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Constitutive Caspase Activation and Impaired Death-Inducing Signaling Complex Formation in CD95-Resistant, Long-Term Activated, Antigen-Specific T Cells

Gudrun Strauss,* Ingrid Knape,* Ingo Melzner,† and Klaus-Michael Debatin1*

Elimination of T cells during an immune response is mediated by activation-induced cell death (AICD) and CD95-mediated apoptosis. Chronic graft-vs-host disease and T cell-mediated autoimmune diseases are caused by the persistence of activated T cells that escaped tolerance induction by deletion or silencing. To mimic the in vivo situation of long-term activated T cells, we generated an in vitro system using HLA-A1-specific T cells, weekly restimulated by Ag. While short-term activated T cells (two to five rounds of stimulation) were CD95 sensitive and susceptible to AICD, T cells stimulated more than eight times acquired constitutive CD95 resistance and exhibited reduced AICD. Phenotypically, these long-term activated T cells could be identified as effector/memory T cells. The expression of the proforms of the CD95 receptor initiator caspases, caspase-8 and -10, and the effector caspase-3 was strongly decreased in these cells, and only active caspase fragments were detected. In contrast to short-term activated T cells, constitutive CD95 receptor clustering was observed on the cell surface, and caspase-8 was bound to the CD95 receptor in the absence of receptor triggering. After further cross-linking of CD95, additional formation of the death-inducing signaling complex (DISC) was strongly impaired. Reduced DISC formation in long-term activated T cells was associated with the loss of PTEN expression and the increased phosphorylation of protein kinase B. Inhibitors of phosphoinositol 3-kinase restored DISC formation (12) or up-regulation of phosphatidylinositol 3'-kinase restored CD95 sensitivity and DISC formation in long-term activated T cells. These data suggest that defective CD95 signaling in effector/memory T cells may contribute to the apoptosis resistance toward physiological stimuli in T cells mediating tissue destruction in vivo. The Journal of Immunology, 2003, 171: 1172–1182.

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2 Abbreviations used in this paper: AICD, activation-induced cell death; CD95L, CD95 ligand; DISC, death-inducing signaling complex; FADD, Fas-associated death domain; FSC/SSC, forward scatter/side scatter; GrzB, granzyme B; PI3-K, phosphoinositol-3,4,5-triphosphate; XIAP, X-linked IAP.

CD95-sensitive cells, triggering of the CD95 receptor leads to tri- merization of the receptor and recruitment of the adapter molecule Fas-associated death domain (FADD) and of procaspase-8 and -10 into a complex called death-inducing signaling complex (DISC) (9, 10). Subsequent self processing of the initiator caspases into active caspase fragments induces cleavage of effector caspases such as caspase-3 and -7, which finally execute the death program (11). Impaired DISC assembly in CD95-resistant T cells can be induced by either increased recruitment of c-FLICE-like inhibitory protein (FLIP)short to the DISC (12) or up-regulation of phosphatidylinositol 3'-kinase (PI3-K) activity (13). Also constitutive activation of protein kinase Bα (PKBα/Akt) prevents efficient DISC formation (14). Studies in CD95-resistant T cell clones from patients with autoimmune diseases, however, showed that resistance mostly correlates with the change in the expression of pro- and antiapoptotic molecules. In CD95-resistant T cell lines from patients with multiple sclerosis, up-regulation of the apoptosis inhibitors FLIP and survivin was found (15, 16). Extensive studies were also performed in T cell clones from patients with rheumatoid arthritis. These patients have an expanded pool of CD4+CD28− T cells, which are functionally active and persist over many years (17). Altered responses to apoptosis-inducing signals such as anti-CD95 ligation, induction of AICD, and growth factor withdrawal...
have been described in these cells and were associated with elevated Bcl-2 levels (18–20).

The establishment of autoaggressive T cell clones comprises the development from naïve T cells, triggered by autoantigen, into effector and memory T cells. These distinct T cell subgroups can be efficiently characterized by the expression of surface markers, e.g., CD45RA, CD45RO, CD28, CD27, CD62L, CCR7, or integrin family members and the intracellular expression of cytotoxic molecules such as perforin, granzymes, and CD95L (21, 22). While naïve T cells express costimulatory molecules CD27 and CD28 and are CD45RA*CD27+CD28+, CCR7+, effector cells exhibit a decrease in CD27, CD28, and CD62L expression (23), but dramatically increased the expression of perforin, granzymes, and CD95L (21). Loss of the chemokine receptor CCR7 and L-selectin (CD62L) as well as CD27 and CD28 appears to characterize a subset of memory T cells with immediate effector functions (effector/memory T cells) (22, 24).

In the present study we recapitulated the in vivo situation of continuous Ag stimulation by an in vitro system in which HLA-A1-specific T cells were weekly restimulated with Ag for >12 wk. As expected, CD95 sensitivity and AICD increased with each restimulation up to the fifth round. Interestingly, after this time point CD95 sensitivity decreased again, and T cells became constitutive CD95 resistant and exhibited reduced AICD after eight rounds of stimulation. In contrast to CD95-sensitive, short-term activated T cells, these long-term activated T cells exhibited constitutive caspase-8, -10, and -3 activation and expressed CD95 receptor in clusters on the cell surface. In addition, caspase-8 was associated with the CD95 receptor in the absence of receptor cross-linking. Further cross-linking did not lead to the formation of a functional CD95-DISC, possibly induced by down-regulation of PTEN, activation of PI3-K and constitutive PKBα/Akt phosphorylation. These findings might help to identify novel approaches to sensitize apoptosis-resistant T cells in vivo.

Materials and Methods

Cell lines

All cell lines were grown in RPMI 1640 medium (Life Technologies, Paisley, U.K.) supplemented with 10% heat-inactivated FCS (Biocoordination, Berlin, Germany), 2 mM L-glutamine, and 1 mM sodium pyruvate at 37°C in a humidified atmosphere containing 6.5% CO2. The HLA-A1-expressing lymphoblastoid cell lines C1R.A1 (25) and 721 (26) and the HLA-A1+ cell line C1R (27) as well as the human T cell line H9 were used.

