The dual functions of Fas ligand in the regulation of peripheral CD8$^+$ and CD4$^+$ T cells

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Although Fas ligand (FasL) is well characterized for its capacity to deliver a death signal through its receptor Fas, recent work demonstrates that FasL also can receive signals facilitating antigen (Ag)-specific proliferation of CD8$^+$ T cells. The fact that the *gld* mutation differentially influences the proliferative capacity of CD8$^+$ and CD4$^+$ T cells presented the intriguing possibility that a single molecule may play opposing roles in these two subpopulations. The present study focuses on how these positive and negative regulatory roles are balanced. We show that naive CD4$^+$ T cells are responsive to FasL-mediated costimulation on encounter with Ag when Fas-mediated death is prevented. Thus, the machinery responsible for transducing the FasL positive reverse signal operates in both CD4$^+$ and CD8$^+$ T cells. Instead, differential control of FasL expression distinguishes the role of FasL in these two T cell subpopulations. FasL costimulation occurs immediately on T cell receptor ligation and correlates with the up-regulation of FasL expression on CD8$^+$ and naive CD4$^+$ T cells, both of which are sensitive to the FasL costimulatory signal. Conversely, FasL-initiated death occurs late in an immune response when high levels of FasL expression are maintained on CD4$^+$ T cells that are sensitive to Fas-mediated death, but not on CD8$^+$ T cells that are relatively insensitive to this signal. This careful orchestration of FasL expression during times of susceptibility to costimulation and conversely, to death, endows FasL with the capacity to both positively and negatively regulate the peripheral T cell compartment.

costimulation | homeostasis | peripheral T cell

The interaction of Fas (CD95/APO-1) with FasL (CD95L) performs many different functions in the regulation of peripheral lymphocytes, perhaps foremost of which is the induction of cell death mediated through the Fas receptor (reviewed in ref. 1). Fas-mediated cell death is one of the mechanisms by which cytolytic T cells lyse their targets (2, 3), the primary means by which CD4$^+$ T cell numbers are reduced after Ag activation (4–6), and the mechanism by which B cell homeostasis is maintained (7, 8).

The role of Fas–FasL interactions in regulating CD8$^+$ T cells has been less clear, given that the primary deletional mechanism for activated CD8$^+$ T cells is triggered through the tumor necrosis factor receptor rather than through Fas (9). In fact, CD8$^+$ T cells are inherently resistant to Fas-mediated cytotoxicity (10). Recent work from our lab has identified a new role for the Fas–FasL interaction in positively regulating the CD8$^+$ T cell proliferative response by generating a costimulatory signal (11). CD8$^+$ T cell lines derived from *gld* mice that express a nonfunctional form of FasL (hereafter designated FasL$^-$ for simplicity) are depressed in Ag-specific proliferation compared with FasL$^+$ CD8$^+$ T cell lines, even in the presence of exogenous IL-2 (11). Because FasL$^-$ CD8$^+$ T cells can proliferate to wild-type levels on optimal stimulation, this attenuated proliferation is not a reflection of an intrinsic T cell receptor (TCR) signaling defect but is more likely due to the lack of a costimulatory signal during antigenic stimulation (11).

FasL was identified as the source of this costimulatory signal in CD8$^+$ T cells by using a fusion protein consisting of the extracellular domain of murine Fas joined to the hinge and constant regions of human IgG1 (FasIgG, ref. 4). In soluble form, FasIgG inhibits proliferation of wild-type CD8$^+$ T cells to the level attained by FasL$^-$ cells. In plate-bound form, FasIgG amplifies proliferative signals when used in conjunction with suboptimal levels of immobilized anti-CD3 (11). Both FasL.1 and FasL.2 allelic products (12) can perform this function, because cells from C57BL/6 (FasL.1) and BALB/cByJ (FasL.2) both demonstrate FasL costimulation (ref. 11 and unpublished observations).

In stark contrast is the role played by Fas–FasL interactions in the CD4$^+$ T cell compartment. FasL$^-$ CD4$^+$ T cells previously have been characterized by more vigorous Ag-specific proliferation than their wild-type counterparts (11, 13, 14), and there is one report that FasL engagement can inhibit the proliferation of wild-type CD4$^+$ T cells (15). To understand the differential effect of Fas–FasL interaction on CD4$^+$ and CD8$^+$ T cells, we have examined CD4$^+$ T cells incapable of undergoing Fas-mediated cell death. We demonstrate that the apparent lack of FasL costimulation in CD4$^+$ T cells is not caused by the inability of FasL to positively reverse signal in this T cell subpopulation. Instead, the positive and negative regulatory roles for FasL in CD8$^+$ and CD4$^+$ T cells, respectively, are dictated in part by the timing of the up-regulation of FasL expression on Ag encounter.

