

# Fas Ligand Mediates Activation-induced Cell Death in Human T Lymphocytes

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## Summary

A significant proportion of previously activated human T cells undergo apoptosis when triggered through the CD3/T cell receptor complex, a process termed activation-induced cell death (AICD). Ligation of Fas on activated T cells by either Fas antibodies or recombinant human Fas-ligand (Fas-L) also results in cytolysis. We demonstrate that these two pathways of apoptosis are causally related. Stimulation of previously activated T cells resulted in the expression of Fas-L mRNA and lysis of Fas-positive target cells. Fas-L antagonists inhibited AICD of T cell clones and staphylococcus enterotoxin B (SEB)-specific T cell lines. The data indicate AICD in previously stimulated T cells is mediated by Fas/Fas-L interactions.

Mature peripheral T cells generally undergo activation and proliferation when stimulated through the CD3/TCR complex. Under certain circumstances, however, thymocytes, T cell hybridomas, and both CD4<sup>+</sup> and CD8<sup>+</sup> T cell clones (TCC)<sup>1</sup> undergo cell death when stimulated through the TCR with CD3 antibody in the absence of APC (1–6). This process is rapid and exhibits classic characteristics of apoptosis such as membrane blebbing, chromatin condensation, and the formation of DNA fragments of ~200 bp. Deletion of T cells by apoptosis appears to be important not only in regulating autoreactive T cells in the thymus, but also in regulating the peripheral T cell pool (7, 8). Little is known, however, about the mechanism that mediates the lytic process that has been termed activation-induced cell death (AICD).

Fas/APO-1 (CD95) is a protein expressed on the surface of a variety of transformed cell lines and chronically stimulated T cells that can mediate apoptosis after ligation with a Fas-specific antibody (9–12). Under appropriate conditions Fas also transduces a stimulatory signal to certain B cell lines (13) and to freshly isolated human peripheral blood T cells and thymocytes (14). To investigate a possible relationship between CD3-stimulated AICD and Fas-mediated T cell apoptosis, we have used a mAb directed against human Fas (Fas M3). Immobilized Fas M3 mAb is able to lyse Fas-expressing tumor cell lines in a manner analogous to Fas-ligand (Fas-L) or the prototypic Fas mAb, CH-11, whereas soluble Fas M3 blocks Fas-mediated killing (15).

## Materials and Methods

**T Cell Lines and Clones.** The alloreactive TCC used in this study were generated by establishing MLC in bulk culture for 7 d followed by limit dilution cloning in 96-well round-bottomed plates in the presence of 10<sup>5</sup> irradiated allogeneic PBMC and 10 ng/ml of IL-2. TCC were maintained by stimulation with irradiated PBMC and soluble CD3 antibody (10 ng/ml) approximately every 2 wk and maintenance in IL-2 (10 ng/ml) between stimulations. Short-term staphylococcal enterotoxin B (SEB)-specific T cell lines were established by stimulation of PBMC (10<sup>6</sup>) with 5 µg/ml SEB (Sigma Chemical Co., St. Louis, MO) for 3 d followed by expansion of cells in IL-2 (5 ng/ml). Cells were maintained in IL-2 for 2 wk before use.

**Fas.Fc and Fas mAbs.** A Fas fusion protein consisting of the extracellular domain of human Fas coupled to the Fc region of human IgG1 (Fas.Fc) was generated as described (14). The anti-human Fas mAbs, M3 and M31, were derived from mice immunized with human Fas.Fc. When added in solution, Fas M3 blocks Fas-mediated lysis, whereas Fas M31 binds to Fas but has no agonistic or antagonistic properties (15).