Generation of short- and long-term activated alloreactive T cell lines

Ficoll-Hypaque-separated PBMC from healthy HLA-A1+ donors (1 × 10^7/ml) were incubated with mitomycin C-treated HLA-A1+ 721 stimulator cells (1 × 10^6/ml) and human rIL-2 (Biochrom; 30 U/ml, first stimulation). After 1 wk viable cells were separated by Ficoll-Hypaque gradient and restimulated with mitomycin C-treated 721 cells at a ratio of 10/1 in medium containing 30 U/ml rIL-2 (second stimulation). Separation of viable cells and restimulation were repeated weekly. Short-term activated CTL presented T cells between the second and fifth stimulations, while long-term-activated CTL were stimulated at least eight times.

Lymphocyte isolation and biomagnetic separation

PBMC were obtained from peripheral blood of healthy donors. Isolation was done by density centrifugation of blood on Ficoll (Amersham Pharmacia Biotech, Uppsala, Sweden). CD4+ and CD8+ T cells were isolated from PBMC by depletion of monocytes by plastic adherence for 2 h at 37°C. Nonadherent cells were stained with supernatants from hybridomas A9 (anti-CD16; provided by M. Pfreundschuh, Hamburg/Saar, Germany), HD37 (anti-CD19) (28), and HD26 (anti-CD4) (29) to obtain CD8+ cells or OKT8 (anti-CD8) (30) to isolate CD4+ T cells. Depletion of T cells was performed with BioMag goat anti-mouse IgG Beads (PAESEL+LOREL, Hanau, Germany). The purity of the population was determined by flow cytometry with CD4-FITC and CD8-FITC mAb on a FACScan cytometer (BD Biosciences, Heidelberg, Germany).

Cytotoxicity assay

Target cells (2 × 10^3) were labeled with 200 μCi of Na^251CrO4 (American-Buchler, Braunschweig, Germany) for 1 h. Increasing numbers of effector cells were titrated to 5 × 10^3 target cells and incubated for 4 h at 37°C. The percentage of supernatants of supernatant was assayed for ^51Cr release in a Top CountNX counter (Packard BioScience, Dreieich, Germany). Maximum release was determined by incubation of target cells in 100 μl of 10% SDS, and spontaneous release was determined by addition of medium. The percentage of specific release was calculated as % specific release = (experimental release – spontaneous release)/(maximum release – spontaneous release) × 100. Assays were set up in triplicate. Cytotoxicity assays were performed on day 6 after the last T cell stimulation.

Induction and analysis of apoptosis

To determine CD95 sensitivity, cells were treated with either Apo-I IgG3 Ab (anti-CD95) (31) or soluble recombinant human CD95L (Alexis, Germany). Cell death was determined by measuring forward/side scatter (FSC/SSC) or by annexin V-FITC staining to externalized phosphatidylserine (Annexin V-FITC Kit; Bender Med Systems, Vienna, Austria) on a FACScan cytometer (BD Biosciences). To analyze AICD, T cells (2 × 10^6/ml) were cultured in triplicate for 24 h on 48-well plates coated with OKT3 (10 μg/ml; anti-CD3-ε chain, obtained from American Type Culture Collection, Manassas, VA) in the presence of rIL-2 (30 U/ml), and cell death was determined by FSC/SSC measurement. ZVAD-fmk (Bachem, Heidelberg, Germany) and Apo-I Fab were used to inhibit AICD. Specific apoptosis was calculated according to the formula: induced (experimental) cell death (%) – spontaneous cell death [%]/100 – spontaneous cell death (%). Data always represent the mean of triplicate determinations.

Flow cytometry

To determine CD95 surface expression, 5 × 10^5 T cells were double stained with CD95-FITC (31) and CD4-PE or CD8-PE (BD Bioscience, Heidelberg, Germany). To analyze surface marker expression of short- and long-term activated T cells, cells were double stained with CD27-PE, CD56-PE, CD62L-PE (BD Biosciences, Heidelberg, Germany), CD28-PE, CD49-PE, and CD8-PE (BD Bioscience). To exclude dead cells, cells were always counterstained with 1 μg/ml propidium iodide (Sigma-Aldrich, Steinheim, Germany), and surface marker expression was only measured on the propidium iodide-negative population. Flow cytometry was performed on a FACScan cytometer.

Western blot analysis and DISC assays

For Western blot analysis, 5 × 10^5 cells were lysed for 15 min at 4°C in lysis buffer (30 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM PMSF, and 0.1% Tween 20. Membranes were hybridized in Odyssey blocking buffer (LI-COR, Lincoln, NE) at 60°C for 1 h. Membranes were washed four times in 1 ml of washing buffer (1% Tween 20, 100 mM NaCl, and 50 mM Tris-HCl (pH 8)), followed by high speed centrifugation. Twenty micrograms of lysate was separated on a 10–20% gradient SDS page and electroblotted onto Hybrid ECL nitrocellulose membrane (Amersham-Buchler). Membranes were blocked for 1 h in PBS supplemented with 5% milk powder and 0.1% Tween 20. Membranes were stained for 2 h (mouse mAb) or overnight (rabbit and goat polyclonal IgG) with the first Ab, followed by 1-h incubation with the HRP-conjugated second Ab, and detection was performed by ECL (Amersham Bioscience, Freiburg, Germany).

For DISC analysis 1 × 10^7/ml T cells were treated with cross-linking CD95 mAb Apo-I (IgG3; 1 μg/ml) (31) for 10 min at 37°C. Control cells were incubated at 37°C in the absence of CD95 Ab. Cells were once washed with ice-cold PBS and lysed in lysis buffer (1% Triton X-100, 150 mM NaCl, and 50 mM Tris-HCl (pH 8)), followed by high speed centrifugation for 10 min at 4°C. Apo-I (1 μg/ml) was added to the lysates of the control cells. To precipitate CD95, 10 μl of Pan mouse IgG Dynabeads (Dynal Biotech, Hamburg, Germany) were added and incubated for 4 h at 4°C. Beads were then washed four times with 1 ml of washing buffer (1% IGEPAL CA-630, 500 mM NaCl, and 50 mM Tris-HCl (pH 7.8)) and once with 25 mM Tris-HCl (pH 7.5). Beads were resuspended in 6× SDS-reducing sample buffer, boiled for 5 min at 95°C, and separated by Dylam Magnetic Particle Concentrators, and the supernatant was separated by 10–20% gradient SDS page, followed by Western blotting as described above. To analyze DISC formation after wortmannin treatment, T cells

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were incubated for 3.5 h in the presence of wortmannin (10 μM; Sigma-Aldrich) and subsequently analyzed for DISC formation as described above.