Materials and Methods

Mice and Reagents. C57BL/6 (B6.wt), B6.MRL-*Fas*^{lpr} (B6.*lpr*), B6Smn.C3H-*Fasl*^{gld} (B6.*gld*), B6.C-*H2*^{bm12}/KhEg (B6.bm12), C3H/HeJ (C3H), and C57BL/6-*scid*/SzJ (B6.SCID) mice all were purchased from The Jackson Laboratory and used at 6–9 weeks of age. Reagents include rat antibodies specific for murine CD4 (RL172.4R6) and for murine CD8 (3.168.8), and a hamster antibody specific for murine CD3ε (145–2C11, PharMingen). NIH 3T3 transfectants expressing the FasIgG fusion protein were derived by B. Stanger (4) and generously provided by A. Marshak-Rothstein (Boston University, Boston, MA). FasIgG was used as dialyzed sera from B6.SCID mice injected 5 weeks earlier with the transfectants. The FasIgG concentration in each preparation was determined by an anti-IgG sandwich ELISA using isotype-matched human IgG1 (HuIgG) as the standard.

Cell Preparation and the Generation of T Cell Lines. CD8$^+$ T cells were purified to >99%, and alloreactive H-2^k-specific cytotoxic T lymphocytes (CTLs) were generated and maintained as described (11). All CTL lines were routinely monitored by flow cytometry and CTL assay. CD4$^+$ T cells were purified to >95% as described (11). Anti-H-2^{bm12} CD4$^+$ T cell lines were maintained by restimulation with irradiated B6.bm12 splenocytes

Abbreviations: Ag, antigen; B6.wt, wild-type C57BL/6; B6.*gld*, B6Smn.C3H-*Fasl*^{gld}; B6.*lpr*, B6.MRL-*Fas*^{lpr}; CTL, cytotoxic T lymphocyte; FasIgG, fusion protein of murine Fas with human IgG1; FasL, Fas ligand; HuIgG, human IgG1; TCR, T cell receptor; MFI, mean fluorescence intensity.

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every 12–14 days, and media were supplemented on day 2 of the third and subsequent stimulations with 50 units/ml of recombinant human IL-2.

Proliferation Assays. To measure Ag-specific proliferation, 2×10^4 cells from long-term lines or 2×10^5 primary T cells were cultured with 5×10^5 irradiated allogeneic stimulator cells in a total volume of 200 μl. $CD8^+$ T cells were supplemented with 50 mM α-methyl mannoside and supernatant from Con A-stimulated rat spleen cells (5% for long-term lines and 1.3% for primary cells). Long-term $CD4^+$ T cell lines were supplemented with 50 units/ml of recombinant human IL-2. For proliferation to a suboptimal concentration of anti-CD3 in the presence of a FasL costimulatory signal, responders were added to wells prepared as follows: overnight coating at 4°C with both 15 μg/ml goat anti-hamster IgG and 15 μg/ml goat anti-HuIgG followed after washing by coating 4 hr with a mixture of 0.2 μg/ml anti-CD3 with either 9.4 μg/ml FasIgG or isotype-matched HuIgG (11). Proliferation levels were measured by thymidine incorporation 18 hr after pulsing on day 3 with 1 μCi [^{3}H]dT per well (1 Ci = 37 GBq). Background counts (responders cultured on first-stage antibodies alone) were subtracted from the values. For FasL blocking assays, soluble FasIgG or HuIgG was added to cultures at 10 μg/ml on the day of Ag stimulation or on each indicated day. We previously have demonstrated that soluble FasIgG-mediated attenuation of Ag-specific proliferation is maximal at this dose (11).

Flow Cytometry. FasL expression was determined by staining 1×10^6 cells with 2 μg of anti-murine FasL antibody (Kay-10, PharMingen) conjugated to phycoerythrin or to biotin followed by streptavidin–phycoerythrin and analyzed by flow cytometry on a FACScan using CELLQUEST software (Beckton Dickinson). The geometric mean fluorescence intensity (MFI) was normalized to the 0-hr time point for $CD8^+$ T cells and to the day 1 time point for $CD4^+$ T cells to accommodate considerable lot-dependent differences in antibody brightness. Apoptotic cells among anti-CD3-stimulated $CD4^+$ and $CD8^+$ splenic T cell cultures were detected by double staining 1×10^5 cells with phycoerythrin-conjugated Annexin-V (PharMingen) and 0.5 μg of 7AAD (Molecular Probes), according to the manufacturer's protocol. Cells were analyzed by flow cytometry; live-gated cells within the Annexin-V^+ 7AAD^- compartment were identified as early apoptotic cells and ungated cells within the Annexin-V^+ 7AAD^+ compartment were identified as late apoptotic/dead cells.

Results

FasL Can Costimulate the Ag-Specific Proliferation of $CD4^+$ T Cells. In response to Ag, $FasL^+$ $CD8^+$ T cell lines proliferate better than do $FasL^-$ $CD8^+$ T cell lines, whereas the opposite is true for $CD4^+$ T cell lines (11). However, from this finding it is unclear whether the enhanced proliferation of $FasL^-$ $CD4^+$ T cells is a result of the insensitivity of $CD4^+$ T cells to FasL-mediated costimulation or whether the removal of Fas-mediated death, to which $CD4^+$ T cells are sensitive, masks the effects of FasL costimulation.