**Fas-L Bioassay.** For detection of Fas-induced killing, <sup>51</sup>Cr-labeled Jurkat cells were incubated with varying numbers of effector cells for 18 h in the presence or absence of PMA (10 ng/ml) and ionomycin (500 ng/ml) or immobilized CD3 mAb (10 µg/ml), as described previously (15). Cultures were performed in 96-well round-bottomed plates and harvested using an SCS harvesting system (Skatron, Sterling, VA). <sup>51</sup>Cr content of supernatants was determined using an ME Plus gamma scintillation counter (Micro-medics, Huntsville, TN). Percent specific <sup>51</sup>Cr release was calculated according to the formula  $100 \times [(experimental\ cpm) - (spontaneous\ cpm)] / [(maximum\ cpm) - (spontaneous\ cpm)]$ , where spontaneous cpm = cpm released in the absence of effector cells and maximum cpm = cpm released in the presence of 1 N HCl.

<sup>1</sup> Abbreviations used in this paper: AICD, activation-induced cell death; Fas-L, Fas-ligand; SEB, staphylococcal enterotoxin B; TCC, T cell clone.

**Cloning of Human Fas-L.** A 180-bp fragment of murine Fas-L DNA was isolated by PCR as described (16). This PCR product was labeled with  $^{32}\text{P}$  by random priming and used to probe a phage cDNA library prepared from activated human lymphocytes (17). Hybridization was at  $37^\circ\text{C}$  and the filters were washed at  $55^\circ\text{C}$  in  $2\times$  SSC. The purified phage cDNA inserts were amplified by PCR, digested with EcoRI and subcloned into pBluescript SK (Stratagene Cloning Systems, La Jolla, CA). For expression of the human Fas-L, one of the isolated cDNAs (GenBank accession number U08137) was excised by digestion with SalI/NotI and cloned into the mammalian expression vector pDC409 (18), which had been similarly digested. The human Fas-L cDNA was transfected into 293 cells and supernatants collected after 3 d and concentrated tenfold before assay for biological activity.

**Flow Cytometric Detection of Apoptotic Cells.** Detection of apoptotic cells by multiparameter flow cytometry used the fluorophores Hoechst 33342 and propidium iodide in combination with forward light scatter (19). Cells were analyzed using an Epics Elite cytometer (Coulter Corp., Hialeah, FL) equipped with argon (488 nm emission) and helium-cadmium (325 nm emission) lasers. Immediately before analysis, Hoechst 33342 and propidium iodide were added to single-cell suspensions ( $10^6$  cells/ml) such that final concentrations were 10 and  $30\ \mu\text{M}$ , respectively. Forward light scatter, log scale red fluorescence (propidium iodide, 675 nm) and log scale blue fluorescence (HO342, 525 nm) were measured at a rate of 700–800 cells/s, and the data stored in list mode. Cell debris and doublets were excluded by light scatter, and dead and necrotic cells by red fluorescence. The data are plotted as blue fluorescence vs. forward light scatter contour histograms.

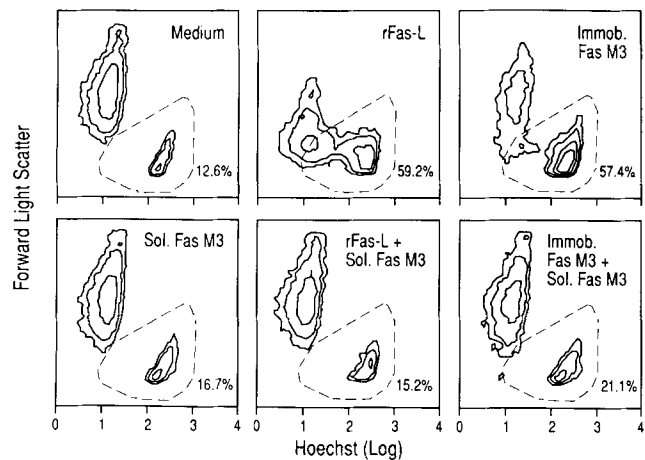
**Northern Blot Analysis.** PL-1 cells were stimulated with immobilized CD3 mAb for varying time periods and total RNA was prepared and Northern analysis performed, as described (20), using a human Fas-L antisense riboprobe. Ribosomal RNA was stained with methylene blue to ensure equal loading in each lane.

