IL-12 Decreases Activation-Induced Cell Death in Human Naive Th Cells Costimulated by Intercellular Adhesion Molecule-1. I. IL-12 Alters Caspase Processing and Inhibits Enzyme Function

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IL-12 Decreases Activation-Induced Cell Death in Human Naive Th Cells Costimulated by Intercellular Adhesion Molecule-1. I. IL-12 Alters Caspase Processing and Inhibits Enzyme Function

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Th cells can receive costimulatory signals through the LFA-1/ICAM-1 accessory pathway that are sufficient to induce early Th cell proliferation, but not subsequent cell expansion and maintenance of cell viability. To investigate the regulatory role for IL-12 in ICAM-1-mediated costimulation, human naive Th cells were stimulated with coimmobilized anti-CD3 mAb and ICAM-1 Ig in the presence or absence of IL-12. The ICAM-1-mediated costimulatory signals in this model resulted in early Th cell proliferation followed by cell death that was partially mediated by Fas and involved loss of mitochondrial membrane potential, processing of procaspase-9 and -3, and activation of caspase-3. Addition of IL-12 prevented activation-induced cell death and promoted late proliferation. ICAM-1 + IL-12-costimulated Th cells were resistant to Fas-mediated cell death through a mechanism that did not appear to involve a decrease in either Fas or Fas ligand expression. IL-12 did not inhibit the loss of mitochondrial membrane potential induced by ICAM-1-mediated costimulation, and this finding was consistent with the inability of IL-12 to increase expression of the antiapoptotic Bcl-2 family members, Bcl-2 and Bcl-xL. Interestingly, IL-12 promoted an altered processing of procaspase-9 and -3 and a decrease in the percentage of cells displaying caspase-3 catalytic function. In conclusion, we now describe a novel regulatory function for IL-12 in preventing Th cell death and, as a result, in greatly increasing Th cell viability and expansion. Together, our findings indicate that IL-12 may perform this regulatory role by preventing Fas-mediated activation-induced cell death through inhibition of caspase-3 enzyme activity. The Journal of Immunology, 2001, 167: 749–758.

Naive Th cell activation leading to proliferation and clonal expansion requires not only Ag-specific recognition by the TCR-CD3 complex, but also costimulatory signals provided by the APC. These costimulatory or second signals are generally generated as a result of interaction between T cell surface-expressed receptors with their counter structures on the APC. Although the LFA-1/ICAM-1 pathway is capable of providing costimulatory signals sufficient for the induction of naive Th cell proliferation (1–4), the CD28/B7 family pathway has been shown to additionally provide costimulatory signals that lead to clonal expansion (5). A critical event that can prevent effective clonal expansion is activation-induced cell death (AICD),4 which results in a lack of cell viability after the first few rounds of proliferation. AICD in primary Th cells is an apoptotic process that can be mediated by the Fas ligand (FasL)/Fas pathway (6) or by a pathway that results in the production of reactive oxygen species (ROS) (7). Loss of mitochondrial membrane potential (Δψm) across the inner membrane of the mitochondria is one early indicator of apoptosis that can occur in many death pathways, including the Fas/FasL and ROS pathways (7–10). Caspases, a family of cysteine proteases (11), are downstream effectors of both the FasL/Fas and ROS pathways that promote DNA fragmentation. In the Fas/FasL death pathway, caspases are activated either directly or indirectly following a loss of mitochondrial integrity. The proteolytic activity of caspases is tightly regulated. Effector caspases exist as inactive precaspases that require specific cleavage by upstream initiator caspases to initiate processing that will result in catalytic activity (12). The effectiveness of the CD28/B7 pathway in costimulation has been attributed, in part, to the induction of the anti-apoptotic Bcl-xL protein, which is involved in maintaining Δψm (13, 14).

Biologically active IL-12 (p35/p40) has a critical role in promoting the differentiation of naive Th cells into Th1 cells (15). In addition, IL-12 is known to have a strong adjuvant effect in promoting T cell-mediated immune responses (16). This adjuvant effect is likely due, in part, to the capacity of IL-12 to enhance IL-2 secretion and responsiveness (17, 18), which can augment proliferation, and/or to induce IFN-γ secretion, which can up-regulate expression of APC costimulatory molecules, such as B7 family members (19) and ICAM-1 (20). Using an in vitro model of Th cell costimulation by accessory cells, we have shown that IL-12 can promote ICAM-1-mediated, CD28-independent costimulaton of human naive Th cells by preventing cell death and enhancing cell expansion (E. Palmer and G. van Seventer, unpublished observations, and Ref. 21). Similarly, a regulatory role for IL-12 in

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preventing apoptosis has also been reported by other investigators studying different models, including HIV-induced Th cell apoptosis (22), Fas-mediated apoptosis in murine and human Th cells (22, 23), and γ-irradiation-induced apoptosis in monocytes (24). However, the mechanism by which IL-12 can prevent apoptosis in all these models remains unknown.

The goal of this study was to characterize the mechanism(s) by which IL-12 prevents AICD in human naive Th cells costimulated by ICAM-1 in the absence of CD28-mediated costimulation. To do this, we used an in vitro model of costimulation by ICAM-1 in the absence of CD28-dependent costimulation to both measure the effects of IL-12 alone in preventing AICD and to directly compare the effects of IL-12- and CD28-mediated costimulation on regulating AICD. Using this model system, we found that the AICD following ICAM-1 costimulation of naive Th cells correlated with a loss of ΔΨm, cleavage and processing of procaspase-9 and -3, and generation of caspase-3 catalytic activity. This cell death was partially inhibited by agonistic anti-Fas mAb. Addition of IL-12 strongly promoted the expansion and viability of ICAM-1-costimulated Th cells through effects that predominately, if not exclusively, inhibited Fas signaling without restoring mitochondrial integrity. Indeed, the most striking effects of IL-12 on apoptotic pathways were the induction of altered processing of procaspase-9 and -3 and inhibition of caspase-3 catalytic activity.

