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Effector CD4+ T Cells Generate Intermediate Caspase Activity and Cleavage of Caspase-8 Substrates

Ravi S. Misra,* Dawn M. Jelley-Gibbs,† Jennifer Q. Russell,* Gail Huston,‡ Susan L. Swain,§ and Ralph C. Budd‡*†

Caspase-8 activation promotes cell apoptosis but is also essential for T cell activation. The extent of caspase activation and substrate cleavage in these divergent processes remains unclear. We show that murine effector CD4+ T cells generated levels of caspase activity intermediate between unstimulated T cells and apoptotic populations. Both caspase-8 and caspase-3 were partially activated in effector T cells, which was reflected in cleavage of the caspase-8 substrates, c-FLIP1, receptor interacting protein 1, and to a lesser extent Bid, but not the caspase-3 substrate inhibitor of caspase-activated DNase. Th2 effector CD4+ T cells manifested more caspase activity than did Th1 effectors, and caspase blockade greatly decreased initiation of cell cycling. The current findings define the level of caspase activity and substrates during initiation of T cell cycling. The Journal of Immunology, 2005, 174: 3999–4009.

It is becoming increasingly evident that ligation of death receptors and activation of cellular caspases does not necessarily always result in apoptosis. Involvement of the death receptor, Fas, in processes other than cell death has been revealed by the ability of Fas ligation to enhance neural outgrowth (1), cardiac hypertrophy (2), liver regeneration (3), fibroblast proliferation (4), dendritic cell maturation (5), and T cell activation (6). More directly, caspase-8 plays an integral role in T cell function, as humans bearing nonfunctional caspase-8, as well as caspase-8 conditional knockout mice, exhibit severe defects in T cell activation (7, 8). However, there is currently no clear picture of which caspases or which substrates are involved with T cell activation and how far down the caspase cascade these signals propagate.

Traditionally, activation of the upstream initiator caspases that possess large prodomains, such as procaspases-8, -9, and -10, had been thought to require proteolytic cleavage of the zymogen, resulting in the release of large and small subunits (9). These fragments then heterodimerize to form an active caspase molecule. Typically, the cleavage occurs by auto- and cross-proteolysis in response to the ligation of death receptors, which increases the local concentrations of caspases (9–11). The upstream initiator caspase signal is propagated to the effector procaspases, such as procaspase-3, by proteolytic cleavage of the procaspase. As part of this caspase cascade, various caspase substrates are cleaved, including the DNase inhibitor inhibitor of caspase-activated DNase (ICAD)3 (12) and structural proteins, such as actin (13), fodrin (14), and lamin (15).

The earlier view that caspase activation occurs only through its cleavage has been revised for certain upstream caspases. An alternative method of caspase activation is through a conformational change. For example, when caspase-9 interacts with cytochrome c complexed to Apaf-1, a conformational change of caspase-9 exposes its active site (16, 17). In a similar manner, procaspase-8 can induce a structural change in a neighboring procaspase-8 molecule, resulting in active full-length caspase-8 (10). In addition, the enzymatically inactive caspase-8-like molecule c-FLIP1 can also associate with and activate full-length caspase-8 (18). As c-FLIP1 also contains a known caspase cleavage site, it represents potentially one of the earliest substrates of caspase-8 (19).

The significance of caspase activity in nonapoptotic effector T cells is still poorly understood. It is not known how the levels of active caspases or cleavage of their substrates in viable effector T cells compare with those in resting T cells or those undergoing apoptosis after Fas ligation. We observe that intermediate levels of caspase activity are generated at the effector stage of T cell activation, which are proportional to cell cycling, and that blockade of caspase activity inhibits cell cycling. Proximal caspase-8 substrates such as c-FLIP1 and receptor interacting protein 1 (RIP1) are cleaved in effector T cells, but not the downstream caspase-3 substrate ICAD. Furthermore, the caspase activity and cleavage of substrates is more extensive in Th2 effector cells than in Th1 effectors. Collectively, these results define the caspase activity profile in viable T cells and underscore the functional requirements of such activity.

Materials and Methods

Mice

C57BL/6 mice were housed and bred in the University of Vermont animal facility and were used at 2–6 mo of age. B10.Br Vo(11V/β3 AND TCR transgenic (pigeon cytochrome c/Eβ responsive) mice were used at 2–3 mo of age and were bred in the animal facilities at the Trudeau Institute. Both facilities are American Association of Laboratory Animal Care approved and protocols were approved by the Institutional Animal Care and Use Committee at the respective institutions.

T cell purification

C57BL/6 spleens and lymph nodes were isolated and disrupted through nylon mesh in RPMI 1640 (MediaTech) containing 5% (v/v) FCS (HyClone). Erythrocyte lysis of splenocytes was performed using Gey’s solution. Lymphocytes and splenocytes were combined and CD4+ T cells were...
isolated by negative selection. C57BL6 cells were incubated with anti-CD8 (Tib 105), anti-MHC class II (M5/114/15/2; a kind gift of M. Rincón, University of Vermont, Burlington, VT), anti-CD11b (M1/70), and anti-NK1.1 (PK136), and anti-B220 (RA3-6B2) on ice for 45 min. For total T cell isolation, anti-CD8 was omitted from Ab mix. Cells were washed three times and rocked with goat anti-mouse and goat anti-rat conjugated magnetic beads at a 10:1 ratio of beads to cells (Qiagen) at 4°C for 45 min. Magnetic depletion was used to remove bead-bound cells, routinely yielding >90% CD4+ T cells. Cells were washed and resuspended in culture medium (RPMI 1640 supplemented with 25 mM HEPES, 2.5 mg/ml glucose (Sigma-Aldrich), 10 μg/ml folate (Invitrogen Life Technologies), 110 μg/ml pyruvate (Invitrogen Life Technologies), 5 × 10−3 M 2-ME (Sigma-Aldrich), 292.3 μg/ml glutamine (Invitrogen Life Technologies), 100 μM penicillin-streptomycin (Invitrogen Life Technologies), and 5% FBS).