**Assessment of DNA Fragmentation.** For assessment of DNA fragmentation, cell pellets were lysed in 10 mM Tris-HCl/10 mM EDTA/0.2% Triton X-100 and centrifuged ( $13,000\ g$ ) for 10 min (21). Supernatants containing RNA and fragmented DNA were extracted with phenol followed by phenol/chloroform/isoamyl alcohol. The DNA/RNA was precipitated with ethanol, dried with a SpeedVac for 6 min and dissolved in  $15\ \mu\text{l}$  of 10 mM Tris/HCl/1 mM EDTA. Samples were treated with  $600\ \mu\text{g/ml}$  of RNase and electrophoresed in 1% agarose gels and DNA was visualized under ultraviolet light after staining with ethidium bromide.

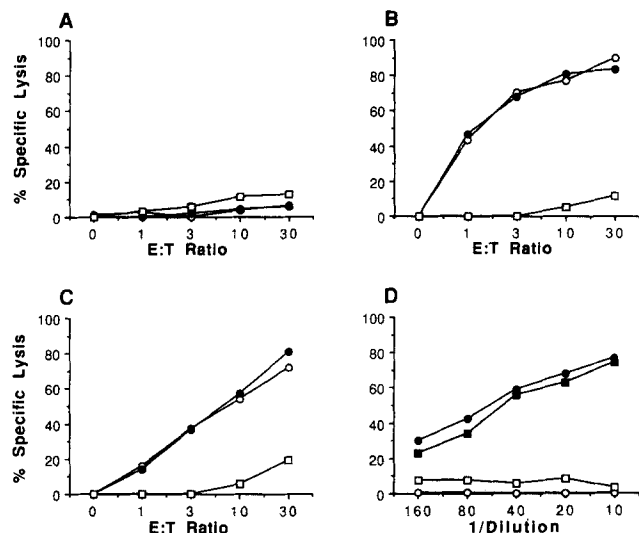
**Confocal Microscopy.** Cells were stained with a live/dead viability/cytotoxicity kit as recommended by the manufacturer (Molecular Probes Inc., Eugene, OR). Live cells were distinguished by their ability to convert nonfluorescent calcein AM to green fluorescent calcein via intracellular esterase activity. Ethidium homodimer stains the DNA of cells with damaged membranes and fluoresces red. Cultures were examined with a confocal laser scanning microscope (Molecular Dynamics, Sunnyvale, CA).

## Results

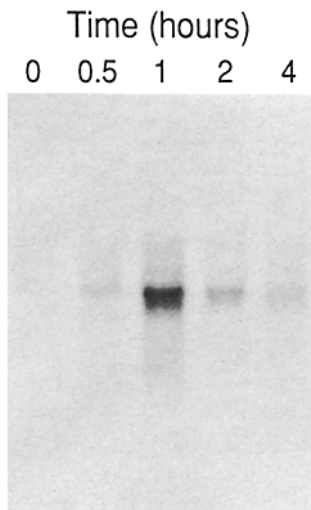
**Fas-L Induces Apoptosis in Human T Cell Clones.** Initially, we compared the ability of immobilized Fas M3 mAb and recombinant human Fas-L (rFas-L) to induce apoptosis in chronically stimulated alloreactive  $\text{CD4}^+$  and  $\text{CD8}^+$  human TCC. Cell viability was assessed using a multiparameter flow cytometric technique that discriminates apoptotic from necrotic cell death based upon measurement of forward light



**Figure 1.** Immobilized Fas M3 mAb and rFas-L induce apoptosis in  $\text{CD4}^+$  TCC which is blocked by soluble Fas M3 mAb. PL-1 cells were cultured for 18 h in medium alone, rFas-L (1:5 dilution of  $10\times$  supernatant), or in wells that contained immobilized Fas M3 mAb either in the presence or absence of soluble Fas M3 mAb ( $10\ \mu\text{g/ml}$ ). Fas M3 mAb was immobilized in 24-well culture plates for 4 h at room temperature at  $10\ \mu\text{g/ml}$  in PBS followed by six washes with PBS. Cells were recovered and analyzed for apoptosis by multiparameter flow cytometry. The percent apoptotic cells is indicated. Data are representative of ten experiments.