For Western blotting the following Abs were used: caspase-8 (clone 12F5; Alexis, Grunberg, Germany), caspase-10 (clone 4C1; MBL, Gottingen, Germany), perforin (clone KMS58/P1–8; Kamiya Biomedical, Seattle, WA), granzyme B (GrzB; clone 2C5/F5; Serotec, Eching, Germany), β-actin (clone AC-15; Sigma-Aldrich), lamin B (Ab-1; Oncogene, Bad Soden, Germany), phospho-Akt (Ser473; New England Biolabs, Frankfurt, Germany), caspase-3 (clone 19), caspase-7 (clone 51), caspase-9 (polyclonal rabbit), FADD (clone 1), death receptor-interacting protein (RIP) (clone G722-2), Bcl-2 (clone Bcl-2/100), Bcl-x (clone 2H12), CD95L (clone G247-4), TRAIL (B35-1), DFF45 (clone 19), PKB/Akt (clone 55; BD Transduction Laboratories, Heidelberg, Germany), c-inhibitor of apoptosis (c-IAP2; H-85), Bak (N-20), poly-(ADP-ribose)-polymerase (PARP) (F-2), FAS (C20), PTEN (A2B1), goat anti-mouse IgG-HRP, goat anti-rabbit IgG-HRP, anti-goat IgG-HRP (Santa Cruz Biotechnology, USA), X-linked IAP (XIAP) (MAB822), cIAP-1 (AF8189), survivin (AF886), TNF-α (MAB610), Bid (AF846), Bad (AF819), and Bax (2282-MC-100; R&D Systems, Wiesbaden, Germany). FLIP-Ab was provided by P. Krammer (Heidelberg, Germany). HRP-conjugated goat anti-mouse IgG, goat anti-rabbit IgG, and anti-goat IgG were obtained from Santa Cruz Biotechnology.

Caspase-3 and -8 assay
To detect active caspase-3 and -8 in nonlysed cells, 5 × 10⁷ T cells were incubated in the absence or the presence of the caspase substrate DEVD-R110 (caspase-3 specific; 505 nm; Roche, Mannheim, Germany) or IETD-AFc (caspase-8 specific; BioVision, Palo Alto, CA) in PBS on 96-well plates and incubated at 37°C. Medium without cells was used as a control. After 1 h, free R110 or AFc was measured fluorometrically at 535 nm and 505 nm on a microplate fluorescence reader (1420 Victor Multilabel Counter; Wallac, Rodgau-Jugesheim, Germany). Experiments were set up in triplicate. X-increase is the mean emission DEVD-R110/mean emission PBS.

Immunofluorescence
T cells were cultured to adherence in collagen I-coated culture slides (Falcon, Heidelberg, Germany) overnight and fixed with methanol for 5 min.

Cells were stained with FTTC-labeled APO-1 for 1 h at room temperature and embedded with fluorescent mounting medium HC08 (Calbiochem-Novabiochem, Schwabach, Germany). APO-1 binding was monitored by confocal laser scanning microscopy (Leica TCS; Leica Microsystems, Wetzlar, Germany).

Results
Continuous Ag stimulation induced CD95-resistant, long-term activated T cells of the effector/memory phenotype
Changes in the apoptosis sensitivity of T cells characterize the course of an immune response. While naive T cells are CD95 resistant, activated T cells become CD95 sensitive after 3–6 days of stimulation. To mimic the course of an immune response in vitro, we established HLA-A1-specific T cells, weekly restimulated with the alloantigen HLA-A1-expressing lymphoblastoid cell line 721. CD95 sensitivity was monitored after each round of stimulation (Fig. 1A). T cells were incubated with agonist CD95 Ab APO-1 (100 ng/ml) on day 5 after each stimulation, and apoptosis was measured after 24 h in the CD4⁺ and CD8⁺ T cell population. CD95 sensitivity developed between the second and fifth rounds of stimulation. These CD95-sensitive T cells (two to five rounds of activation) are called short-term activated T cells. After the fifth stimulation, however, apoptosis sensitivity decreased again, and T cells became progressively CD95 resistant. CD95-resistant T cells obtained after the eight stimulation are designated long-term activated T cells. Staurosporine mediating apoptosis via mitochondrial death pathways equally induced apoptosis in long- and short-term activated T cells (data not shown). Apoptosis resistance was not due to the loss or down-regulation of CD95 expression on the surface of CD4⁺ and CD8⁺ T cells (Fig. 1B). To verify that T cells are dying by apoptosis, we compared two different methods to determine programmed cell death. Apoptosis induction after CD95 treatment was measured either by changes in cell size (FSC/SSC) or staining of annexin V-FITC to externalized phosphatidylserine. Values are the mean of triplicate determinations.