Materials and Methods

Abs and other reagents

The following Abs and purified ligands were used to stimulate or block T cells: anti-CD3 mAb OKT3 (IgG2a), obtained from American Type Culture Collection (Manassas, VA); anti-CD28 mAb 9.3, provided by Dr. C. June (University of Pennsylvania, Philadelphia, PA); and recombinant human ICAM-1 Ig (extracellular domain of ICAM-1 fused to Fc portion of IgG), a gift of P. Hoffman (ICOS, Bothwell, WA). Stimulatory Abs and ligands were indirectly immobilized with goat anti-human IgG-coated and sheep anti-mouse IgG-coated wells (ICN Pharmaceuticals, Aurora, OH). Fas Abs CH-11 (agonist, cross-linking) and ZB4 (antagonist, blocking) were purchased from Kamiya Biomedical (Seattle, WA). Isotype control Abs MOPC21 (IgG1), MOPC195 (IgG2b), and TEP183 (IgM) were purchased from ICN Pharmaceuticals. The following Abs to human proteins were used for Western blotting: caspase-3 (rabbit), caspase-9 (rabbit), Bcl-2 (mouse), Bax (mouse), and Bak (mouse), all purchased from BD Pharmingen (San Diego, CA); Bcl-xL (rabbit) Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary reagents anti-mouse-HRP, anti-rabbit-HRP, and HRP-conjugated NeutrAvidin were purchased from Pierce (Rockford, IL). Biotinylated mouse mAbs to the following molecules were used for flow cytometry: CD25 (clone B-B10, BioSource International, Camarillo, CA) and Fas and FasL (BD Pharmingen); streptavidin-PE (BD Biosciences, San Jose, CA) was used to recognize the biotinylated Abs.

Recombinant human IL-12 (p35/p40) was generously provided by Hoffman-LaRocque (Nutley, NJ). CFSE was purchased from Molecular Probes (Eugene, OR). The following reagents were used to assess cell death: mitochondrial dye 3,3'-dihexyloxacarbocyanine iodide (DiOC6 (3); Molecular Probes, Eugene, OR), a gift of P. Hoffman (ICOS, Bothwell, WA). Stimulatory Abs and ligands were indirectly immobilized with goat anti-human IgG-coated and sheep anti-mouse IgG-coated wells (ICN Pharmaceuticals, Aurora, OH).

Isolation of T cells

Human PBMC from anonymous donors (buffy coats received from LifeSource Blood Services, Glenview, IL, and United Blood Service, Chicago, IL) were isolated by lymphocyte separation medium (Ficoll) density-gradient centrifugation. Resting CD4+CD45RA+CD45RO– naive T cells were obtained by negative selection with mouse mAbs to MHC class II, CD19, CD16, CD14, glycophrin, CD8, and CD45 and magnetic beads (Polysciences, Warrington, PA) as previously described (25). Selected CD45RA+ cells were consistently 99% pure by FACS analysis.

Accessory cell-independent T cell stimulation

The 48-well plates were coated with 0.25 ml of 5 μg/ml each sheep anti-mouse Ig and goat anti-human Ig in PBS overnight at 4°C, washed three times with PBS, and coated with 0.25 ml of 0.3 μg/ml OKT3 and with 0.25 μl of either 0.3 μg/ml of ICAM-1 Ig or anti-CD28 mAb 9.3 in PBS for 1 h at room temperature. After being washed three times with PBS, 0.25 × 10⁶ T cells were added in culture medium (RPMI 1640 supplemented with 10% FBS, 20 mM-L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin; all from BioWhittaker, Walkersville, MD), in a final volume of 0.5 ml, to coated wells with or without 1 ng/ml IL-12. On day 4 after initiation of culture, cells and supernatants were transferred to 12-well plates for expansion with 1 ml fresh culture medium. T cell counts and viability were determined by no-wash flow cytometry on days 4–7 (see below). To determine cell death, naive Th cells were labeled with 1.7 μM CFSE for 10 min at room temperature, quenched with FCS, and washed two times with culture medium before addition to the assay. Cell division analysis was performed using Modfit software (Verity Software House, Topsham, ME).

Flow cytometric analysis was performed on a FACScan (BD Biosciences) as previously described (25) and was analyzed with Lysys II and CellQuest (both from BD Biosciences) software.