Naive AND CD4+ T cells were enriched from pooled spleen and lymph node cells as previously described (20). In brief, spleen and lymph node cells were passed through a nylon wool column, and nonadherent cells were treated with a panel of CD8, HSA, and MHC class II depleting Ab and complement followed by Percoll (Amersham Biosciences) centrifugation. CD4 purity was routinely >95%, 90–95% of which had a naive phenotype (CD45RBlow, CD62 ligandhigh, CD44low, CD25low) and expressed the TCR transgene. AND transgenic cells were cultured in RPMI 1640 plus penicillin (200 μg/ml; Sigma-Aldrich), streptomycin (200 μg/ml; Sigma-Aldrich), glutamine (4 mM; Sigma-Aldrich), 2-ME (50 μM; Sigma-Aldrich), HEPES (10 mM; Sigma-Aldrich), and 5% FBS (Intergen).

**T cell culture**

C57BL6 CD4+ T cells were activated in culture medium by plate-bound anti-CD3 (5 μg/ml; 145-2C11), anti-CD28 asctes (1:500), and recombinant human IL-2 (50 U/ml; Cet rant) for 2 days. Cells were then removed from anti-CD3 and fed with fresh medium plus IL-2. AND effectors were generated by culturing transgenic CD4+ cells as previously described (20). Alternatively, AND CD4+ T cells were stimulated with DCEK-ICAM APCs (1.5 × 106 cells/ml) pulsed with 5 μM pigeon cytochrome c fragment (PCCF; KAERA2LIALYQKAT) and IL-2 (11 ng/ml). The DCEK-ICAM cell line was originally generated by R. Germain (National Institutes of Health, Bethesda, MD) and expresses B7.1, ICAM-1, and class II MHC (H-2b). APCs were treated with 100 μg/ml mitomycin C (Sigma-Aldrich) for 45 min at 37°C, extensively washed, and then used at a 2:1 T cell/APC ratio.

Murine IL-2 and IL-4 were obtained from culture supernatant of X63.Ag8.653 cells transfected with cDNA for the respective cytokines. Th1 culture conditions included 10 μg/ml anti-IL-4 neutralizing Ab (11B11; R&D Biosciences) and 4 ng/ml anti-IFN-γ neutralizing Ab (XMG1.2; BD Biosciences) and 30 ng/ml IL-4 (R&D Systems). Recombinant murine IL-12 used in AND T cell experiments was a gift of S. Wolf (Massachusetts, Worcester, MA) and were grown in DMEM supplemented with Hams 3F12 (Invitrogen Life Technologies), 5 × 10−3 M 2-ME (Sigma-Aldrich), 292.3 μg/ml glutamine (Invitrogen Life Technologies), 100 μM penicillin-streptomycin (Invitrogen Life Technologies), and 10% FBS.

**RIP1-deficient 3T3 cell culture**

RIP1-deficient 3T3 cells were a kind gift of M. Kelliher (University of Massachusetts, Worcester, MA) and were grown in DMEM supplemented with Hams 3F12 (Invitrogen Life Technologies), 5 × 10−3 M 2-ME (Sigma-Aldrich), 292.3 μg/ml glutamine (Invitrogen Life Technologies), 100 μM penicillin-streptomycin (Invitrogen Life Technologies), and 10% FBS (HyClone).

**Caspase activity assays**

Relative caspase activities were determined per vendor recommendation using the Apo-ONE Caspase-3/7 Assay (Promega). Viable cells were isolated and resuspended in complete medium at 10 × 106 cells/ml. Cells were serially diluted in complete medium and then mixed with 100 μl of Caspase-Glo 3/7 Detection Reagent (Promega) and manufacturer’s instructions. Competition experiments included 100 μM z-VAD in the Caspase-3/7 Reagent mix. Spectrophotometric readings were taken over a range of times and data shown represent readings at 90 or 150 min.

**Immunoblot analysis**

Viable cells were lysed in buffer containing 0.2% Nonidet P-40, 20 mM Tris-HCl (pH 7.4), 2 mM sodium orthovanadate, 10% glycerol, 150 mM NaCl, complete protease inhibitor (Roche Diagnostics), and 10 μM z-VAD (Enzyme System Products). Protein concentration was determined by Bradford assay (Bio-Rad). Protein lysates were boiled for 5 min in loading buffer containing 2-ME and were separated using SDS-PAGE on 12.5% gels. Proteins were transferred onto a polyvinylidene difluoride membrane (Bio-Rad) and blocked using 4% milk in TBS plus 0.1% Tween 20 at room temperature for 1 h. Membranes were incubated at 4°C overnight in milk containing one of the following primary detection Abs: 1 μg/ml actin (I-19; Santa Cruz Biotechnology), Bid (R&D Systems), caspase-3 (a kind gift of Y. Lazebnik, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY), caspase-8 (a kind gift of A. Stass, The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia), ICAD (BD Biosciences), RIP1 (BD Biosciences), or 2 μg/ml FLIP (Dave-2; Apotech). Blots were washed three times for 15 min each and incubated for 90 min with 0.5 μg/ml (caspase blots) or 1 μg/ml (all others) of appropriate secondary Ab conjugated to HRP (Santa Cruz Biotechnology). Blots were washed again and developed using LumiGLO (Kirkgaard & Perry Laboratories).

**Biotin-VAD-fmk caspase precipitation assay**

Viable T cells were incubated with z-FA-fmk control peptide or 100 μM nonbiotinylated z-VAD at 37°C for 15 min followed by another incubation with 10 μM biotin-VAD (Enzyme System Products). Cells were lysed in buffer containing 20 μM biotin-VAD. A total of 600 μg of lysate was then precleared by rocking with 40 μl of Sepharose 6B agarose beads (Sigma-Aldrich) at 4°C for 2 h. Supernatant was then rocked with 30 μl of Strep-tag-II agarose beads (Zymed Laboratories) at 4°C overnight. Beads were washed five times in lysis buffer without protease inhibitor, and then were boiled in loading buffer. Beads were removed by centrifugation, and immunoblot analysis was then performed on supernatants.