**Figure 2.** PL-1 cells stimulated with either PMA plus ionomycin or immobilized CD3 mAb are active in a bioassay for Fas-L activity.  $^{51}\text{Cr}$ -labeled Jurkat target cells were cultured for 24 h with PL-1 cells cultured in (A) medium, (B) PMA ( $10\ \text{ng/ml}$ ) plus ionomycin ( $500\ \text{ng/ml}$ ), or (C) immobilized CD3 mAb ( $10\ \mu\text{g/ml}$ ) in the absence (open circles) or in the presence of soluble Fas M3 (open squares) or Fas M31 (solid circles) mAb ( $10\ \mu\text{g/ml}$ ). (D) Jurkat target cells were cultured with titrated concentrations of control supernatant (open circles), or rFas-L supernatant either alone (solid circles) or with Fas M3 mAb (open squares) or Fas M31 mAb (solid squares). Neither PMA plus ionomycin nor CD3 mAb affected the spontaneous release of  $^{51}\text{Cr}$  by Jurkat cells cultured in the absence of PL-1 effector cells. Data are representative of five experiments.



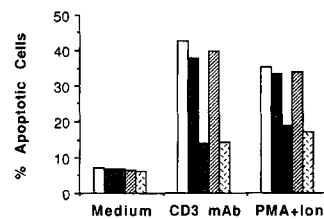
**Figure 3.** Regulation of Fas-L transcription in CD3 mAb stimulated PL1 cells. PL1 cells were cultured for varying time periods, RNA was prepared and Northern analysis of total RNA performed using a human Fas-L antisense riboprobe. Ribosomal RNA was stained with methylene blue to ensure equal loading in each lane.

scatter and the DNA binding fluorophores propidium iodide and Hoechst 33342. The alloreactive CD4<sup>+</sup> TCC, PL-1, showed little evidence of apoptotic cell death when cultured for 18 h in medium alone (Fig. 1). Upon culture with either rFas-L or immobilized Fas M3 mAb, however, almost 60% of cells were determined to be undergoing apoptosis (Fig. 1). In addition, soluble Fas M3 mAb, which had no effect on PL-1 viability when added alone, efficiently blocked apoptosis induced by either rFas-L or immobilized Fas M3 mAb. Similar results were obtained when cell viability was assessed in a panel of TCC (six CD4<sup>+</sup> and two CD8<sup>+</sup> TCC generated from three individual donors) and by a variety of other techniques, including trypan blue dye exclusion, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) conversion, propidium iodide uptake, and by a DNA fragmentation assay (data not shown).

**Human T Cell Clones Express Fas-L.** We have recently developed a sensitive bioassay to detect the presence of Fas-L which uses the Fas-sensitive human T cell line Jurkat as a target cell in a <sup>51</sup>Cr-release cytotoxicity assay (15). This assay was demonstrated to be specific for Fas-L because Jurkat cells are not lysed by either TNF or lymphotoxin- $\alpha$  and because soluble Fas M3 mAb blocked cytolysis by effector cells induced to express Fas-L. PL-1 cells cultured in the presence of either PMA plus ionomycin or immobilized CD3 mAb induced lysis of Jurkat cells, whereas nonactivated TCC had little effect (Fig. 2). The lysis of Jurkat target cells induced by either of these stimuli was almost completely inhibited by the addition of soluble Fas M3 mAb, but not by the non-neutralizing Fas M31 control mAb. In addition, rFas-L was strongly lytic toward Jurkat target cells, and its activity was blocked completely by soluble Fas M3 but not by Fas M31 mAb (Fig. 2 D).

The kinetics of Fas-L expression by PL-1 cells after activation were determined by Northern analysis. Little or no Fas-L-specific mRNA was detected in nonstimulated PL-1 cells (Fig. 3). Within 30 min of stimulation with immobilized CD3 mAb, however, PL-1 cells expressed significant Fas-L mRNA which peaked at  $\sim$ 1 h after stimulation.