To clarify this ambiguity, we analyzed the sensitivity to FasL costimulation of B6.*lpr* (Fas^-) $CD4^+$ T cells. Soluble FasIgG fusion protein binds to FasL and disrupts Fas–FasL interaction on B6.wt and B6.*lpr* $CD8^+$ T cells, resulting in the loss of a positive signal and attenuation of proliferative capacity (Fig. 1*A*; ref. 11). In contrast, disruption of Fas–FasL interactions on B6.wt $CD4^+$ T cells prevents delivery of a negative signal through Fas, thereby blocking the primary mode of death in Ag-activated $CD4^+$ T cells (13). Therefore, we tested the influence of FasL costimulation on B6.*lpr*-derived $CD4^+$ T cells that are incapable of undergoing Fas-mediated death. The addition

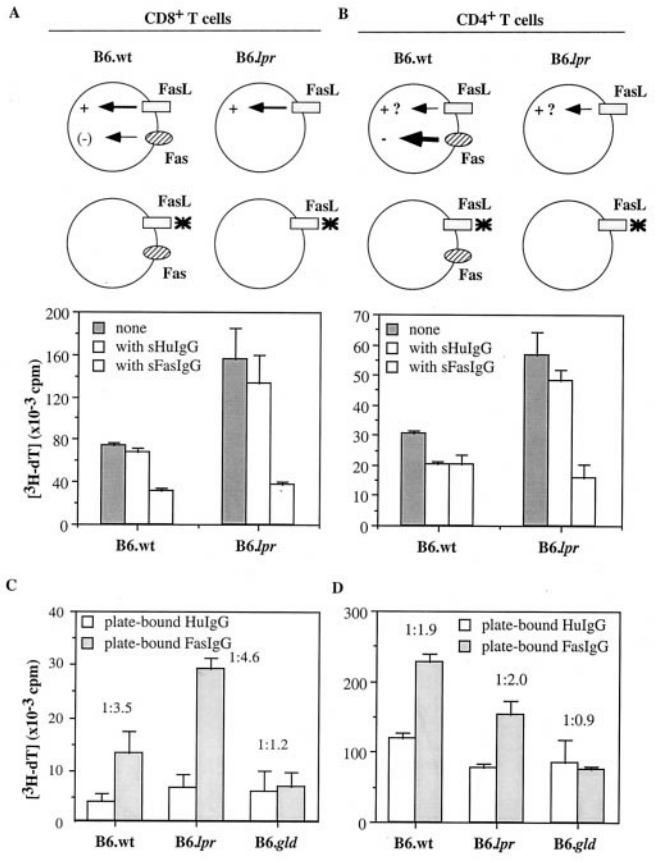

Fig. 1. Both $CD4^+$ and $CD8^+$ T cells are sensitive to FasL costimulation. (*A* and *B*) Schematic diagram of the blocking assay for each cell type is presented above the appropriate panel of data. Soluble FasIgG (denoted by *) blocks both positive costimulatory and negative death signals by binding to FasL. The sizes of the arrows in each diagram correlate with the putative strength of each signal. (*A*) [^{3}H]dT uptake by purified $CD8^+$ T cells from B6.wt and B6.*lpr* mice cocultured with allogeneic H-2^k stimulators alone or in the presence of soluble HuIgG or soluble FasIgG during the entire culture period. Ag-stimulated $CD8^+$ T cells receive a positive signal through FasL and a weak negative signal through Fas, a result of their reduced sensitivity to Fas-mediated death. Only the positive signal is received by *lpr* $CD8^+$ T cells. (*B*) [^{3}H]dT uptake by purified $CD4^+$ T cells from B6.wt and B6.*lpr* mice cocultured with H-2^{bm12} stimulators alone or in the presence of soluble HuIgG or soluble FasIgG during the entire culture period. For $CD4^+$ T cells, the sensitivity to Fas-mediated death (indicated by the large arrow and the negative sign) may mask the positive signal delivered on the interaction of FasL and Fas. The negative signal is missing from *lpr* $CD4^+$ T cells. (*C*) [^{3}H]dT uptake by B6.wt, B6.*lpr*, and B6.*gld* CTL lines seeded over suboptimal amounts of plate-bound anti-CD3 with either plate-bound FasIgG or HuIgG. (*D*) Same as *C* using $CD4^+$ T cell lines as responders. Experiments were repeated four times, and data are averages of triplicate wells with error bars representing the SD of the mean within each experiment. The ratios indicate the fold increase in proliferation of cultures in FasIgG-coated relative to HuIgG-coated wells.

of soluble FasIgG fusion protein inhibits the Ag-specific proliferation of $CD4^+$ T cells from B6.*lpr* but not from B6.wt mice (Fig. 1*B*), demonstrating FasL costimulation in $CD4^+$ T cells that is masked by Fas-mediated cell death in wild-type cells. In this assay, both $CD8^+$ and $CD4^+$ *lpr* responders proliferate better than do their wild-type counterparts, perhaps because of enhanced cytokine production levels (16, 17) and/or the increased expression of costimulatory molecules by *lpr* cells (18, 19). Interestingly, the influence of FasL costimulation on $CD4^+$ T cells was observed only for naive and not for Ag-experienced B6.*lpr* $CD4^+$ T cells (data not shown).