**CFSE dye labeling of cells**

Cells were washed with PBS plus 0.1% BSA and then were mixed with 5 μM CFSE in PBS plus 0.1% BSA (Molecular Probes). Samples were divided into 1-ml aliquots and incubated for 10 min at 37°C. Cells were then washed three times. A portion of cells was incubated overnight at 37°C to serve as a noncycling control for flow cytometry, and the remaining cells were stimulated.

**Flow cytometry**

For surface staining, cells were washed in PBS and then incubated with 1 μg/ml FITC-conjugated anti-CD4 Ab diluted in PBS/1% BSA for 30 min. Cells were then washed with PBS/BSA and fixed using 1% formaldehyde in PBS. Samples were analyzed using an LSRII flow cytometer (BD Biosciences).

For TUNEL and Bcl-2 staining, surface staining was first completed as above, with the exception that cells were fixed on ice for 15 min using 2% formaldehyde in PBS. Cells were then washed twice with PBS, fixed in 70% ice-cold ethanol for 15 min, and then washed twice with PBS. Nicked DNA was labeled by incubating cells with TdT buffer (2.5 mM CoCl2, 1 U TdT, and 0.5 nmol biotin-dUTP (Roche Diagnostics) in a total volume of 50 μl at 37°C for 1 h. Cells were then washed twice with 1% BSA in PBS and subsequently incubated with Streptavidin-Texas Red for 30 min. Samples were then washed twice with 1% BSA in PBS and fixed using 1% formaldehyde (Ted Pella) in PBS. For further Bcl-2 staining, cells were permeabilized with 1% BSA and 0.03% saponin (Sigma-Aldrich) in PBS on ice for 10 min. Cells were then washed and incubated with FITC-conjugated anti-Bcl-2 (BD Biosciences) on ice for 30 min. Samples were washed three times and fixed using 2% formaldehyde (Ted Pella) in 1% BSA in PBS. For staining of active caspase-3 intracellular staining, surface staining was first completed and cells were fixed. Cells were washed twice after fixation and the Anti-Active Caspase-3 Apoptosis kit 1 (BD Biosciences) was used to detect intracellular active caspase-3, with PBS/0.03% saponin used in place of Perm/Wash buffer. Cells were washed twice with PBS before TUNEL staining.

**Results**

Effect CD4+ T cells possess active caspases

Although caspase activity is essential for T cell activation (7, 21, 22), the extent of caspase activation and their substrate cleavage have not been defined. Caspase activation and cleavage of known substrates were examined during the progression of naive CD4+ T
cells to the effector stage. Fresh polyclonal CD4+ T cells were purified from C57BL/6 mice, a portion were stimulated with anti-CD3 CD28 in the presence of IL-2, and nonviable cells were removed at all time points by Ficoll centrifugation. Caspase activity was then determined using a DEVD-rhodamine substrate (Fig. IA). Although DEVD has a high affinity for active caspase-3, it also has an appreciable affinity for the other caspases, including caspase-8 (23). This assay is therefore best viewed as an indication of overall caspase activity (9, 24). Fresh CD4+ T cells had negligible levels of caspase activity, whereas viable day 4 effectors manifested considerably increased caspase activity (Fig. 1A). Caspase activity in effector CD4+ T cells was greatly diminished in the presence of the pan-caspase blocker z-VAD-fmk (Fig. 1B), a competitive inhibitor of DEVD, confirming the specificity of the assay. In contrast, apoptotic T cells generated by incubating T cell blasts with soluble Fas ligand (FasL) manifested a 4-fold increase in caspase activity compared with viable effector T cells (Fig. 1C). Intracellular levels of active caspase-3 were assessed in fresh day 4 effectors, and day 4 effectors were treated with FasL (Fig. 1D). This demonstrated monophasic peaks of active caspase-3, whose mean fluorescence intensity increased from 82 in fresh cells to 825 in day 4 effectors and 1717 in effectors treated with FasL. Cells were simultaneously stained for TUNEL, which revealed 3% of fresh cells, 9% of effectors, and 63% of FasL-treated cells were TUNEL−. This demonstrated that the level of caspase activity in effector CD4+ T cells was intermediate between that of resting T cells and of those undergoing programmed cell death. To determine the levels of active caspase-3 in truly nonapoptotic TUNEL− effectors, TUNEL− and TUNEL+ populations of effector cells were gated separately and caspase-3 activity was measured. Active caspase-3 levels were clearly higher in TUNEL+ cells, but TUNEL− cells had levels of active caspase-3 that were still substantially above those of fresh T cells (Fig. 1E).

To further define which caspases contributed to the caspase activity of day 4 effector CD4+ T cells, we examined the activation of upstream caspase-8 and downstream caspase-3 as defined by their cleavage at specific aspartate residues. We also determined cleavage of the known caspase-8 substrates c-FLIPL and Bid as well as the caspase-3 substrate ICAD by immunoblot using lysates from purified CD4+ T cells that were either unstimulated or activated by anti-CD3/CD28 (Fig. 2). Lysates from FasL-treated cells were included as a positive control for substrate cleavage products. One means of caspase-8 activation is by cleavage of its full-length 55-kDa form, resulting in p43/41 caspase-8, through the liberation of a C-terminal p10/12 fragment. Further cleavage of p43/41 caspase-8 leads to the generation of a p18 fragment that heterodimerizes with the p10 fragment to form a fully active protease (25). Caspase-8 was largely present in its full-length 55-kDa form in freshly isolated CD4+ T cells (Fig. 2A). Of note, p18 caspase-8 was absent in effector lysates, but was present in lysates from the FasL-treated population (Fig. 2A). Cleavage of caspase-8 to p43/41 caspase-8 was seen 2 days after activation. Evidence of caspase-8 cleavage was apparent as early as 4 h after T cell activation (21). The intensity of the p43/41 complex increased gradually through day 4 and then decreased slightly by day 6 (Fig. 2A). The kinetics of the cleavage pattern closely paralleled CD25 expression and cell cycle rates (Ref. 21 and data not shown).