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FIGURE 1. Continuous Ag stimulation induced CD95-resistant, long-term activated T cells. A, T cells were treated with α-CD95 Ab APO-1 (100 ng/ml) after different rounds of stimulation. After 24 h, cells were double stained for CD4 and CD8 expression, and cell death was determined in each subpopulation by measuring FSC/SSC. Values are the mean of triplicate determinations. B, T cells after the first, fifth, and ninth stimulations were double stained for CD4 or CD8 and CD95 expression (solid line). Dotted lines showed staining of an isotype-matched control Ab. C, T cells after the 5th and 10th stimulations were treated with APO-1 (100 ng/ml) for 24 h. Specific apoptosis was determined by measuring FSC/SSC or binding of annexin V-FITC to externalized phosphatidylserine. Values are the mean of triplicate determinations. D, T cells after the first, fifth, and ninth stimulations were used as effector cells in a standard chromium release assay. The percentage of CD8⁺ T cells increased from 35% (first stimulation) to 60% (fifth stimulation) to 95% (eighth stimulation). The HLA-A1-expressing C1R A1 cell line and the untransfected control line C1R were used as ⁵¹Cr-labeled target cells. Each experiment was performed at least three times.
or by measurement of externalized phosphatidylserine on the membrane (annexin V). Fig. 1C indicated that both methods are comparable, and therefore apoptosis was determined in the following experiments by analyzing FSC/SSC. All experiments were performed in the presence of exogenously added rIL-2. As IL-2 withdrawal induced death in short- and long-term activated T cells (data not shown), we could not investigate the influence of IL-2 on CD95 sensitivity in our system. Short- and long-term activated T cells are functional cytotoxic T cells because they specifically lysed the HLA-A1-expressing target cell line C1R.A1 and not the non-HLA-A1-expressing parental cell line C1R. Only T cells at the beginning of the stimulation process did not exhibit HLA-A1 specificity (Fig. 1D). To further characterize CD95-resistant, long-term activated T cells, the expression of surface markers used to dissect naive T cells from effector and memory T cells was analyzed (Table I). Long-term activated CD4+ and CD8+ T cells are devoid of CD28, CD62L, and CCR7 expression and displayed the phenotype of effector/memory T cells strongly up-regulated CD56 as a marker of increased cytolytic capacity (32). These results demonstrate that continuous Ag stimulation induced the development of a CD95-resistant T cell population of the effector/memory phenotype.

### AICD in long-term activated T cells is decreased, and CD95 and caspase independent

AICD mediated via the CD95/CD95L system serves as the major mechanism to remove activated T cells from the periphery during the termination of an immune response. AICD can be induced in T cells by TCR/CD3 triggering via immobilized mAb OKT3. T cells after the 5th and 10th stimulations were incubated in the presence or the absence of mAb OKT3. Simultaneously, the broad caspase inhibitor ZVAD-fmk or APO-1 Fab, preventing the CD95-CD95L interaction, was added. After 24 h cell death was determined in the CD8+ T cell populations (Fig. 2). Long-term activated CD8+ T cells exhibited decreased AICD compared with their short-term activated counterparts. Interestingly, long-term activated CD8+ T cells were barely protected from AICD by ZVAD-fmk or APO-1 Fab, indicating that AICD in these cells proceeds through a caspase- and CD95/CD95L-independent mechanism. The decline in AICD varied for each experiment performed and ranged between 10 and 50%. As a control for AICD induction and the effects of inhibitors we used the human T cell line H9, which was efficiently protected from AICD in the presence of ZVAD-fmk and APO-1 Fab. These data indicate that CD95 resistance of long-term activated T cells correlates with a caspase- and CD95-independent decrease in AICD.

### Expression of pro- and antiapoptotic molecules in short- vs long-term activated T cells

Differences in the expression of pro- and antiapoptotic molecules in long-term activated T cells could explain CD95 resistance compared with their short-term activated counterparts. The expression of IAP and members of the Bcl-2 family was analyzed by Western blot analysis in T cells after the first, fifth, and ninth rounds of stimulation. No difference in FLIP, XIAP, and CIAP-1 and -2 expression was detected. Survivin, which is weakly expressed after the first stimulation, was up-regulated after the fifth stimulation, but protein expression did not further increase in long-term activated T cells (Fig. 3A). Bcl-2 was also up-regulated after the first stimulation, but expression levels did not differ in short- and long-term activated T cells. Bcl-xL, however, was strongly up-regulated after the first round of stimulation in CD95-sensitive cells, with a slight increase in long-term activated, CD95-resistant T cells. With progressive Bcl-xL induction, the proapoptotic molecules Bak and Bad were down-regulated, while the Bax expression level was unchanged. Bid, which links the receptor-mediated apoptosis pathway to the mitochondrial pathway, disappeared in the beginning of T cell stimulation, but was not differently expressed between short- and long-term activated T cells (Fig. 3B). T cells can kill target cells either by death-inducing ligands such as CD95L, TRAIL, and TNF-α or via the granzyme/perforin system. During the stimulation process, CD95L, GrzB, and perforin were strongly up-regulated, indicating that the cytotoxic capacity increased during repeated stimulation (Fig. 3, C and D). An increase in CD95L.

### Table I. Expression of surface markers used to dissect naive T cells from effector and memory T cells

<table>
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<tr>
<th>Cells</th>
<th>Stimulation</th>
<th>CD28</th>
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* T cells after different rounds of stimulation or without any stimulation (0 stimulation) were double stained for CD4 or CD8 expression and the indicated surface markers and analyzed by flow cytometry. Numbers indicate the percentage of positively stained cells.
Long-term activated T cells up-regulate Bcl-xL and down-regulate the proapoptotic molecules Bak and Bcl-2. After the first, fifth, and ninth stimulations, 20 μg of protein lysates of T cells were subjected to Western blot analysis. Expression of inhibitors of apoptosis (A), pro- and antiapoptotic molecules of the Bcl-2 family (B), death-inducing ligands (C), and GrzB and perforin (D) was determined. Expression of β-actin served as the loading control. The percentage of CD8+ T cells increased from 20% (first stimulation) and 40% (fifth stimulation) to 90% (ninth stimulation). The experiment shown is representative for T cells from one donor of two analyzed.