**Fas Antagonists Block AICD.** Our data indicate that TCC

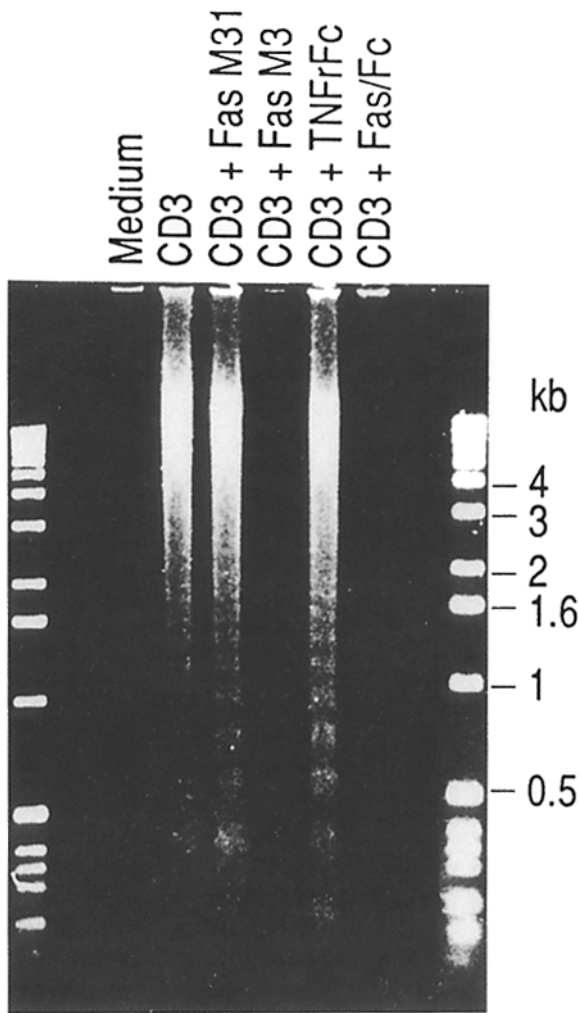


**Figure 4.** Fas antagonists inhibit AICD in TCC. Apoptosis was measured in PL-1 cells by Hoechst 33342 staining after 18 h stimulation. Fas.Fc and TNFR (p75).Fc are fusion proteins consisting of the extracellular domain of either Fas or the p75 TNF receptor coupled to human IgG1 and were used at 10  $\mu$ g/ml. The Fas antibodies (M3 and M31) were used at 10  $\mu$ g/ml. Data are representative of ten experiments.  $\square$  0;  $\blacksquare$  Fas M31;  $\text{■}$  Fas M3;  $\text{▨}$  TNFR.Fc;  $\text{▩}$  Fas.Fc.

are both susceptible to Fas-mediated apoptosis and can be induced to express Fas-L (Figs. 1–3). Therefore, the interaction of this cognate pair may account for the AICD seen upon exposure of TCC to stimuli such as CD3 mAb or phorbol ester plus calcium ionophore. To directly test this hypothesis, we used antagonists of Fas in an attempt to block apoptosis induced by CD3 mAb or PMA plus ionomycin. Soluble Fas M3 mAb blocked AICD in PL-1 cells induced by either of these stimuli, whereas the control mAb, Fas M31, had no effect (Fig. 4). Neither Fas mAb affected cell viability in the absence of stimulation. AICD induced by phytohemagglutinin was also strongly inhibited by soluble Fas M3 antibody, and similar results were obtained with four other TCC (data not shown).

To determine whether blocking of AICD in TCC was due to interference with the interaction of Fas with its ligand, or whether the Fas M3 mAb acted by signaling the T cells directly, we tested the effect of a soluble Fas.Fc fusion protein on AICD. Fas.Fc blocked AICD, whereas the TNFR(p75).Fc had no effect (Fig. 4). Thus, AICD in T cells is dependent upon Fas-L but appears to be independent of TNF. To confirm that the AICD observed using the Hoechst staining method was apoptotic cell death, we isolated DNA from cells stimulated as in Fig. 4 and confirmed that AICD was associated with oligonucleosomal DNA degradation (Fig. 5). CD3 mAb-induced DNA fragmentation was specifically blocked by the Fas antagonists Fas M3 and Fas.Fc but not by the control antibody or fusion protein (Fig. 5).