c-FLIPL heterodimerizes with caspase-8 and is a caspase-8 substrate (19, 26). In a manner similar to caspase-8, cleavage of c-FLIPL from its full-length 55-kDa form to p43/41FLIPL was considerably more apparent by day 2 (Fig. 2B). This corresponds with a known caspase-8 cleavage site in c-FLIPL at Asp376 (27, 28). Bid was only weakly expressed in fresh T cells, but was considerably up-regulated by day 2 after activation (Fig. 2C). Cleavage of Bid was only slight but mirrored that of caspase-8 activation, becoming apparent at day 2, peaking at day 4, followed by a gradual decrease by day 6 (Fig. 2C). Thus, caspase activation in effector CD4+ T cells led to the cleavage of the known caspase-8 substrate, c-FLIPL, and less extensively downstream, Bid.
Caspase-3 is inactive in its full-length 33-kDa form and is cleaved by upstream caspases, including caspase-8, which liberates a large p20 and a small p12/10 fragment (25, 29). The large and small fragments then dimerize to form the active protease (25). p20 caspase-3 may then be autoproteolytically processed to a p17 fragment, which is considered the mature large caspase-3 subunit (29–31). In fresh CD4⁺ T cells, full-length caspase-3 and p20 caspase-3 were present, whereas active p12/10 caspase-3 was not apparent until day 2 (Fig. 2D). p17 caspase-3 was never observed in the immunoblot of whole cell lysates, even those treated with FasL. This may be explained by the lack of ability of the anti-caspase-3 Ab to strongly detect p17 caspase-3. However, in lysates made from cells treated with biontin-VAD, a 17-kDa band was revealed with HRP-conjugated Strepavidin or anti-biotin Ab and was enriched after the precipitation of active caspases, which provides support for p17 caspase-3 being catalytically active in effector T cells (see Fig. 3, and data not shown). Despite the degree of caspase-3 cleavage, only minimal cleavage of ICAD was detectable (Fig. 2E). Cleavage of each of these caspase substrates was considerably less than that observed in the same cells treated with FasL (Fig. 2).

A conformational change in procaspase-8 induced by c-FLIP₉ interaction results in an active full-length protease (10, 18, 27). Distinguishing active full-length caspase-8 from inactive procaspase-8 is thus not possible by standard immunoblot analysis of whole cell lysates. To better identify the degree of caspase activation and the size of active caspases, we used biontin-VAD-fmk to selectively bind enzymatically active caspase catalytic pockets. Fresh day 0 or day 4 effector CD4⁺ T cells were incubated with biontin-VAD. Lysates were then incubated with avidin-Sepharose beads to affinity purify active caspases. Active caspase-8 and caspase-3 levels were identified using specific Abs in immunoblot analysis of precipitates (Fig. 3A). Whole cell lysates were included to positively identify each caspase band and assess their overall expression (Fig. 3A, lanes 1 and 2). This analysis revealed that full-length caspase-8 bound biontin-VAD in effector T cells (Fig. 3A, top panel, lane 4), but not in unstimulated cells (Fig. 3A, lane 3). A longer exposure of the caspase-8 immunoblot revealed additional p43/41 caspase-8 bands, indicating that this species is also enzymatically active (Fig. 3B, right panel). The binding of biontin-VAD to caspases in effector cell lysates was effectively competed by the addition of 10-fold excess nonbiotinylated z-VAD before incubation of cultures with biontin-VAD (Fig. 3A, lane 5). In addition, no detectable caspase-8 was precipitated using only nonbiotinylated z-VAD (data not shown).

As with caspase-8, more active caspase-3 was present in effector cell lysates than in fresh lysates (Fig. 3A, middle panel, lanes 3 and 4). In contrast with caspase-8, however, there was negligible binding of biontin-VAD to full-length caspase-3, but considerable binding to the small amount of the cleaved p12/10 fragments present in day 4 effector T cell lysates (Fig. 3A, middle panel, lane 4). As with caspase-8, competition with nonbiotinylated z-VAD decreased the amount of precipitated active caspase-3 (Fig. 3A, middle panel, lane 5). These findings are consistent with the accepted model that upstream caspases (e.g., caspase-8) can be active in their full-length form, whereas downstream effector caspases (e.g., caspase-3) require cleavage and dimerization to be active (9, 32). The results demonstrate that activated nonapoptotic CD4⁺ T cells possess more caspase activity than do unstimulated CD4⁺ T cells.

Of additional interest was that death domain kinase RIP1 (Fig. 3A, bottom panel, lane 4), but not Fas-associated death domain protein (data not shown), was found to be associated with active caspases precipitated from effector cells. RIP1 is involved with the generation of apoptotic signals by TNFR2 (33) and Fas (34, 35), as well as with the activation of NF-κB in response to TNF-α (36, 37), and thus acts as a link between NF-κB activation and apoptosis. RIP1 both associates with c-FLIP and is a known caspase-8 substrate (33, 38, 39). Reinforcing the observation that RIP1 was associating with active caspases, RIP1 was not seen in precipitates from fresh T cells (Fig. 3A, bottom panel, lane 1). RIP1 association was specifically reduced upon the addition of excess nonbiotinylated z-VAD (Fig. 3A, bottom panel, lane 5). In addition to RIP1, both full-length c-FLIP₉ and p43FLIP were also precipitated with the active caspase complex (Fig. 3B). Thus, RIP1 and c-FLIP become complexed with active caspases.