In addition, plate-bound FasIgG fusion protein can augment

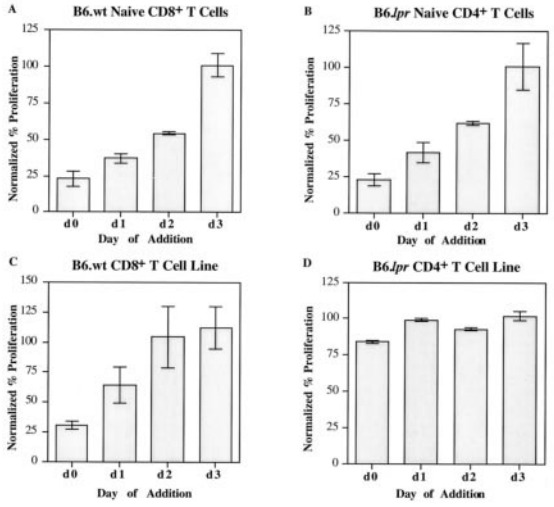

Fig. 2. FasL costimulation requires concomitant Ag-mediated TCR stimulation in naive $CD8^+$ T cells, previously stimulated $CD8^+$ T cells, and naive $CD4^+$ T cells, but not in previously stimulated $CD4^+$ T cell lines. [^{3}H]dT uptake by B6.wt $CD8^+$ splenocytes (*A*) and H-2^k-specific CTLs (*C*) pulsed on day 3 of coculture with H-2^k stimulators and either soluble HuIgG or FasIgG added on the indicated day. [^{3}H]dT uptake by B6.*lpr* $CD4^+$ splenocytes (*B*) and an H-2^{bm12}-specific B6.*lpr* CD4 line (*D*) pulsed on day 3 of coculture with H-2^{bm12} stimulators and either soluble HuIgG or FasIgG added daily between days 0 and 3. Percentages were determined by normalizing the counts per minute of incorporated thymidine in cultures with soluble FasIgG to those in which soluble HuIgG was added on the given days. Experiments were repeated 1–3 times. The cpm incorporated in cultures with HuIgG ranged from 30,000-40,000 cpm in each of three experiments and data are averages of triplicate wells with error bars representing the SD of the mean.

proliferation of $FasL^+$ (B6.wt and B6.*lpr*) $CD8^+$ T cells but not $FasL^-$ (B6.*gld*) $CD8^+$ T cells when used with suboptimal levels of plate-bound anti-CD3 (Fig. 1*C*; ref. 11). In contrast to our inability to detect FasL costimulation in wild-type $CD4^+$ T cells using the blocking assay (Fig. 1*B*), costimulation is readily apparent on cross-linking FasL on $CD4^+$ T cells of both wild-type and *lpr* origin (Fig. 1*D*).

FasL-Mediated Costimulation of Both $CD8^+$ and $CD4^+$ T Cells Occurs Early During the Course of a Mixed Lymphocyte Culture. To determine the timing of the FasL-mediated costimulatory signal in relation to the TCR stimulus in $CD4^+$ and $CD8^+$ T cells, we added soluble FasIgG either at the initiation of Ag encounter or at the indicated time points thereafter. Because B6.wt $CD4^+$ T cells are insensitive to blocking by soluble FasIgG (Fig. 1*B*), we used purified $CD4^+$ T cells derived from B6.*lpr* mice for this assay. The results show inhibition of proliferation on days 0–2 of the initiation of coculture for both naive $CD8^+$ and $CD4^+$ T cells (Figs. 2 *A* and *B*), with the strongest influence on the day of initial Ag stimulation (day 0). Therefore, FasL costimulation of naive T cells occurs early during antigenic stimulation. For Ag-experienced $CD8^+$ T cell lines, inhibition of FasL costimulation occurs within the first 2 days of stimulation (days 0–1; Fig. 2*C*), with the strongest influence again on day 0. Strikingly, the B6.*lpr*-derived $CD4^+$ T cell line that previously had encountered Ag is insensitive to soluble FasIgG blocking of proliferation and does not demonstrate any dependence on FasL costimulation during the time assayed (Fig. 2*D*).