Staining cells for cell death analysis

To determine plasma membrane breakdown, ~10 μl of 50 μg/ml PI was added to cell samples immediately before analysis in FL3 parameter on FACS. For ΔΨm, 0.1 ml cultures of T cells were collected, and 0.4 ml of 40 μM DiOC₆(3) (3) diluted in culture medium was added to the cells, which were then incubated at 37°C for 30 min. Samples were immediately analyzed in FL1 parameter on FACS. To determine phosphatidylerine (PS) exposure, T cells were collected, washed twice in bead separation buffer, resuspended in annexin V binding buffer (10 mM HEPES, 10 mM NaCl, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4) at 1 × 10⁶/ml, and incubated with directly labeled annexin V for 15 min at room temperature, in the dark, according to the manufacturer’s specifications. Samples were then diluted in annexin V binding buffer and analyzed on FACSScan within 1 h of staining. To determine caspase activity in living cells, 0.5–1 × 10⁵ T cells were collected in 1.5-ml Eppendorf tubes and centrifuged to remove all supernatant. A total of 50 μl of 10 μM PhiPhiLux-G-D₂ substrate and 5 μl of FCS were added to each sample, and cells were resuspended by gently tapping the tube with the fingers. Open tubes were incubated in 5% CO₂/100% air for 60 min. Finally, cells were detached from the tube by addition of 1 ml ice-cold flow cytometry diluting buffer (provided by manufacturer) and gently resuspended in 0.5–1 ml flow cytometry diluting buffer. Samples were analyzed on FACSScan within 60–90 min of the final wash.

ELISA

The mAb pairs obtained from BD Pharmingen were used at 1–4 μg/ml in a sandwich ELISA to measure IL-2 (sensitivity, 0.2 U/ml) in supernatants. Avidin-peroxidase and ABTS substrate were purchased from Sigma and used as described in the BD Pharmingen protocol.

Cell lyses

Stimulated T cell were collected at various time points, washed one time in PBS, and snap-frozen in LN₂ and stored at ~80°C until detergent lysis. Cell pellets were thawed on ice and lysed in either buffer containing 1% Triton X-100, 50 mM Tris, 300 mM NaCl, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM pefabloc, 1 mM sodium orthovanadate, 2 mM EDTA, and 10 mM NaF, or in modified Laemmlıı buffer containing 60 mM Tris (pH 6.8), 10% glycerol, and 2% SDS, a buffer reported to prevent caspase processing as result of cell lysis (26). Cell membranes were pelleted by microcentrifugation, and the protein content of each lysate-containing supernatant was determined by bicinechonic acid protein assay (Pierce). Next, 5% loading dye (125 mM Tris (pH 6.8), 25% glycerol, 4% SDS, 10% 2-ME, and 0.5% bromophenol blue) was added to samples before boiling and loading, or boiling, snap-freezing, and storage at ~80°C.

SDS-PAGE and Western blotting

Equal microgram amounts (30–50 μg) of cell lysate protein were separated by SDS-PAGE through a 15% polyacrylamide gel and were electroblotted

750 IL-12 DECREASES AICD IN NAIVE ICAM-1-COSTIMULATED Th CELLS

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on to polyvinylidene difluoride membranes. Membranes were blocked with 5% milk in TBST for 1 h at room temperature and blotted overnight at 4°C with primary Abs diluted, as recommended by the manufacturer, in 1% BSA in TBST. After washing, membranes were incubated with appropriate HRP-conjugated secondary reagent, and chemiluminescence detection was performed according to the manufacturer’s instructions (Amersham Pharmacia Biotech, Piscataway, NJ). Densitometry analysis was done with ImageQuant (Molecular Dynamics, Sunnyvale, CA).

**Results**

**IL-12 maintains the proliferation of ICAM-1-costimulated naive T cells**

To study the regulation of T cells by LFA-1-mediated costimulatory signals, we stimulated peripheral blood-derived human naive (CD45RA+CD45RO−) CD4+ Th cells with coimmobilized anti-CD3 mAb and ICAM-1 Ig fusion protein in the absence or presence of recombinant human IL-12, added on day 0. Th cells stimulated with coimmobilized anti-CD3 and anti-CD28 mAbs with or without IL-12 were used as a positive control for cell expansion. The concentrations of anti-CD3 mAb, ICAM-1 Ig (ICAM-1), and anti-CD28 mAb used were chosen to provide consistent and comparable activation of naive Th cells (between both costimulatory conditions and different donors), as determined by [3H]thymidine incorporation at day 4 (data not shown). Th cells were stimulated for 4 days in the presence of Ab and fusion protein (activation phase of the assay) and then were transferred to a larger well to expand for an additional 3 days (expansion phase of the assay) in the absence of Ab and fusion protein.

The relative number of live naive Th cells obtained under the various costimulatory conditions was determined by PI exclusion in a no-wash flow cytometric analysis on days 4–7 of culture. The results are summarized in Fig. 1A. These results indicated that up to day 5 of culture, there was equivalent cell expansion among all four costimulatory conditions (ICAM-1, ICAM-1 + IL-12, anti-CD28, and anti-CD28 + IL-12). However, after day 5, the relative number of live ICAM-1-costimulated Th cells began to plateau, and by day 7, these numbers actually declined to levels significantly lower than that of ICAM-1 + IL-12, anti-CD28-, or anti-CD28 + IL-12-costimulated Th cells. On day 7 of culture, there was no significant difference in the relative number of live Th cells occurring under ICAM-1 + IL-12 and anti-CD28 costimulatory conditions, and both these costimulatory conditions generated approximately four times more live cells than ICAM-1 costimulation, whereas anti-CD28 + IL-12 costimulation generally exceeded the viable cell recoveries of ICAM-1 + IL-12 or anti-CD28. It should be noted that viable cell recovery following stimulation with anti-CD3 alone, IL-12 alone, or anti-CD3 plus IL-12 did not differ significantly from that of unstimulated Th cells (data not shown).