To illustrate that p55 caspase-8 is in fact active, effector cells were treated with FasL for 1 or 2 h, and active caspases were labeled, precipitated, and analyzed by immunoblot (Fig. 3C).
Whole cell lysates and the portion of cell lysates that did not bind to avidin-Sepharose ("Flow Through") were also included in the immunoblot. As expected, anti-caspase-8 Ab revealed that p55 and p43 caspase-8 were precipitated with biotin-VAD (Fig. 3 C, top panel). It is important to note that the proportion of total caspase-8 that was precipitated as active caspase-8 was relatively low, as most caspase-8 in effector T cells did not bind avidin-Sepharose (Fig. 3 C, Flow Through). To confirm that biotin-VAD directly bound to full-length p55 caspase-8 in effector but not fresh naive T cells, and this represented only a small fraction of the total caspase-8 in the effector cells.

**Caspase blockade inhibits CD4+ T cell proliferation**

Given the caspase activity of cycling effector CD4+ T cells, we investigated whether this activity was necessary to initiate cell cycling. CD4+ T cells were labeled with the membrane dye CFSE and stimulated in vitro with anti-CD3/CD28 plus IL-2 in the presence of z-VAD or DMSO vehicle control added at the time of activation. As shown in Fig. 4, both medium control and DMSO-treated cells cycled to a similar degree (36%) by day 2, whereas only 20% of z-VAD-treated cells underwent cell cycling. By day

**FIGURE 3.** Activated T cells possess active full-length caspase-8, active cleaved caspase-3, full-length FLIPα, p43FLIP, and RIP1 complexed with active caspases. Freshly isolated polyclonal T cells (lanes 1 and 3) or day 4 effectors (lanes 2, 4, and 5) were incubated with control peptide z-FA-fmk (lanes 1–4) or 100 μM nonbiotinylated z-VAD (lane 5) at 37°C for 15 min. All cells were then incubated with 10 μM biotin-VAD-fmk for an additional 15 min at 37°C. Subsequently, cells were lysed in buffer containing 20 μM biotin-VAD (lanes 1–5). A portion of lysates were then incubated with avidin-Sepharose beads to precipitate active caspases. Immunoblot analysis was performed on precipitates for caspase-8 (top panel), caspase-3 (middle panel), or RIP1 (bottom panel) to reveal active caspases and RIP1 cleavage products. As a positive control for the presence and size of caspase-8, caspase-3, and RIP1, a portion of nonprecipitated whole cell lysates (WCLs) was included in the analysis from fresh (lane 1) or day 4 effectors (lane 2). Active caspases were precipitated from day 4 effector lysates and analyzed by immunoblot for the indicated molecules. WCLs were included as a reference comparison. A longer exposure of the caspase-8 immunoblot is included to illustrate precipitation of p43/41 caspase-8. C, Day 4 effectors were treated with FasL for 1 or 2 h, followed by biotin-VAD for 15 min. Active caspases were then precipitated and immunoblot analysis was performed for caspase-8. A portion of the fraction not binding to avidin-Sepharose (Flow Through) as well as WCLs were included in analysis. Bands were revealed using anti-caspase-8 Ab (top panel) or HRP-conjugated Strepaavidin (middle and bottom panels). Bands at 55 and 43/41 kDa were identified, which corresponds with the bands revealed in the caspase-8 immunoblots (top and middle panels). In addition, a band of 17 kDa was observed that corresponds with cleaved p17 caspase-3 (bottom panel).
3, 60% of medium control cells cycled and 58% of the DMSO control cells, compared with only 32% of cells that received z-VAD. These results indicate that a blockade in caspase activation prevents cycling of murine T cells.

**Th2 effectors manifest more caspase activity than do Th1 effectors**

Given the role of caspase activity for both cell growth and cell death, the level of caspase activity in effector T cells could profoundly influence the proportion of various effector subsets. As Th1 and Th2 cells are differentially responsive to Fas-mediated cell death, we characterized caspase activity and cleavage of caspase substrates in these cells (40, 41). We also wished to determine whether our findings using anti-CD3/CD28 stimulation of polyclonal CD4+ T cells extended to Ag-specific CD4+ T cells. For this purpose we used CD4+ T cells from AND TCR transgenic mice, which recognize PCCF presented in the context of I-EK (42).

AND Th1 and Th2 effector CD4+ T cells were produced in vitro, as described in Materials and Methods. Intracellular cytokine staining and ELISA were used to verify correct polarization of the resulting day 4 effectors (data not shown). Viable day 4 effector T cells were purified by Percoll centrifugation, and a portion was then used in the DEVD-rhodamine release assay to determine overall caspase activity (Fig. 5A). AND Th2 effectors had significantly higher overall caspase activity than did Th1 effectors. The remaining day 4 effectors were extensively washed and rested for 3 days in the absence of exogenous cytokines to reduce cell cycling (43), and viable rested effectors were then purified. Concomitant with their reduced cell cycling, rested effectors manifested considerably diminished caspase activity (Fig. 5B). Nonetheless, Th2 rested effector cells possessed higher caspase activity than did Th1 rested effector cells (Fig. 5B, inset). Thus, caspase activity closely paralleled cell cycling.

To better define which caspases were active, and to what degree, in Th1 and Th2 effectors, biotin-VAD was again used to purify active caspases. In agreement with the DEVD-rhodamine caspase activity assay, Th2 effectors possessed more active full-length caspase-8 than did Th1 effectors (Fig. 6, top panel, lanes 3 and 4), despite having identical overall levels and cleavage patterns of caspase-8 in their whole cell lysates. Comparable results were seen with analysis of active caspase-3 (Fig. 6, middle panel). In addition, more RIP1 was seen in complex with active caspases in Th2 cells than in Th1 cells (Fig. 6, bottom panel).