Constitutive caspase activation in long-term activated T cells

Caspases are the main mediators of cell death after CD95 triggering, mitochondrial dysfunction, and granzyme-induced cell death. During death induction the caspase proform is converted into its active caspase fragment. The expression of procaspases and their active cleavage fragments in HLA-A1-specific T cells after the first, fifth, and ninth stimulations was analyzed by Western blot analysis (Fig. 4A). Surprisingly, long-term activated T cells did not express the proforms of caspase-3, -8, and -10. While caspase-8 and -3 were completely converted into their intermediate cleavage products (p43/41 for caspase-8) and the active fragments (p20/18 for caspase-8 and p12 for caspase-3), active caspase-10 fragments could not be detected due to Ab specificities. In contrast, short-term activated T cells expressed procaspase-8 (55/54 kDa), the intermediate cleavage products p43/41, and only low amounts of the active fragment p20/18. Caspase-10 was not processed, and partial processing of caspase-3 was found. Constitutive activation of caspase-9 was not detected in long-term activated T cells. Caspase-7 was processed in all T cells independently of the stimulation status. Expression levels of FADD, the adapter molecule between CD95 and caspase-8 and -10 at the DISC, were unchanged at all stages of stimulation. Down-regulation of RIP, which was recently reported to mediate CD95-induced T cell death (33), could not consistently be detected. Although long-term activated T cells expressed active caspases, no differences in the cleavage of caspase substrates such as PARP, DFF45, and lamin B, were detected between the different stages of T cell stimulation, indicating that cellular substrates were not fully cleaved by the active caspase fragments. To rule out that caspase activation in long-term activated T cells was mediated via GrzB released from cytolytic granule during protein preparation, we measured caspase-3 and -8 activities fluorometrically in non-lysed T cells (Fig. 4B). Medium and isolated nonstimulated CD4+ and CD8+ T cells from healthy donors served as a negative control. While unstimulated T did not process the caspase-3 substrate DEVD-R110, T cells after the fourth stimulation showed enhanced caspase-3 activity, which was further increased in T cells after the 12th stimulation. Caspase-8 activity in long-term activated T cells was less pronounced, possibly reflecting a decreased diffusion of the peptide into the cells. Considering the CD95 resistance of long-term activated T cells, these data indicate that constitutive caspase activation might be associated with apoptosis resistance.

Short-term activated CD4+ and CD8+ T cells do not differ in their protein expression pattern

Our in vitro system of repeated Ag stimulation led to a predominant expansion of CD8+ T cells. After five rounds of stimulation, B and NK cells disappeared, and the ratio of CD4+/CD8+ T cells varied from 1/1 to 1/5. Further stimulation increased the percentage of CD8+ T cells up to 85–98%. We did not observe any relationship between the composition of lymphoid cells in the starting culture and the acquisition of CD95 resistance in long-term activated T cells (data not shown). To rule out that some of the observed differences in apoptosis sensitivity were attributed to the significant amount of CD4+ T cells present in the initial culture, we isolated both subpopulations at the beginning of the stimulation and restimulated them separately. Fig. 5A shows that proforms and active fragments of caspase-3, -8, -9, and -10 were comparably expressed in both T cell subsets. No differences were found for PARP, RIP, and FADD expression. Bcl-xL, Bcl-2, survivin,

**FIGURE 3.** Long-term activated T cells up-regulate Bcl-xL and down-regulate the proapoptotic molecules Bak and Bcl-2. After the first, fifth, and ninth stimulations, 20 μg of protein lysates of T cells were subjected to Western blot analysis. Expression of inhibitors of apoptosis (A), pro- and antiapoptotic molecules of the Bcl-2 family (B), death-inducing ligands (C), and GrzB and perforin (D) was determined. Expression of β-actin served as the loading control. The percentage of CD8+ T cells increased from 20% (first stimulation) and 40% (fifth stimulation) to 90% (ninth stimulation). The experiment shown is representative for T cells from one donor of two analyzed.

**FIGURE 4.** Constitutive caspase activation in long-term activated T cells. A, Cell lysate (40 μg) of T cells after different rounds of stimulation was used for Western blot analysis to detect the expression of procaspases and their cleavage products. Arrows indicate the m.w. of procaspases and cleavage products. To detect the expression of FADD, RIP, PARP, lamin B, and DFF45, 20 μg of cell lysate was loaded. β-Actin served as the loading control. Blots shown are representative of two different experiments. B, Medium, nonstimulated, isolated CD4+ and CD8+ T cells from healthy donors and T cells after the 4th, 9th, and 12th stimulations were incubated with caspase-3 substrate DEVD-R110 or the caspase-8 substrate IETD-AFC, and caspase-3 and -8 activity was determined fluorometrically. Values are the mean of triplicate determinations, and the experiment was performed twice.
CD95L, and GrzB were up-regulated after the first stimulation in both T cell subsets, and Bid was down-regulated. Perforin, however, strongly expressed in CD8^+ T cells after the fifth stimulation was nearly absent in the CD4^+ T cell population (Fig. 5B), consistent with the observation that short-term activated CD4^+ T cells do not lyse HLA-A1-expressing target cells (data not shown). These data indicate that short-term activated CD4^+ and CD8^+ T cells do not differ in the expression of caspases and apoptosis regulators. For additional experiments short-term activated T cells were considered a unique cell population.

**Analysis of the CD95 signaling complex**

As long-term activated T cells exhibited an impaired CD95 signaling pathway, we analyzed CD95 receptor expression by confocal microscopy on isolated short-term activated CD8^+ T cells and their long-term activated counterparts (Fig. 6A). While short-term activated T cells displayed a regular distribution of the CD95 molecules on the cell surface with minimal CD95 clustering, extensive CD95 receptor clustering was found in long-term activated T cells. CD95 clusters are completely absent during the early phase of the activation process (first and second stimulations; data not shown). Although Fig. 1B showed that CD95 expression on short and long term activated T cells is similar, Fig. 6A might indicate that long-term activated T cells express lower levels of CD95 than their short-term activated counterparts. To exclude that CD95 is down-regulated on apoptosis-resistant cells, we incubated T cells of the third and ninth stimulations with soluble CD95L for 24 h and measured CD95 expression on the resistant CD8^+ population. No difference in CD95 expression was detected (Fig. 6B), indicating that clustering of the CD95 receptor might cause quenching of the fluorescence intensity by confocal microscopic analysis, leading to relatively less intense staining of clustered CD95 receptors.