To further examine the increase in expansion induced by the addition of IL-12 to ICAM-1-costimulated Th cells, we analyzed the response of individual Th cells to ICAM-1 and ICAM-1 + IL-12 costimulation. This was done by labeling naive Th cells with the fluorescent vital dye CFSE, which segregates equally between daughter cells upon cell division such that loss of fluorescence corresponds with cell division (27). The proliferative responses of CFSE-labeled Th cells to ICAM-1, ICAM-1 + IL-12, and anti-CD28 costimulation were compared by performing no-wash flow cytometry on CFSE-stained cells to determine the relative number of live Th cells (based on forward scatter vs side scatter characteristics) in each generation during the expansion phase of the assay. The representative CFSE experiment depicted in Fig. 1B shows that, under all three costimulatory conditions, most naive Th cells entered the cell cycle and underwent a similar pattern of cell division while in contact with stimulating Ab and ligand (day 4). However, ICAM-1-costimulation maintained proliferation only until day 5 or 6, whereas both ICAM-1 + IL-12- and anti-CD28-costimulated Th cells continued to proliferate until day 7 (Fig. 1B).

The Th cell proliferation data suggested that ICAM-1 + IL-12 and anti-CD28 costimulation may induce Th cells to produce levels of autocrine growth factors that support late proliferation. Consequently, we determined the amount of the T cell growth factor, IL-2, detectable in the culture supernatant at day 4 and the expression levels of the inducible IL-2Rα-chain (CD25) induced by each costimulatory condition on days 4–6. We found that the differences in late expansion of the various costimulatory populations could not be explained by differences in the amounts of IL-2 secreted or CD25 expressed. In all populations, similar levels of IL-2 were detected in supernatants on day 4, and comparable levels of CD25 were expressed on days 4, 5, and 6 (data not shown). Together, these results show that ICAM-1, ICAM-1 + IL-12, and anti-CD28 costimulation induced similar levels of early naive Th cell expansion and IL-2 production, and that IL-12 enhanced late proliferation of Th cells costimulated with ICAM-1.

It should be noted that Th cells costimulated with ICAM-1 + IL-12 increased expression levels of CD28 ligands (CD80 and CD86) compared with ICAM-1-costimulated Th cells (data not shown). However, blocking studies with a combination of anti-CD80 and anti-CD86 mAbs demonstrated that CD28-mediated costimulation did not contribute to the IL-12-mediated effects on Th cell viability and late proliferation (data not shown).

**IL-12 decreases the portion of ICAM-1-costimulated T cells that die**

In other model systems, ICAM-1 costimulation has been shown to induce death in T cells (4, 28). Thus, we were interested in determining whether ICAM-1 costimulation also induced Th cell death in our model and whether IL-12 may affect the viability of ICAM-1-costimulated T cells. To address these issues, the death of CFSE-labeled Th cells was assessed by PI exclusion after stimulation with ICAM-1, ICAM-1 + IL-12, and anti-CD28. The results of no-wash flow cytometry showed that, after the cells have divided multiple times, cell death occurs in all populations, although to varying extents (Fig. 2). In particular, the percentage of PI+ dead cells (Fig. 2, upper sections of dotplots) increased in ICAM-1-costimulated cultures throughout expansion, resulting in the death of the majority of cells by day 7. In contrast, although ICAM-1 + IL-12 and anti-CD28 costimulation cultures also accumulated dead cells after a few rounds of cell division, these costimulatory signals induced cell death to a much lesser extent than ICAM-1-costimulatory signals. Taken together with our previous results, these data indicate that although ICAM-1 can costimulate the early activation and expansion of Th cells, it cannot sustain late proliferation or Th cell survival but, rather, results in Th cell death. Furthermore, they show that the addition of IL-12 to ICAM-1-costimulated Th cells can rescue late proliferation and promote cell survival, resulting in a comparable viable Th cell recovery at day 7 as with anti-CD28 costimulation. We found that these effects of IL-12 on ICAM-1-costimulated Th cells are dose dependent and optimal at concentrations of 1 ng/ml (data not shown). In addition, the effects are obtained if IL-12 is added to the culture as late as day 3 of stimulation, but they do not occur with the addition of IL-12 on day 4 of culture or later (data not shown).

**Fas-mediated signals contribute to ICAM-1-costimulated death**

Our observations thus far corroborated the reports of others (4, 28) indicating that costimulation with ICAM-1 results in Th cells undergoing cell death. However, it was not known whether the Fas/FasL death pathway, which is involved in some forms of Th cell AICD (6), was also involved in the Th cell death that follows...
activation by ICAM-1 costimulation. Consequently, we performed experiments to examine the contribution of Fas signaling to the death of ICAM-1-costimulated Th cells. Our initial studies focused on determining the expression of Fas and FasL on the surface of Th cells costimulated with ICAM-1, ICAM-1 + IL-12, or anti-CD28. We found that expression of Fas on the vast majority of cells in each costimulatory condition persisted throughout expansion (days 4–7) (Fig. 3A). Furthermore, the percentage of cells expressing low levels of FasL was comparable on day 4, after which it decreased similarly for all populations (Fig. 3B). Together, these results indicated that there was the potential for Fas-mediated death in all culture conditions. In addition, coculture with IL-12 did not appear to either lower the percentage of cells expressing Fas or FasL (Fig. 3, A and B) or the intensity of Fas and FasL staining (data not shown). Interestingly, the expression of FasL rapidly decreases in all conditions after day 5, a point at which the percentage of nonviable cells is still increasing in the ICAM-1 costimulatory condition.