To determine the downstream targets of increased caspase activity in Th2 effectors, we examined cleavage patterns of candidate caspase substrates in viable AND naive cells as well as in viable polarized AND CD4+ effector T cells stimulated with PCCF. AND naive CD4+ T cells contained predominantly full-length c-FLIP1, whereas activated day 4 Th1 and Th2 effectors expressed cleaved p43FLIP in addition to full-length c-FLIP1 (Fig. 7A). No difference was observed in FLIP RNA levels between Th1 and Th2 effectors (data not shown). However, Th2 cells possessed an additional p27FLIP product with proportionally less full-length c-FLIP1 and p43FLIP. The p7FLIP fragment that we observed was of a molecular mass similar to that of an alternative splice product of FLIP mRNA (FLIPshort), which has been reported to be up-regulated in activated T cells and during restimulation events (44, 45). The p27FLIP fragment that we observed corresponds with a second putative caspase cleavage site at Asp254. In this instance, it would appear that p27FLIP was more likely a further cleavage product than an alternative splice form. Evidence for this was observed in CD4+ T cell lysates from full-length c-FLIP1 transgenic mice. In the case of the transgenic animal, the transgene cannot be alternatively spliced (Fig. 7A). This finding is also consistent with the observation of elevated caspase...
activity in T cells from c-FLIP<sub>L</sub> transgenic mice (R. C. Budd, unpublished observations). Consistent with the concept that p27FLIP can be generated by active caspases, wild-type CD4<sup>+</sup> T cells treated with FasL also manifest a p27FLIP fragment (Fig. 2B). Rested effectors showed a return of full-length c-FLIP<sub>L</sub> (Fig. 7A), consistent with the observed decrease in the caspase activity of these cells (Fig. 5). Nonetheless, the residual higher level of caspase activity in Th2 rested effectors (Fig. 5) was reflected in an increased amount of p27FLIP compared with that seen in Th1 rested effector cells.

To assess the extent of propagation of the caspase cascade in polarized effector T cells, we examined cleavage of the caspase-8 substrates RIP1 and Bid and the downstream caspase-3 substrate ICAD (Fig. 7, B–D). Given our findings with c-FLIP cleavage, RIP1 was a particularly attractive candidate as it both associates with c-FLIP and is a known caspase-8 substrate, yielding a p38RIP1 fragment (33, 38). We observed that RIP1 cleavage patterns in Th1 and Th2 effectors mimicked that of c-FLIP<sub>L</sub> AND naive CD4<sup>+</sup> T cells expressed mainly full-length RIP1, whereas effector cells manifested the p38RIP1 cleavage product with concomitant reduction of full-length RIP1 (Fig. 7B). In each sample, full-length RIP1 appeared as three distinct bands, consistent with recent reports that RIP1 can be ubiquitylated (46). Th2 effectors possessed less full-length RIP1 than did Th1 effectors (Fig. 7B). As with c-FLIP cleavage products, levels of p38RIP1 diminished in both Th1 and Th2 rested effector cells, but Th2 rested effectors manifested less full-length RIP1 (Fig. 7B). The specificity of the full-length RIP1 and p38RIP1 bands was confirmed using lysates from both RIP1-deficient 3T3 cells as a negative control (Fig. 7B).

Unlike c-FLIP<sub>L</sub> and RIP1, Bid is not known to form a complex with caspase-8, although Bid can be cleaved by caspase-8. Interestingly, in contrast with c-FLIP<sub>L</sub> and RIP1, Bid showed only minimal levels of cleavage in either polarized effector or rested effector populations (Fig. 7C). This finding closely paralleled the earlier observations with nonpolarized CD3/CD28-stimulated effector cells (Fig. 2C). In addition, no observed cleavage of the caspase-3 substrate ICAD was detected in either Th1 or Th2 effector or rested effector populations. Collectively, these data show that Th2 cells manifest greater caspase activity and cleavage of upstream caspase substrates than do Th1 cells at the effector and rested effector stages.

**FIGURE 7.** AND Th2 effector CD4<sup>+</sup> T cells show more extensive cleavage of selective upstream caspase substrates than do Th1 effectors. Viable naive (Fresh) day 4 Ag-activated Th1 and Th2 effectors and Th1 and Th2 rested effector populations were purified, and immunoblot analysis of lysates was performed for c-FLIP<sub>L</sub> (A), RIP1 (B), Bid (C), and ICAD (D). Lysates from c-FLIP<sub>L</sub> transgenic CD4<sup>+</sup> T cells were included in A to demonstrate the p43FLIP and p27FLIP cleavage fragments of overexpressed full-length c-FLIP<sub>L</sub>. B, RIP1 represents noncleaved protein and p38RIP1 represents a cleavage product generated by cleavage at Asp<sup>324</sup>. NS, Nonspecific band. C, t-BID is generated by caspase-mediated cleavage at Asp<sup>224</sup>. D, Cleavage of ICAD at Asp<sup>224</sup> results in p18ICAD. Lysates of RIP1<sup>−/−</sup> 3T3 fibroblasts were included as negative controls. Bid (C) and ICAD (D) remained as mostly noncleaved proteins under these culture conditions. Results are representative of four experiments.

Th2 effectors may better tolerate elevated caspase activity due to higher levels of Bcl-2

Conceivably, the higher levels of caspase activity of Th2 cells might result in greater cell death over Th1 cells. However, TUNEL analysis of polarized Th1 and Th2 effector cells actually revealed higher levels of apoptosis in Th1 effectors on days 3 and 4 (Fig. 8A). Similar results were found in polarized polyclonal CD4<sup>+</sup> T cell populations (data not shown). The differences between TUNEL<sup>+</sup> cells in Th1 and Th2 populations on day 4, but not day 3, reached statistical significance (p < 0.05, n = 2). This finding is consistent with an earlier report that a higher proportion of Th1 cells die compared with Th2 effectors (40).