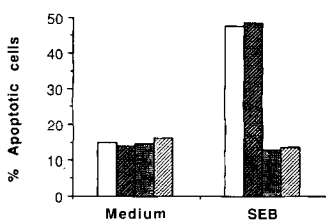
**Fas Antagonists Block Superantigen AICD.** Superantigens stimulate a large percentage of T cells via their ability to simultaneously bind to class II MHC and V $\beta$  TCR (22, 23). In animal models, superantigens, which include many bacterial toxins such as SEB, cause an initial expansion of T cells expressing the appropriate V $\beta$  gene followed by deletion of these cells to amounts lower than in unchallenged animals (24–27). Deletion of superantigen-specific human T cell lines has also been observed in vitro and occurs by an apoptotic process (28, 29). To assess whether superantigen-induced T cell deletion also involves Fas/Fas-L interactions, we established short-term SEB-specific T cell lines and tested whether SEB-induced AICD could be blocked by Fas antagonists. We found both soluble Fas M3 mAb and Fas.Fc completely inhibited AICD induced by SEB (Fig. 6). These Fas antagonists also blocked



**Figure 5.** Fas antagonists block DNA fragmentation in TCC induced by CD3 ligation. Cells were analyzed for DNA fragmentation after 8 h stimulation. Data are representative of four experiments.

AICD stimulated by CD3 mAb in the SEB-specific T cell lines (data not shown).

**Visualization of AICD by Confocal Microscopy.** To directly visualize the effect of soluble Fas M3 mAb on AICD, the process was examined by confocal microscopy using fluorophores that discriminate live (*green fluorescence*) from dead (*red fluorescence*) cells. PL-1 cells cultured overnight in medium alone were >98% viable (Fig. 7 A), whereas widespread cell death was evident in cells cultured with rFas-L (Fig. 7 B). This lytic effect was completely inhibited by addition of soluble



**Figure 6.** Fas antagonists block AICD in SEB-specific T-cell lines. Short-term SEB-specific T cells were stimulated with SEB (5  $\mu$ g/ml) for 18 h and apoptosis was measured by Hoechst staining. Data are representative of four experiments.  $\square$  0;  $\blacksquare$  Fas M31;  $\square$  Fas M3;  $\square$  Fas.Fc.

Fas M3 mAb (Fig. 7 C). PL-1 cells cultured with CD3 mAb formed tight clusters of cells and within the clusters were apposing live and dead cells (Fig. 7 D). With the addition of soluble Fas M3 mAb, cell clusters were not disrupted and few, if any, dead cells were detected within the clusters (Fig. 7 E). These results suggest that the lytic processes of AICD induced by CD3 stimulation require direct cell-cell contact and are completely inhibitable by addition of antagonists of Fas/Fas-L interactions.