To determine whether signaling through Fas might actually contribute to the death of ICAM-1-costimulated Th cells, the Fas-blocking (antagonistic) mAb ZB4 was added to the various costimulatory cultures on day 4, at the time of Th cell transfer to expansion cultures. Induction of cell death was assessed in live Th cells on day 6 by staining cells with annexin V, a protein that binds to the PS that moves to the outer membrane of cells undergoing apoptosis (29). The amount of ZB4 mAb added to cultures was saturating, as it was able to completely block agonistic Fas Ab CH-11-induced annexin V binding to Th cells in all culture conditions (data not shown). The results shown in Fig. 3C confirm that ICAM-1-costimulated Th cells undergo apoptosis, as demonstrated by binding of annexin V. ZB4 mAb inhibited the percentage of live Th cells positive for PS by ~35% in ICAM-1-costimulated cultures compared with cultures containing isotype control Ab, and the mAb had no inhibitory effect on the PS expression of live Th cells costimulated with ICAM-1 + IL-12 (Fig. 3C). Thus, although Fas-blocking experiments indicate that Fas signaling contributes to the death of ICAM-1-costimulated Th cells, these combined results (Fig. 3, B and C) also suggest that other death mechanisms must be involved in the induction of ICAM-1-dependent AICD. Furthermore, the data indicate that the major, if not only, mechanism by which IL-12 inhibits the death of ICAM-1-costimulated Th cells is by inhibition of Fas-mediated death.

Expression of Bcl-2 family members does not correlate with survival of ICAM-1 + IL-12-costimulated Th cells

Antia apoptotic Bcl-2 family members are effective in inhibiting Fas-mediated death in certain types of T cells (10). Bcl-2 family members regulate both the loss of Δψm and release of cytochrome c from mitochondria in cells undergoing apoptosis (14, 30, 31). Consequently, we investigated whether IL-12 may inhibit ICAM-1-costimulated T cell death by up-regulating expression of the antia apoptotic Bcl-2 family members, Bcl-xL and Bcl-2, and/or by down-regulating expression of the proapoptotic members, Bax and Bak. As shown in Fig. 4A, Bcl-xL was detected at low levels in ICAM-1-costimulated Th cells and at high levels in anti-CD28-costimulated Th cells at 48 h of stimulation. However, the results also show that addition of IL-12 to ICAM-1-costimulated T cells

determined by no-wash flow cytometric analysis over 1 min, followed by further analysis using Modfit software. Undivided cells are represented in the parent (P) generation. Results depict the mean and SE of duplicate cultures derived from one representative of six tested donors different from those donors in A.

FIGURE 1. IL-12 increases the proliferation and yield of ICAM-1-responsive naive Th cells. A, The relative number of live Th cells was determined by PI exclusion in a no-wash flow cytometric analysis on days 4–7 for unstimulated (○), ICAM-1 (■), ICAM-1 + IL-12 (■), anti-CD28 (▲), and anti-CD28 + IL-12 (■) costimulated Th cells. Results represent the mean and SEM of results derived from five donors. B, Naive Th cells were labeled with CFSE and stimulated with coimmobilized anti-CD3 mAb and ICAM-1 Ig with (□) or without (■) IL-12 or with coimmobilized anti-CD3 and anti-CD28 mAbs (■). Th cells were transferred to new wells with supernatant and fresh culture medium on day 4, and Th cells were collected for no-wash flow cytometry on days 4–7. Analysis of cell division was performed on live cells based on forward and side scatter characteristics. The number of live events collected in each generation was determined by no-wash flow cytometric analysis over 1 min, followed by further analysis using Modfit software. Undivided cells are represented in the parent (P) generation. Results depict the mean and SE of duplicate cultures derived from one representative of six tested donors different from those donors in A.
FIGURE 2. IL-12 decreases the portion of ICAM-1-costimulated T cells that die. Naive Th cells were labeled with CFSE and were left unstimulated or stimulated as described in Fig. 1. Cells were collected for no-wash flow cytometry on days 4–7. PI was added immediately before flow cytometric analysis. CFSE (x-axis) vs PI (y-axis) staining of total cells is shown in dot plots. Decreasing CFSE staining indicates cells undergoing an increasing number of divisions. The percentage of PI− cells is noted in each dot plot. One representative of 20 donors tested is shown.

does not alter Bcl-xL expression. Thus, the survival advantage of costimulation with ICAM-1 + IL-12 did not correlate with early enhancement of Bcl-xL expression. Surprisingly, IL-12 was found to inhibit expression of Bcl-xL in Th cells costimulated with either ICAM-1 or anti-CD28 for 96 h (Fig. 4B). Furthermore, this negative influence of IL-12 on Bcl-xL expression in ICAM-1-costimulated cells was maintained through days 4–7 of culture, during which time a similar IL-12-inhibitory effect was found for Bcl-2 expression (Fig. 4C).

Western analysis for the proapoptotic Bcl-2 family members, Bak and Bax, showed mixed results. In both ICAM-1- and ICAM-1 + IL-12-costimulated Th cells, the expression of both Bak and Bax proteins consistently increased with culture time (Fig. 4C). However, whereas coculture with IL-12 was found to actually up-regulate Bak expression in all experiments, the expression of Bax was decreased in the majority of experiments (4 of 7; Fig. 4C). However, the IL-12-induced relative decrease in expression of the antiapoptotic Bcl-2 family members, Bcl-xL and Bcl-2, was observed to be greater than the IL-12-induced relative decrease in Bak expression (Fig. 4C). Thus, together, these data strongly suggest that IL-12 does not inhibit AICD in ICAM-1-costimulated human naive Th cells through effects on the expression of Bcl-2 family members.