Because the increased caspase activity in Th2 effectors did not result in increased apoptosis, we considered that there might be a mechanism by which Th2 effectors better tolerate elevated caspase activity. To explore this possibility, we examined expression of a major protector against caspase-driven death, Bcl-2, which functions to protect the outer mitochondrial membrane from caspase-initiated cell death (47). Day 4 Th1 and Th2 effectors were analyzed by simultaneous staining for apoptotic cells by TUNEL assay and for intracellular Bcl-2. Th1 effectors expressed a higher proportion of Bcl-2<sup>−/−</sup> cells (23%) than did Th2 effectors (12%) (Fig. 8B). Th2 AND effectors manifest a statistically higher proportion of cells of the phenotype Bcl-2<sup>−/−</sup>/TUNEL<sup>−</sup> and lower levels of Bcl-2<sup>low</sup>/TUNEL<sup>−</sup> cells (p < 0.05, n = 2). In addition, the apoptotic cells were almost entirely confined to the Bcl-2<sup>−/−</sup> subpopulation, suggesting that the presence of Bcl-2 was protective against the caspase activity in CD4<sup>+</sup> effector T cells. This was reinforced by the addition of z-VAD to cultures at day 0, which reduced the proportion of TUNEL-positive cells in the Bcl-2<sup>−/−</sup> population from 22% to 15% in Th1 cultures and from 17% to 8% in Th2 cultures (Fig. 8B). Caspase blockade also yielded an overall increase in the proportion of Bcl-2<sup>−/−</sup> cells. Th2 effector cells are thus potentially better adapted to sustain higher levels of caspase activity, in part through maintenance of higher levels of Bcl-2 expression.

An additional, though not mutually exclusive, possibility is that Th2 effector cells may have a greater requirement for caspase activity for cell cycling. To examine this, CFSE-labeled AND CD4<sup>+</sup> T cells were stimulated with PCCF under Th1 and Th2 polarizing
conditions with or without z-VAD added at day 0 of culture. Equivalent fractions of Th1 (10%) and Th2 (7%) cells proliferated by day 2 in the absence of caspase blockade (Fig. 9, DMSO). However, caspase blockade reduced the percentage of proliferating cells to 1% in both the Th1 and Th2 populations. By day 4, 94% of Th1 and 95% of Th2 cells had proliferated, whereas caspase blockade at day 0 decreased proliferation to 58% in Th1 and 55% in Th2 cells. Furthermore, a delay in the addition of z-VAD until day 2 had no effect on subsequent proliferation by AND CD4 T cells (data not shown). These findings suggest that caspase activation is equally necessary for initial activation of the majority of Th1 and Th2 populations, but that it is not required for continued proliferation during the Ag-independent, cytokine-driven phase of CD4 T cell growth (20).

Discussion

Our results show that viable effector CD4 T cells generate caspase activity levels that are between those of fresh CD4 T cells and FasL-treated effector T cells. This is reflected in the cleavage of caspase-8 substrates c-FLIPL, RIP1, and to a lesser extent Bid, but not the downstream caspase-3 substrate ICAD. In addition, RIP1, full-length c-FLIPL, and p43FLIP were precipitated with active caspases in effector T cells. Furthermore, this intermediate caspase activity is necessary for the initiation of cell cycling by the majority of T cells. Caspase activity is also higher in Th2 vs Th1 viable effector CD4 T cells and subsides in each population as they decrease cell cycling and become rested effector cells. Activated T cells thus run a delicate balance of generating sufficient caspase activity to promote cell cycling, but not so much as to induce cell death. The levels of caspase activity may profoundly affect the population of T cell effector subsets.

Because there is a fine balance of caspase activity between proliferation and cell death, it is important to restrain and target caspase activity to specific substrates in living cells. The recent concept that noncleaved caspase-8 can manifest intermediate activity is fully consistent with our results (18). Our findings further suggest that this form of active caspase-8 leads to the cleavage of molecules known to associate with it, including c-FLIPL and RIP1 (39), with less extensive cleavage of more distal substrates such as Bid. This would suggest that molecules that complex with caspase-8 are most efficiently cleaved in effector cells. Although we speculate that RIP1 is binding to caspase-8, it remains formally possible that RIP1 is binding to and cleaved by active caspases other than caspase-8. Furthermore, the limited degree of caspase-3 activity in effector T cells does not lead to any appreciable ICAD cleavage.

Recent reports show that caspase-3 and caspase-8 can localize in lipid rafts of nonactivated peripheral blood lymphocytes and that caspase-3 levels increase in lipid rafts upon activation (48). In addition, these and other investigators have shown that p20 caspase-3 is kept inactive by X-linked inhibitor of apoptosis protein (XIAP) and is activated upon further cleavage in which the prodomain of caspase-3 is released, thereby generating p17 caspase-3 (29, 48). If second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low iso-electric point protein is released from the mitochondria, then XIAP
is inactivated, thereby enabling the autoproteolytic processing of p20 caspase-3 to p17 caspase-3. Bcl-2 protein present in the mitochondria inhibits release of second mitochondria-derived activator of caspase, thereby maintaining the association of XIAP and p20 caspase-3 and preventing further processing of caspase-3 (29–31). Studies have suggested that other active caspasas, such as caspase-8 or caspase-9, can process caspase-3 to its fully mature 17-kDa fragment (49). Thus, the likely need to maintain mitochondrial integrity to prevent excessive caspase activity at the effector T cell stage is reflected in the high percentage of apoptotic cells in the Bcl-2− subset. In agreement with this concept, caspase blockade allowed greater survival of Bcl-2− cells.

Further support for the notion that p20 caspase-3 is inactive derives from its presence in freshly isolated T cells, in which negligible levels of caspase activity were detected. However, in day 4 effector T cells, where caspase activity was detected, the intensity of the p20 caspase-3 band decreased concomitantly with an increase of detectable p12/10 caspase-3. By day 6, there was a decrease in p12/10 caspase-3 with a proportional increase in p20 caspase-3. Taken together, these results suggest that p20 caspase-3 is likely inactive, consistent with it being complexed with XIAP (29). Furthermore, our results showing negligible binding of biotin-VAD to either full-length pro-caspase-3 or p20 caspase-3, but binding to a 17-kDa fragment, are consistent with this view and with other studies (29, 48).

