore the defective AICD in IL-4−/− T cells (Fig. 4B). Interestingly, IL-4-mediated restoration of AICD in IL-4−/− T cells correlates with increased degradation of c-FLIP. These findings indicate that IL-4 is an enhancer of AICD and that IL-4-mediated enhancement of AICD is through the regulation of c-FLIP expression. Note that our observations were similar to those described by Refaeli et al. (37) who found that in IL-2−/− T cells, c-FLIP expression was maintained in activated T cells. This raised the possibility that the enhancement of AICD in IL-4−/− T cells by IL-4 might be mediated by IL-2. Although IL-2 production during the primary stimulation with anti-CD3 and anti-CD28 was equivalent in Wt and IL-4−/− T cells, it remains possible that in the absence of IL-4, T cell sensitivity to IL-2 might decrease. This possibility is supported because 1) the addition of rhIL-2 can restore the susceptibility of IL-4−/− T cells to AICD (Fig. 4B) and favor c-FLIP degradation (Fig. 5A); 2) IL-4-mediated enhancement of AICD in IL-4−/− T cells could be blocked completely by a neutralizing anti-IL-2 mAb (Fig. 4B); 3) IL-4 significantly augments IL-2-mediated IL-2R expression during induction of AICD (Fig. 4C); and 4) induction of FasL expression and c-FLIP degradation may require different thresholds for IL-2 signaling (Fig. 5). Taken together, our observations suggest that the enhancement of AICD in IL-4−/− T cells by IL-4 is mediated by the up-regulation of T cell sensitivity to IL-2. Although the regulation of IL-2R by IL-4 remains controversial (46–50), our data are consistent with those that find that IL-4 can up-regulate the expression of IL-2Rα and IL-2Rβ (46, 47).

The role of c-FLIP in Fas-mediated cell death is still controversial. It was reported that IL-2 can sensitize T cells without affecting expression levels of c-FLIP in primary human T cells (40). Studies using c-FLIP knockout transgenic mice revealed that c-FLIP suppresses FasL-induced T cell apoptosis but not AICD (51). Several possibilities may explain why AICD is not impaired in c-FLIP transgenic mice. First, c-FLIP−/− may be the major isoform responsible for regulating the susceptibility of T cells to AICD (29, 42). In support of this notion, c-FLIPL−/− but not c-FLIPK−/− completely blocks the cleavage of pro-caspase-8 at the DISC (52). Consistent with this notion, our data showed that c-FLIPL Tg T cells are not completely resistant to FasL-induced T cell death (Fig. 3E). Second, the levels of transgenic c-FLIPL in T cells may not be sufficient to block caspase-8 activation required for AICD. It has been shown that c-FLIPL expression at low levels enhances procaspase-8 processing at the DISC, whereas at high levels inhibits...
Fas-mediated apoptosis (53). The role of c-FLIP in the regulation of AICD in vitro and in vivo has also been strongly supported by the following evidence: retrovirus-mediated overexpression of c-FLIP blocks Fas-induced apoptosis of activated T and B cells, which leads to the production of autoantibodies and to the development of autoimmune diseases (54). Consistent with this finding, it has recently been shown that c-FLIP may be involved in controlling lymphodegeneration in IL-2−/− mice (55). These observations suggest that the modulation of c-FLIP is necessary to maintain self-tolerance. In support of these findings, c-FLIP−/− cells are specifically sensitive to death receptor-induced apoptosis (56). Most importantly, we have shown that T cells overexpressing c-FLIPpL are resistant to Fas-mediated T cell death, supporting the notion that c-FLIP plays a role in Fas-mediated AICD.

The regulation of c-FLIP expression is not fully understood. While c-FLIP is encoded on chromosome 1q25, its expression is induced by several cytokines which regulate T cell susceptibility to AICD, and under activation that a STAT-6 inducible gene can enhance the responsiveness of T cells to AICD. IL-4 potentiates AICD by an IL-2-dependent mechanism.

In summary, our observations indicate that IL-4 is involved in the regulation of the susceptibility of T cells to AICD. IL-4 promotes AICD via inducing degradation of c-FLIP, which then increases the susceptibility of T cells to AICD. These two pieces of evidence clearly indicate that the JAK/STAT-6 signaling pathway is required for IL-4-mediated enhancement of AICD.

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