Since long-term activated T cells expressed processed caspase-8 and -10 and clustered CD95, we next analyzed whether formation of the CD95-DISC was intact (Fig. 7A). T cells were incubated with the cross-linking agonistic CD95 Ab anti-APO-1 or left untreated. After cell lysis, CD95 was precipitated, and subsequently Western blot analysis was performed to determine compounds bound to the receptor. In the absence of CD95 cross-linking, no association between caspase-10 and the CD95 receptor was detected in short-term activated T cells, and only small amounts of caspase-8 were bound to CD95. Long-term activated T cells, however, displayed a strong constitutive association between CD95 and caspase-8. Also, small amounts of caspase-10 were bound to the receptor in the absence of cross-linking. After CD95 triggering, short-term activated T cells recruited caspase-8, caspase-10, and FADD to the DISC. Recruitment of caspase-8 and -10 and FADD, however, was impaired in long-term activated T cells. We did not observe differences in FLIP_short or processed FLIP_long (p43) recruitment in short- vs long-term activated T cells. Unprocessed FLIP_L was not found in the DISC. RIP, however, was not present in the DISC of long-term activated CD8^+ T cells independently of anti-APO-1 treatment. In addition, full-length procaspase-8 (55/54 kDa) was hardly detectable in any of the DISC assays performed, showing that even in short-term activated T cells caspase-8 is rapidly converted into the intermediate cleavage product p43/41 after receptor triggering. As short-term activated T cells after the fifth stimulation presented a mixture of CD4^+ and CD8^+ T cells, DISC formation of both subsets, isolated via biomagnetic cell separation before the experiment, were analyzed (Fig. 7B). Differences in

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**FIGURE 5.** Comparison of protein expression in short-term activated, isolated CD4^+ and CD8^+ T cells. CD4^+ and CD8^+ T cells were isolated by biomagnetic sorting from PBL of an HLA-A1-negative donor. Each subpopulation was separately stimulated up to five times. Proteins were isolated after the first and fifth stimulations and were subjected to Western blot analysis. A, Expression of caspases, PARP, RIP, and FADD. B, Expression of pro- and antiapoptotic molecules, PTEN, CD95L, perforin, GrzB, and the loading control β-actin.

**FIGURE 6.** Expression of CD95 clusters on the surface of long-term activated T cells. A, CD8^+ short- and long-term activated T cells were grown on collagen-coated culture slides slides overnight. Methanol-fixed slides were stained with APO-1-FITC, and the fluorescence images were analyzed by confocal laser scanning microscopy. Magnification: a, ×630; b, ×2400. B, T cells after the third and ninth stimulations were incubated with soluble CD95L (200 ng/ml). After 24 h, cells were double stained for CD95 and CD8, and apoptosis-resistant cells were gated via FSC/SSC. The expression of CD95 or isotype control (dotted lines) was analyzed on the resistant CD8^+ population.
Impaired DISC formation in long-term activated T cells is associated with the loss of PTEN expression and increased PI3-K activity, and PKBα/Akt phosphorylation. DISC assays were always performed on day 6 after the last stimulation. DISC analysis was performed after cross-linking of the CD95 receptor by APO-1 Ab (+APO-1) or in the absence of the Ab (−APO-1). After CD95 precipitation, proteins were subjected to Western blot analysis, and association of caspase-8 and -10, FADD, FLIP, and RIP to the CD95 receptor was determined. CD95 expression served as the loading control.

A, DISC formation of T cells after the 5th and 10th stimulations. Ninety-five percent of T cells were CD8+ after the 10th stimulation, and 70% were CD8+ after the fifth stimulation. B, DISC formation of isolated CD4+ and CD8+ T cells after the third stimulation. C, Proteins isolated from T cells of two donors after different rounds of stimulation were subjected to Western blot analysis, and the expression of PTEN, PKBα/Akt, and phospho-Akt (Ser473; pAkt) was analyzed. The percentage of CD8+ long-term activated T cells was >95%, while 50–60% of CD8+ T cells were present in short-term activated T cells. D, Short- and long-term activated T cells after the fourth and ninth stimulations were incubated with APO-1 (100 ng/ml) or medium in the absence or the presence of wortmannin (10 μM). After 24 h cells were stained for CD8 expression, and the percent specific apoptosis was calculated in the CD8+ T cell population using FSC/SSC analysis. Data are representative of three different experiments performed with T cells from different donors. E, DISC formation of T cells after the fourth and ninth stimulations in the presence or the absence of wortmannin (10 μM). The blots shown are representative of at least three independent experiments with T cells from different donors.
DISC formation after CD95 triggering were not observed in both subsets. In all assays CD95 served as a loading control. Taken together, CD95 resistance of long-term activated T cells might be due to a defective CD95 signaling pathway, including constitutive caspase activation, CD95 cluster formation, and impaired DISC formation.

Down-regulation of PTEN expression, elevated PI3-K activity, and increased phosphorylation of PKBα/Akt in long-term activated T cells

Activation of PKBα/Akt has been shown to mediate the inhibition of CD95-DISC formation and apoptosis in mouse T cells. Hyper-phosphorylation and activation of PKBα/Akt may be consequences of an increase in the intracellular phospholipid phosphatidylinositol-3,4,5-triphosphate (PIP3) (14, 34). PIP3 is generated by PI3-K (35) and is negatively regulated by the lipid phosphatase PTEN, which cleaves the D3 phosphate from PIP3 (36). Since long-term activated T cells exhibited a CD95-resistant phenotype with defective DISC assembly, we analyzed the expression of PTEN and phosphorylated PKBα/Akt in short- vs long-term activated T cells from two donors (Fig. 7C). PTEN was strongly down-regulated, and elevated levels of phosphorylated PKBα/Akt were detected in long-term activated T cells from both donors. PTEN down-regulation in long-term activated T cells was not due to the significant amount of CD4+ T cells still present after early stimulations, as PTEN expression did not vary in the isolated CD4+ and CD8+ short-term activated subpopulations (Fig. 5B). The amount of unphosphorylated inactive PKBα/Akt was attenuated between the different stages of T cell stimulation. Activation of PI3-K also leads to increased phosphorylation of PKBα/Akt. Short- and long-term activated T were incubated in the presence of the PI3-K inhibitor wortmannin, and CD95 sensitivity and DISC formation were analyzed. After wortmannin treatment, long-term activated T cells exhibited CD95 sensitivity comparable to that of untreated short-term activated T cells. A modest increase in CD95 sensitivity was also observed in short-term activated T cells after PI3-K inhibition (Fig. 7D). To further clarify whether inhibition of PI3-K restored caspase-8 and-10 recruitment to the DISC, we compared DISC formation of short- and long-term activated T cells in the presence or the absence of wortmannin. Caspase-10 recruitment to the DISC was increased in short- and long-term activated T cells, while caspase-8 association to the DISC was only marginally increased in long-term activated T cells (Fig. 7E). These results suggest that impaired DISC formation of long-term activated T cells might be due to the loss of PTEN expression and the increased activation of PI3-K leading constitutive activation of PKBα/Akt and preventing the formation of a functional DISC.