The Accumulation of Apoptotic Cells Among Activated $CD4^+$ T Cells Is Delayed in the Absence of Fas Expression. To determine the temporal relationship between FasL costimulation and Fas-mediated death, we compared the kinetics of Fas-mediated

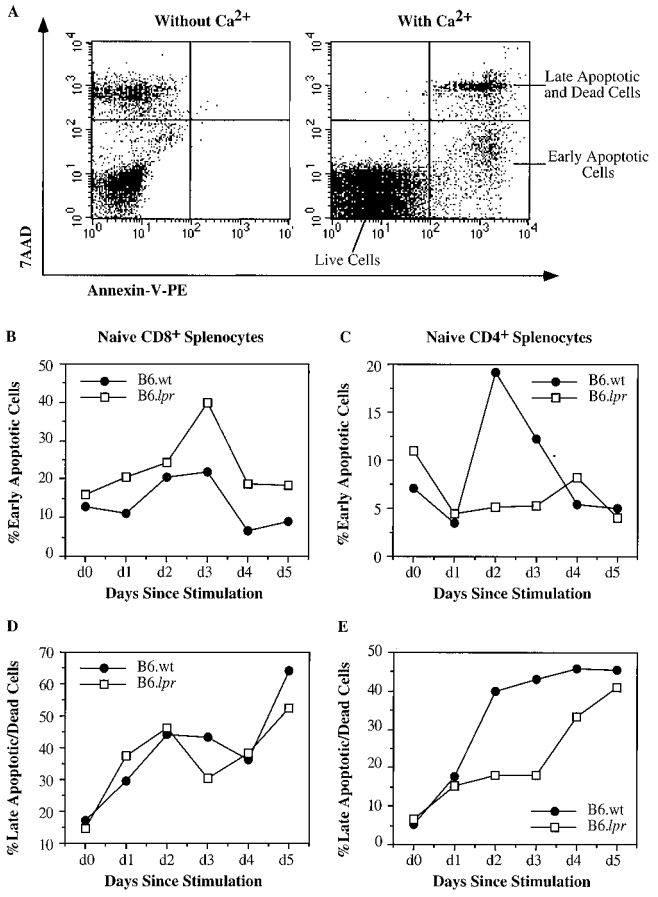

Fig. 3. In the absence of Fas-mediated cell death, the accumulation of apoptotic and dead cells is delayed in populations of $CD4^+$ but not $CD8^+$ T cells. (*A*) Early apoptotic and late apoptotic/dead cells were distinguished by 7AAD and Annexin-V staining (*Right*). Markers were set by control staining in the absence of calcium (*Left*). (*B* and *C*) The percent early apoptotic cells (live-gated Annexin-V^+ $7AAD^-$) is depicted from days 0 to 5 of culture among B6- and B6.*lpr*-derived anti-CD3 stimulated splenic $CD8^+$ and $CD4^+$ T cells, respectively. (*D* and *E*) The percent late apoptotic/dead cells (ungated Annexin-V^+ $7AAD^+$) is depicted from the same cultures as in *B* and *C*. Experiments were repeated twice.

activation-induced cell death in B6.wt- and B6.*lpr*-derived cells. Representative profiles of 7AAD and Annexin-V stained populations of anti-CD3 activated wild-type and B6.*lpr*-derived cells are shown in Fig. 3 *A* (*Right*), as is the sample used to set the relevant markers (*Left*). The results illustrate that the removal of Fas-mediated death from stimulated $CD8^+$ T cells does not reduce the number of early apoptotic cells and late apoptotic/dead cells, nor does it alter the kinetics of their formation (Fig. 3 *B* and *D*). These data confirm that $CD8^+$ T cells can undergo Fas-independent cell death (9). For unknown reasons, $CD8^+$ T cell cultures protected from Fas-mediated death accumulate higher levels of early apoptotic cells throughout the culture period, even though the percentage of late apoptotic/dead cells is the same as in the cultures of B6.wt cells (Fig. 3 *B* and *D*). For $CD4^+$ T cells, however, the absence of Fas reduces and delays the accumulation of early apoptotic cells and slows the accumulation of late apoptotic/dead cells (Fig. 3 *C* and *E*). However, it should be noted that in the absence of Fas-mediated death, other death pathways are used by $CD4^+$ T cells (20, 21), because late apoptotic/dead cells eventually accumulate to wild-type levels (Fig. 3*E*). Thus, Fas-mediated death plays a role in the accumulation of apoptotic and dead $CD4^+$ T cells,