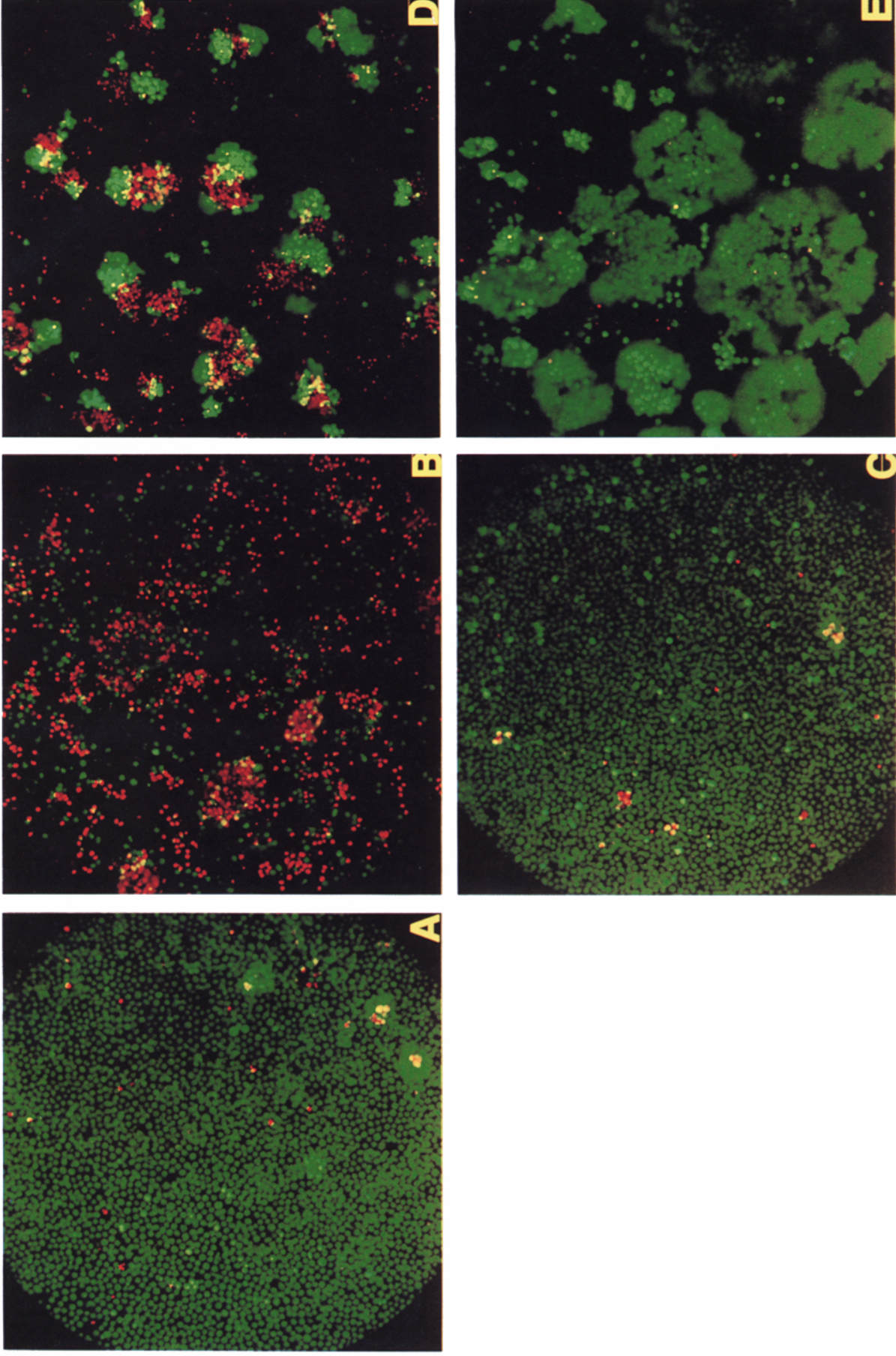
## Discussion

Apoptosis is believed to play an important role in the deletion of autoreactive or unwanted T cells in two different phases during the ontogeny of the immune response. First, the encounter of self-antigens in the thymus leads to T cell deletion characterized by apoptotic cell death. Thymic T cell apoptosis, however, appears to be independent of Fas, because mice homozygous for the *lpr* mutation, which results in expression of a defective Fas molecule (30, 31), appear to delete autoreactive T cells in a normal manner (32). Second, chronically stimulated mature T cells can be eliminated in the periphery by the process of AICD (1-6). The data presented in this paper suggest that Fas-L is critically involved in AICD of mature T cells, and it therefore seems likely that Fas-L is the prime mediator of the peripheral deletion of T cells and maintenance of peripheral self-tolerance. Thus, it may be possible that antagonists of Fas will block peripheral deletion and tolerance induction in vivo, and perhaps lead to the development of autoimmune reactivity.