Induction of loss of Δψm is similar in ICAM-1- and ICAM-1 + IL-12-costimulated T cells

Loss of Δψm across the inner membrane of the mitochondria is an early indicator of apoptosis generated by many death stimuli (8, 9), including some types of AICD-inducing stimuli (10, 31). We investigated whether the death of ICAM-1-costimulated T cells might correlate with a loss of Δψm during expansion, despite the enhanced expression of Bcl-2 and Bcl-xL during this period. Loss of Δψm was determined by staining with the lipophilic cationic dye DiOC6(3). High DiOC6(3) staining is detected in cells in which Δψm is intact, whereas low staining is detected in cells in which Δψm has dissipated. Live cells were analyzed to determine whether the ICAM-1-costimulatory signal induced a loss of Δψm before the ability to exclude PI was lost. We found that 20–30% of live ICAM-1- and ICAM-1 + IL-12-costimulated Th cells stained low for DiOC6(3) on days 4, 5, and 6, whereas negligible staining was observed in anti-CD28-costimulated live Th cells (Fig. 5). These results indicate that ICAM-1 costimulation initiated a loss of Δψm regardless of the addition of IL-12, whereas CD28 costimulation did not. Based on these results, dead cells would be predicted to accumulate in both ICAM-1- and ICAM-1 + IL-12-costimulated Th cell cultures. However, the results depicted in Fig. 2 show that only ICAM-1-costimulated Th cells accumulate a majority of dead cells. These combined results suggest that IL-12 can inhibit death pathways downstream of mitochondria in ICAM-1-costimulated Th cells.

Pro caspase-9 and procaspase-3 are differentially processed in ICAM-1- and ICAM-1 + IL-12-costimulated Th cells

To determine the potential downstream effectors of Fas signaling that may be affected by mitochondrial dysregulation in ICAM-1-costimulated Th cells, we investigated the cleavage of initiator caspase-9 and effector caspase-3 in the costimulated Th cell populations. Caspases are synthesized as inactive proenzymes that require cleavage and subsequent heterodimerization of large and small subunits for activity (11). Pro caspase-9 processing results from the interaction of Apaf-1 with mitochondria-derived cytochrome c (32). Western blot analysis of cellular lysates collected on day 6 demonstrated that although a portion of caspase-9 is processed in all populations, the resulting fragments of caspase-9 are unique depending on the stimulus (Fig. 6A). Two aspartic acid residues near the end of the large subunit of caspase-9 result in cleavage fragments sized 35 kDa (p35) and 37 kDa (p37), both of which include the prodomain and large subunit (33). In Th cells costimulated with ICAM-1, only p35 was generated (Fig. 6A, lane 1). In contrast, ICAM-1 + IL-12 costimulation resulted in the generation of predominantly p37 (Fig. 6A, lane 2). p35 was generated in anti-CD28-costimulated Th cells, although substantial levels of uncleaved procaspase-9 remained (Fig. 6A, lane 3). We also investigated the effect of IL-12 on caspase-9 processing in anti-CD28-costimulated T cells and found that, similar to its effects on ICAM-1-costimulated Th cells, IL-12 promoted the processing of procaspase-9 to primarily the p37 fragment (Fig. 6A, lane 4).
Effector caspases, such as caspase-3, are activated by initiator caspases and, once activated, the effector caspases cleave cellular substrates, resulting in cell death (12). Effector caspases are characterized by short prodomains. Two aspartic acid residues are associated with the prodomain of procaspase-3, one contained in the prodomain at residue 9 and the other at the junction between the prodomain and the large subunit (11). Investigation of caspase-3 cleavage from its proform (p32) to its large (p17) and small (p12) subunits revealed that although procaspase-3 was processed in every T cell population, the relative proportion of the p21 (prodomain plus large subunit), p19 (partial prodomain plus large subunit), p17, and p12 fragments differed depending on the stimulus. Significantly, the putatively active subunits of caspase-3, p17, and p12, were the most prevalent fragments formed only in ICAM-1-costimulated Th cells (Fig. 6B, lane 1). Addition of IL-12 to ICAM-1-costimulated Th cells did not inhibit the processing of procaspase-9 and -3 but, rather, it inhibited the generation of the p17 and p12 subunits in favor of the p21 subunit (Fig. 6B, lane 2).

In summary, these results show that IL-12 can alter processing of procaspase-9 and -3 in both ICAM-1- and anti-CD28-costimulated Th cells. Naive Th cells were stimulated with coimmobilized anti-CD3 mAb and either ICAM-1 Ig with or without IL-12 or anti-CD28 mAb. Cells were stained for cell surface expression of Fas (A) and FasL (B) on the day indicated. The percentage of cells positive for cell surface proteins is plotted for each stimulation condition. The mean and SEM of results derived from three or more donors is shown. C, Fas blocking during expansion. Naive Th cells were stimulated as described above. On day 4, expansion cultures were distributed over microtiter wells in 100-μl aliquots after addition of fresh medium, and 3 μg of MOPC21 (isotype control) or ZB4 (Fas antagonist Ab) was added to each culture. The percentage of annexin V-PE-binding live cells was determined by forward vs side scatter characteristics on day 6. The mean and SEM of results from three donors tested is shown.
costimulated Th cells. Specifically, IL-12 can induce the processing of procaspase-9 to the p37 fragment and inhibit the generation of the active p12 and p17 subunits of procaspase-3 while increasing the generation of a p21 fragment. Taken together, the results suggest the possibility that IL-12 may inhibit the occurrence of death in ICAM-1-costimulated Th cells by inducing the generation of the catalytically active p12 and p17 subunits of caspase-3.