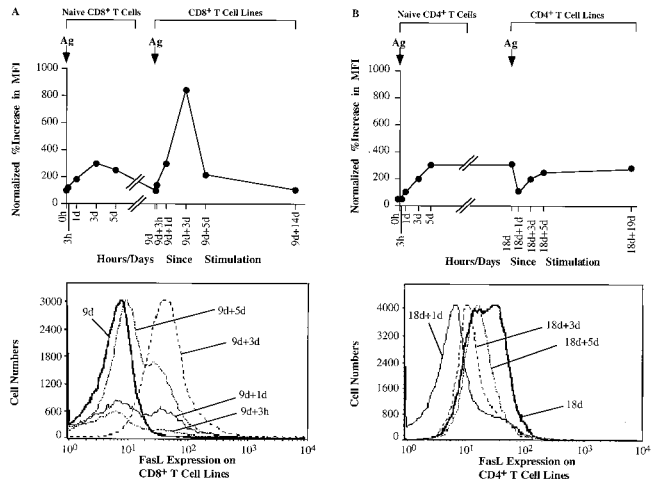

Fig. 4. Ag-induced alterations in FasL expression differ both in tempo and degree in naive and Ag-experienced $CD8^+$ and $CD4^+$ T cells. (*A*) FasL staining of B6.*lpr*-derived $CD8^+$ splenocytes and anti-H-2^k CTL lines cultured with irradiated C3H stimulators. (*B*) FasL staining of B6.*lpr*-derived $CD4^+$ splenocytes and anti-H-2^{bm12} $CD4^+$ T cell lines cultured with irradiated B6.bm12 stimulators. Results from *lpr*-derived cells are shown, because they have the same kinetic patterns of FasL expression as wild-type cells (data not shown), but express this molecule at higher levels (42, 43). Stained populations were gated on cells of high forward scatter to exclude the stimulator cells, and at least 100,000 gated events were analyzed for phycoerythrin staining. (*Upper*) The normalized MFI plotted against time after Ag stimulation. The time of Ag addition is indicated by an arrow and the time points are designated as the number of hours/days after the Ag stimulation plus the hours/days after the subsequent Ag stimulation. (*Lower*) A representative histogram of staining at the indicated time points. The histograms were normalized to allow comparison of the independent peaks.

consistent with previous data indicating the importance of Fas-mediated death in the down-regulation of the $CD4^+$ T cell response (4–6). Fas-mediated death occurs from 24 hr after TCR stimulation onward (Figs. 3 *C* and *E*). Thus, Fas-mediated death, at least with naive $CD4^+$ T cells, occurs later than does FasL costimulation.

The Kinetics of FasL Expression on Ag Encounter Differs Between $CD8^+$ and $CD4^+$ T Cells. We then examined how the kinetics of FasL expression in the two T cell subpopulations correlates with the timing of FasL costimulation and Fas-mediated death. Unlike Fas expression (22, 23), FasL expression is tightly controlled and restricted largely to populations of activated lymphocytes and to sites of immune privilege (22, 23). FasL expression on $CD8^+$ naive splenocytes and Ag-experienced T cell lines follows the same tempo: up-regulation of FasL expression occurs by 3 hr after Ag encounter, peaks on day 3, and begins to decrease by day 4 (Fig. 4*A* and data not shown). In our hands, long-term $CD8^+$ cytolytic lines express higher levels of FasL compared with their naive $CD8^+$ counterparts (Fig. 4*A*). FasL expression on $CD4^+$ T cells encountering Ag for the first time demonstrates an initial phase of up-regulation that occurs with kinetics similar to that of $CD8^+$ T cells (Fig. 4*B*). This up-regulation continues past day 3 and is maintained at a plateau until Ag restimulation. After Ag encounter, FasL expression on previously activated $CD4^+$ T cells is first rapidly decreased, and then up-regulated on day 3. Notably, naive $CD8^+$ and $CD4^+$ T cells and long-term $CD8^+$ cytolytic T cell lines all up-regulate FasL levels at a time when FasL-mediated costimulation is most readily apparent (day 0, Figs. 2 *A–C*). In contrast, lines of previously activated $CD4^+$ T cells down-regulate FasL expression during this time, perhaps explaining their relative insensitivity to FasL costimulation. Moreover, $CD4^+$ T cells maintain high levels of FasL expression late into culture, whereas $CD8^+$ T cells do not, providing one explanation for the more prevalent role Fas-mediated death plays in the homeostasis of $CD4^+$ compared with $CD8^+$ T cells.

Discussion

The recent identification of FasL as a costimulatory receptor for CTLs has added a positive twist to the well-studied Fas–FasL interaction, known primarily for its negative regulatory role mediated through the Fas death receptor (1). As the absence of functional FasL influences the proliferative capacity of $CD8^+$ and $CD4^+$ T cells in distinct ways (11), we hypothesized that FasL may have the capacity to receive alternate signals in the two T cell compartments. Deciphering the distinct effects of FasL costimulation in $CD8^+$ and $CD4^+$ T cells was the goal of the current study.

Contrary to our earlier prediction, current data demonstrate the capacity of $CD4^+$ T cells to receive and translate costimulatory signals through FasL, a demonstration that required the removal of Fas-mediated death using the *lpr* mutation (Fig. 1*B*). This alteration was unnecessary in the cross-linking assay, perhaps because the two-dimensional configuration of the plate-bound cells impeded self and cell–cell contact-based Fas-mediated death, allowing the detection of FasL costimulation even in wild-type $CD4^+$ T cells (Fig. 1*D*). Conveniently, the reduced sensitivity of $CD8^+$ T cells to the Fas-mediated death pathway (Fig. 3 *B* and *D*; ref. 9) permitted the detection of FasL-mediated costimulation in wild-type $CD8^+$ T cells using both blocking and cross-linking assays (Fig. 1 *A* and *C*).