In addition to eliminating self-reactive T cells in the periphery, Fas-L may serve to limit the expansion of antigen-activated lymphocytes by mediating AICD in a proportion of cells that reencounter their antigen or in cells that remain activated after elimination of their cognate antigen. In this sense, Fas-L would prevent excess accumulation of antigen-reactive T cells. This is consistent with the pathophysiology observed in *lpr/lpr* mice which display a progressive development of an autoimmune disease process characterized in part by the accumulation of T cells with an unusual phenotype (33). Thus, a defect in Fas would result in the failure of T cells to undergo AICD and result in their gradual accumulation, including those cells specific for self-antigens.

A recent analysis of cell surface molecules involved in superantigen AICD suggested that LFA-1/intercellular adhesion molecule 1 (ICAM-1) interactions are involved because an antibody to LFA-1 partially blocked cell death (34). Our data do not preclude an accessory involvement of LFA-1/ICAM-1 in the adhesion of cells undergoing AICD, but Fas-L most likely provides the signal that actually initiates the apoptotic process.

The involvement of Fas in AICD was recently suggested by experiments that demonstrated that T cells derived from *lpr/lpr* mice are defective in AICD (35, 36). In addition, *gld/gld* mice show a similar defect in AICD and thus it was suggested that the *gld* product links the CD3/TCR and Fas apoptotic pathways (37). These observations, together with the recent demonstration that *gld/gld* mice have a mutation in



**Figure 7.** Confocal microscopic visualization of AICD. PL-1 cells were cultured for 24 h with (A) medium alone, (B) rFas-L, (C) rFas-L plus soluble Fas M3 mAb, (D) immobilized CD3 mAb, and (E) immobilized CD3 mAb plus soluble Fas M3 mAb and stained with reagents from a viability kit. Live cells were distinguished by esterase activity (*green*), whereas dead cells were stained with ethidium homodimer (*red*) and were examined with a confocal laser scanning microscope.

the Fas-L gene (16, 38), support a role for Fas and Fas-L in AICD. Their precise mechanism of action in AICD in normal T cells, however, was not addressed. Data presented here not only directly demonstrate that AICD in normal activated human T cells involves Fas-L/Fas but further demonstrate the process by which this interaction occurs. Thus, we show the induction of expression of Fas-L by activated T cells upon activation, susceptibility to recombinant Fas-L-mediated killing by these same T cells, and finally that although antagonists of Fas do not block clustering of T cells induced by TCR ligation, they virtually completely inhibit the induction of apoptosis. In contrast, an antagonist of TNF had no effect on AICD suggesting that this process is independent of the TNF/TNFR apoptotic pathway.

Fas-L expression by activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells may shed light on the mechanism of TNF-independent cytotoxicity mediated by CD4<sup>+</sup> cytotoxic T cells and non-MHC-restricted cytotoxicity mediated by CD8<sup>+</sup> T cells. T cells induced to express Fas-L lyse target cells susceptible to Fas antibody-mediated cytotoxicity (Fig. 2), which includes a wide variety of transformed cell lines (9–12). The role of Fas-L

in T cell-mediated cytotoxicity is supported by two recent studies suggesting that Fas is a major target for CD4<sup>+</sup> T cell-mediated cytotoxicity (39) and that a component of CD8<sup>+</sup> CTL killing involves Fas-L (40).

AICD mediated through Fas may have broader implications for the immune response and autoimmune disease in general. For example, AICD has been observed in T cells freshly isolated from HIV-infected, but not from uninfected, individuals (41, 42). Thus, apoptosis may play a role in the diminution of CD4<sup>+</sup> T cells and the progression to AIDS in HIV infected individuals. The AICD seen in activated normal T cells and in freshly isolated T cells from HIV<sup>+</sup> individuals is qualitatively identical, and it is tempting to speculate that Fas/Fas-L may be involved in this process in AIDS patients. If so, therapeutic intervention for HIV-infected individuals with a Fas antagonist may be possible. The recent molecular cloning of a rat and mouse ligands for Fas (16, 38, 43) and the human Fas-L described herein will facilitate the determination of the precise role for Fas ligand in AICD in both normal immune responses and in responses where apoptosis appears to be dysregulated, such as in HIV infection.

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