It was recently reported that c-FLIPs, a 27-kDa alternate splice variant of c-FLIP, blocks caspase-8 activation to a greater extent than does FLIPL (28). Both c-FLIPs and c-FLIPs have been reported to inhibit TRAIL receptor-mediated apoptosis (50–54). c-FLIPs protein is up-regulated within the first day of activation of primary human T cells (45). When cyclohexamide is used during this time frame, FLIPs is down-regulated and cells are sensitive to Fas-mediated mitochondrial membrane potential loss, despite normal expression of Bcl-2 family members (45). Levels of c-FLIPs diminish over time and by day 6 are nearly undetectable (45). Other studies show that restimulation of day 6 effectors with anti-CD3/CD-28 also leads to up-regulation of c-FLIPs protein and partial protection of mitochondrial integrity, compared with restimulation with only anti-CD3 (44). In both studies, increased c-FLIPs protein expression correlated with decreased caspase-8 processing at the Fas-mediated death-inducing signaling complex (44, 45). Other studies indicate a role for elevated c-FLIPs in the survival of various tumor cells, including human gastric cancers (55, 56) and cells from patients with myelodysplastic syndrome (57). We propose an alternative mechanism of generating p27FLIP by further cleavage of p43FLIP. In addition to the known caspase cleavage site at Asp376 (LEVVD), there is a second potential caspase cleavage site at Asp258 (LIID) that would lead to p27FLIP. We found that p27FLIP is also a cleavage product in T cell lysates from c-FLIPs transgenic mice as well as from Fasl treatment of T cell blasts. The fact that Th2 cells possess more caspase activity and greater p27FLIP than do Th1 cells is also consistent with proteolytic generation of this fragment. In addition, quantitative PCR revealed that c-FLIP message was expressed at very similar levels in Th1 and Th2 cells (data not shown). If the p27FLIP that we observed were to be generated via an alternative splicing mechanism, then we would expect to see an increase in c-FLIP message in Th2 cells. p27FLIP may thus arise by two mechanisms in activated T cells.

Our results expand on other recent studies showing a requirement for caspases in T cell function. In addition, human T cells bearing a mutation in the caspase-8 gene exhibited decreased IL-2 production and CD25 induction upon stimulation (7). Restoration of wild-type caspase-8 in these cells promoted normal induction of CD25 upon TCR stimulation (7). Consistent with these findings, inhibition of caspase-8 expression by siRNA decreased activation of T cells (7). In contrast, caspase-8 conditional knockout mice did not show a defect in IL-2 production, but did exhibit a defect in response to IL-2, which could not be rescued by PMA and ionomycin (8). Thus, multiple studies highlight the importance of caspases in T cell effector function. Yet, it has been uncertain just how much caspase activity is present in cycling vs apoptotic T cells, what the substrates are, and how extensive is the progression of the caspase cascade.

It is not presently clear why Th2 cells exhibit higher caspase activity than do Th1 cells. One potential explanation is that Th2 effectors are better equipped to survive caspase activation. This possibility is supported by the findings of a higher percentage of Bcl-2− cells among Th2 cells compared with Th1 effectors. These results may offer a partial explanation for two reports that find that Th1 cells are more susceptible to Fas-mediated cell death (40, 41). Alternatively, not mutually exclusively, Th2 cells may require higher levels of caspase activity to obtain their fully polarized cytokine phenotype by cleavage of selective substrates important in signal transduction pathways. Studies to explore the latter possibility are currently underway.

The exact contribution of caspase activity and their substrates in T cell activation remains undefined, though the current findings identify logical candidate substrates. One role for caspases could be to cleave c-FLIPs to its p43 form, which more efficiently recruits RIP1 and TNFR-associated factor 2, leading to activation of NF-κB (58). Consistent with this hypothesis, we observed that RIP1 was in a complex with and proportional to levels of active caspases and overall p43FLIP levels (Figs. 3 and 6). Several studies have revealed a role of Fas signaling in neurite outgrowth (1), fibroblast proliferation (4), maturation of dendritic cells (5), and hepatocyte regeneration (59). Conceivably, Fas may engage signal pathways involved with cell growth, such as Erk activation (1). However, we currently have no evidence that Fas is required for the initiation of caspase activity in effector T cells. In fact, preliminary studies show similar caspase activity is generated in Fas-deficient lpr effector T cells (J. Q. Russell, unpublished observations).

The direct connection between TCR ligation and activation of caspases has not yet been identified. A logical consideration is that expression of a death receptor ligand is up-regulated, which stimulates its cognate receptor to activate caspases. This is consistent with previous findings showing that Fas ligation can costimulate T cell proliferation (6, 21, 60). However, as mentioned above, Fas-ligand−/− cells proliferate normally and activate caspases upon TCR stimulation. An alternative view is that TCR activation may promote approximate of caspase-8 with c-FLIPs independently of a death receptor, possibly via lipid raft association as suggested for Fas signaling in activated T cells (61). In this regard, c-FLIPs transgenic mouse CD4+ T cells manifest increased caspase activity (R. C. Budd, unpublished observations) and hyperproliferate in response to TCR engagement (62).

Caspases (especially caspase-8) and c-FLIPL clearly have an important role in the growth and development of potentially many cell types. This is underscored by the cardiac developmental block in mice deficient for either c-FLIPL (63) or caspase-8 (64). Defining the substrates of caspases that are critical for cell growth will represent an important link between the mechanisms of cell growth and cell death and may suggest an important central process for preventing aberrant growth by linking cell cycling to the same pathways used at a higher intensity for cell death.
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