Discussion

Thymic selection (central tolerance) prevents the development of autoreactive lymphocytes leading to chronic tissue damage. This elimination process, however, is often incomplete, and peripheral regulatory mechanisms, such as anergy, ignorance, suppression, or apoptosis (37–39) are needed to control mature self-reactive cells. Activated T cells are deleted from the periphery by either CD95-mediated apoptosis or AICD. Both mechanisms are affected by alterations in CD95 sensitivity during the course of an immune response. According to current concepts, naive T cells are CD95 resistant, whereas activated T cells acquire CD95 sensitivity after 3–6 days of activation (3). AICD, mainly mediated via the CD95/CD95L system, terminates the immune response after repeated TCR triggering (4). Molecular mechanisms of apoptosis defects, however, leading to uncontrolled expansion and prolonged survival of self-reactive T cells, are poorly understood.

To mimic the in vivo situation of an immune response after continuous Ag stimulation we established an in vitro system of HLA-A1-specific T cells that were weekly restimulated with HLA-A1-expressing cells. Short-term activated T cells (stimulated for 2–5 wk) were CD95 sensitive and susceptible to AICD. Surprisingly, long-term activated T cells (>8 wk of stimulation) acquired constitutive CD95 resistance and exhibited decreased AICD. AICD induction could not be inhibited by caspase inhibitors or CD95 Fab that prevent the interaction between CD95 and its ligand. Inhibition of perforin/GranzB (40, 41) and TRAIL (42) activity, described as further mediators of AICD in human T cells, could also not prevent T cell death (data not shown). Phenotypically, long-term activated T cells were identified as effector/memory T cells, characterized by the loss of CD62L and CCR7 expression and their immediate effector function (24). IL-5 and IFN-γ production decreased in long-term activated T cells, while the amount of the immunomodulatory cytokine IL-10, which promotes proliferation and differentiation into effective cytotoxic T cells (43, 44), was significantly increased, underlining their efficient cytotoxic capacity (data not shown). In agreement with other studies (12, 13), only a subpopulation of activated T cells in the early stimulation process (second to fifth stimulations) was CD95 sensitive. CD95 sensitivity varied in T cells from different donors from 30–70%, indicating that the total T cell population always contained CD95-resistant T cells. Changes in CD95 sensitivity might reflect the expansion of CD95-resistant cells or indicate individual changes from CD95-sensitive cells into CD95-resistant cells. The final result of continuous Ag stimulation, however, is the development of CD95-resistant T cells.

Apoptosis resistance of T cells was described in different autoimmune diseases, such as rheumatoid arthritis (18), multiple sclerosis (45), and autoimmune hematologic cytopenias (46). CD95-resistant T cell clones from patients with multiple sclerosis exhibited up-regulation of apoptosis inhibitors survivin or FLIP (15, 16). Patients with rheumatoid arthritis have an expanded pool of long-lived, functional CD4+CD28− T cells that are insensitive to apoptosis signals such as CD95 ligation, AICD, and growth factor withdrawal and express elevated levels of the apoptosis inhibitors Bcl-2 and FLIP (17–19). Also, apoptosis resistance of memory T cells specific for viral peptides and alloantigens was linked to the transcriptional up-regulation of FLIP RNA (47). Up-regulation of FLIP or IAP, however, was not detected in our long-term activated T cells. Also, the differential expression of Bcl-2 family members did not show a clear association between the sensitive and resistant phenotypes. Although the proapoptotic molecules Bak and Bad were decreased during the development of effector/memory cells in vitro, Bax and Bcl-2 levels remained stable. Also, Bcl-2ΔB was up-regulated in apoptosis-sensitive short-term activated cells, with a further increase in apoptosis-resistant, long-term activated T cells. Stauroporine, a mediator of mitochondrial cell death (48), induced apoptosis in short- and long-term activated T cells (data not shown), indicating that differences in the expression of Bcl-2 family members do not predominantly account for apoptosis resistance, but may contribute to an enhanced survival capacity.

Strikingly, we observed that procaspase expression of caspase-3, -8, and -10 was strongly decreased in long-term activated T cells of the effector/memory phenotype, and only active caspase fragments were detected. Caspase activation in the absence of apoptotic stimuli has also been observed in TCR-triggered human T cells (49–53). Caspase inhibitors or Fas-Fc, preventing the interaction between CD95 receptor and endogenously produced and secreted CD95L, inhibited T cell proliferation in the early phase of T cell stimulation, suggesting a model in which T
cell activation via TCR up-regulates CD95L, which binds to surface CD95 receptor and induces caspase activation. In our long-term activated T cells, increased CD95L expression might also be induced by TCR triggering through the stimulator cells, which subsequently leads to caspase activation. Activation of T cells and caspses, however, is always synchronized with the up-regulation of molecules of the intrinsic death machinery, such as perforin, GrzA, GrzB, and CD95L. To rule out that caspase activation in long-term activated T cells is induced by the release of granzymes after protein isolation (54) we measured internal caspase-3 and -8 activity in living cells. While caspase-3 activity was not observed in unstimulated T cells, a 3-fold increase in activity was detected during the development from short- into long-term activated T cells, which was, to a lesser extent, also detected for caspase-8 activity. Although effector caspases were activated, cleavage of nuclear targets such as lamin B or DFF45 was not found, indicating that caspase activation in long-term activated T cells does not lead to nuclear apoptosis. Partial PARP-cleavage was already present after the first stimulation and did not increase after further stimulations, corresponding to published data (50, 51). Caspases, however, may still be cleaved by granzymes that are exported from cytolytic granules into the cytoplasm of T cells (55). In addition, it is not clear how T cells are protected from apoptosis in the presence of active caspase fragments. Safety catch, e.g., is a regulatory peptide, that controls the autocatalytic cleavage of procaspase-3 (56), but to our knowledge no molecules regulating the apoptosis-inducing capacity of active caspase fragments have been described.