**IL-12 prevents the generation of catalytic active caspase-3 in ICAM-1-costimulated Th cells**

The results shown in Fig. 6B suggested that ICAM-1 costimulation may lead to the generation of catalytically active caspase-3, whereas addition of IL-12 may promote incomplete processing of procaspase-3 and the generation of an enzymatically inactive caspase-3 form. To directly determine whether this might be the case, ICAM-1- and ICAM-1 + IL-12-costimulated Th cells were loaded with PhiPhiLux-G$_\text{D}_{\alpha}^\text{2}$, a substrate for caspase-3-like enzymes that contains the DEVD sequence. The fluorescence of the PhiPhiLux substrate increases when it is cleaved by caspase-3. The results in Fig. 7 show that on day 7, ~74% of ICAM-1-costimulated Th cells demonstrated caspase-3-like enzyme activity, whereas only ~21% of ICAM-1 + IL-12-costimulated Th cells exhibited increased fluorescence associated with the PhiPhiLux substrate (Fig. 7, right quadrants). Simultaneous PI staining revealed that virtually all cells positive for caspase activity were also in the late stages of death, as demonstrated by their inability to exclude PI (Fig. 7, upper right quadrants). Similar PI exclusion results were observed on days 5 and 6 (data not shown). These results indicate that caspase-3-like enzyme activation is associated with the late stages of death in primary human Th cells costimulated with ICAM-1. Furthermore, they indicate that IL-12 can inhibit caspase-3-like enzyme activity and cell death in ICAM-1-costimulated Th cells.

**Discussion**

The studies we now present show that although naive human Th cell costimulation through the LFA-1/ICAM-1 accessory pathway leads to early proliferation, it cannot support cell expansion by means of late proliferation and maintenance of cell viability. Furthermore, our findings demonstrate that addition of IL-12 to cultures of ICAM-1-costimulated naive Th cells will promote cell proliferation and viability resulting in cell expansion. In addition, they establish that this effect of IL-12 on cell expansion is accomplished, at least in part, by preventing AICD through a mechanism that involves suppression of caspase-3 activation.

We found that coengagement of the TCR and LFA-1 with anti-CD3 mAb and ICAM-1 Ig is capable of stimulating naive Th cells to secrete high levels of IL-2 and of inducing Th cell division (data not shown and Fig. 1). This was a result inferred from prior studies using [\textsuperscript{3}H]thymidine incorporation to measure proliferation in both murine and human Th cells costimulated by ICAM-1 (1–3, 34, 35). Consistent with reports from Damle et al. (28), we also showed that ICAM-1 costimulation leads to the death of human Th cells. Results obtained with a murine ICAM-1 costimulation model differed from those presented here in that, in the absence of exogenous IL-2, there was little IL-2 production or cell division in murine naive TCR-transgenic Th cells stimulated with antigenic peptide and ICAM-1-positive (B7-1- and B7-2-negative) APC, although there was extensive cell death (4, 36). The nature and
strength of the TCR- and LFA-1-derived signals may account for at least some of the differences between our model and the TCR-transgenic model. Nevertheless, despite differences in IL-2 secretion and early proliferation, it is evident that ligation of LFA-1 by ICAM-1 leads to death in both human and murine naive Th cells.

The activation-dependent cell death seen upon naive Th cell costimulation with ICAM-1 occurs as a consequence of a primary stimulation. The mechanism(s) involved in this type of activation-dependent cell death may therefore differ from that of the AICD that takes place after restimulation of previously activated Th cells. Our data are consistent with a model of activation-dependent Th cell death upon ICAM-1 costimulation in which downstream events resulting from activation of the FasL/Fas pathway and some other apoptotic pathway contribute to Th cell death. Specifically, Fas-blocking studies indicated that ~35% of the death seen in naive Th cells costimulated with ICAM-1 can be attributed to FasL/Fas interaction.

Addition of IL-12 to ICAM-1-costimulated Th cells results in an increase in the number of cell divisions in cycling Th cells, a lower percentage of dead cells, and ultimately, four times more live Th cells than with ICAM-1 costimulation alone (Figs. 1 and 2). These results suggest that IL-12 acts in a manner similar to CD28 in sustaining proliferation and enhancing cell survival (5). Does IL-12 promote proliferation, survival, or both in ICAM-1-costimulated Th cells? Our results show that IL-12 enhanced the proliferative capacity of ICAM-1-costimulated Th cells just as it is reported to enhance the proliferation of anti-CD28 costimulated T cells (37, 38). It is difficult to separate proliferation and survival in our system, because they are always linked. Thus, it is possible that IL-12 may induce the outgrowth of Th cells that survive ICAM-1 costimulation, because the percentages of PI+ cells differed greatly between ICAM-1- and ICAM-1 + IL-12-costimulated Th cells, although the number of PI+ cells did not differ (data not shown). However, our analysis of death indicators supports the notion that IL-12 actively inhibits death induced by ICAM-1 costimulation while promoting proliferation.