Although our data indicate that $CD4^+$ T cells are sensitive to FasL reverse signaling, the signal received by $CD4^+$ T cells may be weaker than that received by $CD8^+$ T cells, because the enhancement in proliferative capacity due to FasL cross-linking was consistently less dramatic for $CD4^+$ T cells (Fig. 1 *C* and *D*). This reduced sensitivity to FasL costimulation may be related to the recently described FasL-mediated cell cycle arrest and cell death in $CD4^+$ T cells (15). The ability of FasL engagement to enhance Ag-specific $CD4^+$ T cell proliferation is likely to depend on adequate cross-linking, achieved in our case by binding FasIgG to anti-Ig coated plates. These earlier experiments used FasIgG bound directly to plates, a configuration that does not trigger costimulation in our hands regardless of the presence of exogenous IL-2 (data not shown). Similarly, antibody-mediated bivalent aggregation of lymphotoxin-β receptor is sufficient to trigger binding of at least one downstream mediator, but is insufficient to induce the rest of the signaling pathway without further receptor aggregation (24).

As is the case for FasL costimulation, previous results have indicated that intercellular adhesion molecule-1- and 4-1BB-mediated costimulation is more apparent in $CD8^+$ than in $CD4^+$ T cells (25, 26). One consequence of weaker FasL costimulation in $CD4^+$ T cells may be increased susceptibility to Fas-mediated cell death, because costimulation through other receptors such as CD28 and 4-1BB have been shown to protect superantigen-activated T cells against Fas-mediated apoptosis (26, 27). Conversely, the reduced sensitivity of $CD8^+$ T cells to Fas-mediated death may be partly a consequence of a strong FasL costimulatory signal. The reduction in the sensitivity of $CD4^+$ T cells to FasL costimulation (Fig. 1 *B* and *D*) and of $CD8^+$ T cells to Fas-mediated cell death (Fig. 3 *B* and *D*; ref. 9) may at least partially explain the differential downstream effects of Fas–FasL interaction within the two T cell subpopulations.

In addition to these inherent sensitivity differences, we hypothesize that the temporal regulation of FasL expression and the timing of the alternate roles played by FasL in $CD4^+$ and $CD8^+$ T cells together hold the key to the evolution of these distinct and opposing roles assumed by a single molecule. In support of this notion, the two opposing functions we charac-

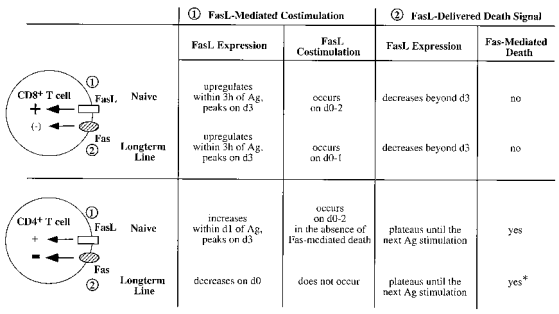

Fig. 5. Model to correlate FasL expression with the dual roles this molecule plays in costimulation and death. The data presented here are most compatible with a model in which costimulation occurs early after Ag stimulation (correlating with increased FasL expression on Ag stimulation in sensitive cells), and Fas-mediated death occurs late (correlating with the maintenance of FasL expression in $CD4^+$ T cells). The numbers ''1'' and ''2'' represent the sequence of the two FasL functions as they occur. The sizes of the arrows correlate with the strength of the overall effect from each signal. * denotes the possibility that long-term $CD4^+$ T cell lines may be less sensitive to Fas-mediated cell death (44–46), possibly because of the up-regulated levels of Fas death signaling inhibitors (44, 45).

terize here are relegated to distinct periods of the immune response. FasL costimulation occurs within 24 hr of TCR cross-linking, according to data from timed blocking experiments (Fig. 2). Staining for apoptotic cells indicates that Fas-mediated death occurs progressively from 24 hr after TCR stimulation onward (Fig. 3), serving to orchestrate the down-regulation of $CD4^+$ T cell responses. Therefore, these two phenomena seem to be regulated temporally, with FasL costimulation occurring before Fas-mediated cell death. The necessity of preventing Fas-mediated cell death to detect FasL costimulation in $CD4^+$ T cells (Fig. 1*B*) is likely due to the fact that proliferation is measured on day 4 of culture, a time when death via the FasL-initiated pathway also can be observed.

More direct data in support of the temporal regulation of opposing FasL functions in $CD4^+$ and $CD8^+$ T cells comes from a comparison of the kinetics of FasL surface expression between the two T cell subpopulations, both for naive and Ag-experienced cells. These data reveal a correlation between FasL expression and function (Fig. 4). Both naive and long-term lines of $CD8^+$ T cells up-regulate surface FasL expression rapidly on TCR stimulation (Fig. 4*A*), thereby increasing the number of FasL molecules available for sending costimulatory signals at this early stage. Down-regulation of FasL levels on $CD8^+$ T cells starts 3 days after interaction with Ag, perhaps tempering a major role for FasL in the homeostatic regulation of $CD8^+$ T cell responses. On the other hand, $CD4^+$ T cells up-regulate FasL expression and maintain this level until their next stimulation cycle (Fig. 4*B*), thereby maintaining the availability of FasL molecules for delivering a death signal through Fas. Interestingly, the pattern of FasL up-regulation differs between naive and Ag-experienced $CD4^+$ T cells. Naive $CD4^+$ T cells up-regulate FasL immediately on TCR stimulation, whereas $CD4^+$ T cell lines down-regulate FasL on stimulation and do not begin to re-express this molecule until day 3. Because FasL costimulation occurs within the first 24 hr after Ag stimulation, the altered tempo of FasL expression may explain why naive $CD4^+$ T cells are sensitive to FasL-mediated costimulation, whereas long-term $CD4^+$ T cell lines are not.