Since caspase-8 and -10 are initiator caspases involved in DISC formation, we further analyzed whether functional DISC formation occurs in the presence of constitutive activated caspases. While short-term activated T cells formed a functional DISC after CD95 cross-linking, long-term activated T cells showed an impaired recruitment of caspase-8 and -10 and FADD to the DISC. Up-regulation of c-FLIPshort and thereby reduced procaspase-8 cleavage at the DISC have been described as being responsible for apoptosis resistance of TCR/CD3-activated, short-term activated T cells (12). However, we did not detect differences in FLIPs or processed FLIPs recruitment to the DISC in uninduced or CD95-triggered T cells. This may be due to differences in the T cell populations used, i.e., short-term activated T cells vs long-term activated effector/memory T cells. Murine T cells, transgenic for active PKBα/Akt or hapolinsufficient for PTEN expression, a phosphatase that negatively regulates PKBβ/Akt phosphorylation, exhibit a CD95-resistant phenotype induced by decreased recruitment of caspase-8 to the DISC (14). In our system impaired recruitment of initiator caspases-8 and -10 in long-term activated T cells was linked to the loss of PTEN expression and increased levels of PKBα/Akt phosphorylation. PKBα/Akt is phosphorylated in response to PIP3, a second messenger generated by PI3-K. Incomplete procaspase-8 processing at the DISC was also observed in the presence of active PI3-K (13), as shown for TCR/CD3-triggered apoptosis-resistant TH2 cells. Inhibition of PI3-K activity restored CD95 sensitivity in long-term activated T cells and increased the recruitment of caspases to the DISC, indicating that active PI3-K might mediate protection from CD95-induced cell death. How PKBα/Akt phosphorylation inhibits recruitment of effector caspases to the DISC, however, is currently unknown. Phosphorylation of Bad and caspase-9 by PKBα/Akt inhibits mitochondrial-mediated apoptosis (57, 58), but nonfunctional Bad or caspase-9 would not influence recruitment of FADD and initiator caspases to the CD95 receptor. More likely, PKBα/Akt acts via regulation of transcriptional factors, such as NF-kB (59, 60) or c-Myb (61), on the up-regulation of molecules preventing the association of caspases and FADD to the CD95 receptor. Loss of PTEN expression in mouse T cells renders them insensitive to different apoptosis stimuli, such as anti-CD95 treatment, IL-2, IL-2 plus serum withdrawal, and AICD (62, 63), reflecting CD95 resistance and decreased AICD induction in our human effector/memory T cells. Transfection of PTEN in long-term activated T cells will show whether CD95 sensitivity and functional DISC formation can be restored.

Defects in the CD95 signaling pathway of long-term activated T cells of the effector/memory phenotype could be further explained by preassociation of caspase-8 cleavage products (p43/41) to the CD95 receptor in the absence of receptor cross-linking and cluster formation of the receptor on the cell surface. It is not clear, however, whether cluster formation induces continuous caspase activation and impaired DISC formation or whether preassociation of caspase-receptor complexes leads to CD95 resistance. Possibly, CD95 receptors on long-term activated T cells are recruited into clusters by extracellular oligomerization domains (64, 65), inducing conformational changes or continuous caspase activation and preventing CD95 signaling. Furthermore, constitutive complex formation of CD95 receptor with its ligand CD95L, which is strongly up-regulated in long-term activated T cells, might explain CD95 resistance. DISC assays, immunoprecipitations, or immunofluorescence analysis, however, did not reveal any complex formation between CD95L and its receptor (data not shown).

In vitro-derived, long-term activated T cells resemble self-reactive T cells in patients with chronic graft-vs-host disease after bone marrow transplantation from unrelated donors. Clinically these T cells can only be controlled by aggressive cytotoxic therapy, but many patients develop resistance to drugs such as corticosteroids, cyclosporin A, or anti-thymocyte globulin, reflecting their apoptosis-resistant status (66, 67). Also, in vitro-generated long-term activated T cells were less susceptible to cyclophosphamide- and methotrexate-induced apoptosis than short-term activated T cells (data not shown). Immunosuppressive therapy that prevents T cell activation and induces T cell apoptosis (25) makes it difficult to identify long-term activated T cells in patients with chronic graft-vs-host disease in vivo. A small proportion of CD8+ T cells from three haplo-identical transplanted patients displayed the phenotype of long-term activated T cells and showed decreased CD95 sensitivity and AICD induction (data not shown). Also, in the synovial fluid of one patient with rheumatoid arthritis, 50% of the CD8+ T cells did not express CD28, CD62L, CD27, and CCR7 and showed up-regulation of CD56, resembling effector/memory T cells in vivo. Further analysis of these T cells will show whether they exhibit CD95 resistance by similar mechanisms as in vitro derived long-term activated T cells.

In conclusion, the present data provide strong evidence that apoptosis resistance of long-term activated T cells is due to an impaired CD95 signaling pathway. Constitutive caspase activation, continuous clustering of CD95 receptors, and impaired DISC formation are associated with the CD95-resistant phenotype. Understanding the molecular mechanisms of internal caspase activation and CD95 clustering in the absence of cell death can provide novel therapeutic strategies to sensitize CD95-resistant T cells in vivo.

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References