Our investigation of the expression of antiapoptotic and proapoptotic Bcl-2 family members during Th cell activation and expansion in our stimulatory models revealed that changes in the relative expression levels of these proteins are unlikely to explain the increased survival of ICAM-1 + IL-12-costimulated Th cells. We found that the relative expression of the antiapoptotic Bcl-2 family members, Bcl-2 and Bcl-xL, in Th cells costimulated with ICAM-1 + IL-12 is decreased compared with Th cells costimulated with ICAM-1 only (Fig. 4C). Furthermore, the expression of the proapoptotic Bcl-2 family member Bak is actually up-regulated following IL-12 coculture (Fig. 4C). The expression of the proapoptotic Bcl-2 family member Bax is often (although not always) down-regulated by IL-12 coculture and is not likely to influence the overall outcome, because the relative decrease in expression of Bax is much less than the IL-12-induced decreases in Bcl-2 and Bcl-xL expression (Fig. 4C). Therefore, these observations, in combination with results showing that a loss of Δψm was not inhibited by IL-12 (Fig. 5), suggest that IL-12 induces a survival factor acting downstream of the mitochondria.

The strongest indications that IL-12 actively inhibited the death of ICAM-1-costimulated Th cells are evident in the ability of IL-12 to inhibit Fas-mediated death and to prevent death events downstream of the loss of Δψm. Although one-third of ICAM-1-costimulated Th cells were susceptible to Fas-mediated signals, ICAM-1-costimulated Th cells exposed to IL-12 showed no evidence of Fas-mediated death (Fig. 3C). Furthermore, we found that IL-12 altered ICAM-1-mediated processing of procaspase-9 and -3, (Fig. 6, A and B) and prevented both the activation of procaspase-3 and the accumulation of dead cells (Fig. 7). Loss of Δψm is an early indicator of apoptosis (39). Cytochrome c release from the mitochondria is an event that is known to promote caspase activation and, thus, lead to cell death (32), and to take place independently of but coordinated with loss of Δψm (31, 40). Although we do not know the status of cytochrome c in ICAM-1-costimulated Th cells, it is possible that IL-12 did not prevent a loss of Δψm but did prevent the release of cytochrome c from the mitochondria of ICAM-1-costimulated Th cells in a Bcl-2/Bcl-xL-independent fashion. Alternatively, we believe it is more likely that cytochrome c was released from the mitochondria in all cells costimulated with ICAM-1, and that IL-12 altered the proapoptotic processing promoted by this cytochrome c and possibly altered procaspase-3 processing, as well, in a manner that prevented caspase-3 activation and cell death.

Certain caspase processing patterns are correlated with death, such as the generation of the p35 fragment of caspase-9 and the p17 and p12 fragments of caspase-3. We detected all of these caspase fragments in ICAM-1-costimulated T cells (Fig. 6, A and B), thus indicating that cell death in our model is caspase-mediated. However, in ICAM-1-costimulated cells exposed to IL-12 during activation, caspase-9 was processed to primarily a p37 fragment and caspase-3 to primarily a p21 fragment, while the cells continued to proliferate. The significance of these alternative cleavage patterns is not known. These cleavage products may have no function as incompletely processed enzymes. Alternatively, if they are capable of pairing with fully processed subunits, they may act as decoys that limit the amount of active enzyme generated. It is also possible that the alternative cleavage products may have a function in promoting cellular proliferation, as has been shown for certain caspases in early Th cell proliferation (41–43).

In addition to inhibiting apoptosis in ICAM-1-costimulated Th cells, IL-12 has been shown to inhibit Fas-mediated death in T cells from HIV+ donors (22), both γ-irradiation-induced and Fas-mediated death in macrophages (24), and death of a human Th1 clone resulting from either IL-2 withdrawal or treatment with HIV gp120 (44). In all of these scenarios, death is executed by effector caspases. The ability of IL-12 to block many different death stimuli...
suggests a model in which IL-12 is capable of modifying apoptotic signals at a distal stage in multiple death pathways. Our results demonstrate an inhibitory effect of IL-12 on caspase-3 activity are consistent with this model.

One way in which IL-12 may influence caspase processing at a distal stage of multiple death pathways is by inducing expression of an inhibitor of apoptosis (IAP), a family of proteins that can inhibit apoptosis through direct interaction with caspases (45). In support of such a mechanism of IL-12 function, IAP family members have been shown to interact with caspase-3, -7, and -9 and to be expressed in activated T cells (46, 47). Furthermore, similar to the effects of IL-12, certain IAPs, such as survivin, are involved in promoting cell division (48).

In conclusion, we have characterized a mechanism by which IL-12 prevents a form of AICD in ICAM-1-costimulated Th cells. One way in which IL-12 may influence normal gut homeostasis; specifically, Th1-type cytokines (49). Our findings suggest another way in which IL-12 was lost in mice deficient for the FasL/Fas pathway. Our results would predict that the expansion effect of IL-12 observed by Marth and colleagues is due to the capacity of IL-12 to prevent Fas/Fas-mediated cell death through inhibition of caspase-3 activity. In addition to its beneficial effect on immune responses, the novel regulatory role of IL-12 in promoting cell expansion can also be envisioned to have detrimental effects. The expression of IL-12 in gut-associated tissues of patients with inflammatory bowel disease has, thus far, been correlated mainly with the presence of Th1-type cytokines (49). Our findings suggest another way in which IL-12 may influence normal gut homeostasis; specifically, by inhibiting cell death and, thus, promoting the local expansion of inflammatory cytokine-secreting Th1 cells in the gut, IL-12 may contribute significantly to the pathogenesis of inflammatory bowel disease.

References