Fig. 5 summarizes the model best supported by our data by correlating the temporal regulation of FasL expression and its positive and negative regulatory roles within $CD4^+$ and $CD8^+$ T cell compartments. The model that emerges from our work suggests that the net effect of the interaction of Fas and FasL differs between the $CD4^+$ and $CD8^+$ compartments and that this encounter is carefully regulated kinetically by controlling the expression of FasL and the timing of the opposing FasL functions.

This kinetic pattern of FasL protein expression at the cell surface differs from previous reports of expression at the transcriptional level, in which mRNA levels decline within a day of stimulation (5, 6). The difference in timing may be a reflection of multiple factors, including delayed protein synthesis, differences in the control of FasL expression in the distinct cell types analyzed, or cell surface expression of preformed FasL, characterized by the recruitment to the surface after TCR stimulation of molecules stored in intracellular vesicles (28–31). It also should be noted that in contrast to the human, the membrane-bound molecule is the primary functional form of murine FasL (32). In addition, we find that inclusion of a molecule that inhibits the family of FasL-cleaving metalloproteinases (33) does not appreciably shift FasL surface staining levels on murine cells (data not shown). Thus, assays of surface FasL expression serve as relevant guides for its potential role as a receptor.

Separate from the temporal compartmentalization of the two opposing FasL functions, another level of regulation controls FasL costimulation early after Ag encounter. Recently, we have found that TCR coengagement is required for FasL costimulation (unpublished data). Thus, proliferation induced by anti-Thy-1 antibodies is not susceptible to FasL costimulation, despite the fact that FasL expression is up-regulated on the responding T cells with the predicted kinetics. These data are compatible with the notion that TCR coengagement is required to initiate the recruitment of signaling molecules to the cytoplasmic tail of FasL. The structure of the murine FasL cytoplasmic tail and the identity of several highly conserved domains provide some clues as to its possible role in reverse signaling. Of particular note is a domain in which 25 of the 78 residues are prolines (34, 35), a region that contains at least three potential src-homology 3 binding sites (36). There is some evidence that at least one of these sites mediates the specific interaction between the cytoplasmic tail of FasL and the src-homology 3-containing protein kinase $p59^{fyn}$ (37). At the time this interaction was documented, the $p59^{fyn}$-FasL association was interpreted as a means either to modulate surface expression of newly synthesized FasL or to achieve a high local concentration of FasL in the proximity of the TCR complex, perhaps enabling delivery of a localized lethal hit (37). However, these data are also compatible with the notion that TCR engagement facilitates the colocalization of $p59^{fyn}$ and FasL. In addition, recent data indicate that the cytoplasmic tail of FasL contains two motifs for the serine/threonine kinase casein kinase I, sites whose phosphorylation status may change on receptor binding (38). It remains speculative which, if any, of these domains in the FasL cytoplasmic tail are crucial for delivering a costimulatory signal.

Although we have demonstrated FasL costimulation in both peripheral $CD8^+$ and naive $CD4^+$ T cells, the *in vivo* function of this signal is still being explored. For $CD4^+$ T cells, our data predict that FasL will function mainly as a negative regulator of wild-type $CD4^+$ T cells. However, recent reports of differences in FasL expression (23) and in the susceptibility to Fas-mediated cell death of Th1 and Th2 cells (39, 40), as well as the predisposition of *gld* mice for Th2 cytokine secretion on *Trypanosoma cruzi* infection (41), suggest an intriguing role for FasL in the regulation of Th1 and Th2 responses. For this reason, it may be interesting to explore the possibility of differential effects of FasL costimulation among the different mice strains. For $CD8^+$ T cells, we know that CTLs require FasL costimulation for achieving maximal levels of proliferation, but do not require it for optimal cytolysis (11). Because FasL costimulation requires concomitant TCR engagement, it will be interesting to explore whether FasL positive reverse signaling plays any role in regulating the decision made by activated Ag-specific $CD8^+$ T cells to become effector cells versus memory cells.

In summary, FasL performs two opposing roles that generally can be divided by cell type. FasL costimulation is required for maximal proliferation of $CD8^+$ T cells but initiates Fas-mediated death in activated $CD4^+$ T cells. Overlying the inherent sensitivities of $CD8^+$ and $CD4^+$ T cells to these two different functions is the temporal regulation of these distinct roles played by FasL, events that are correlated with the kinetics of the Ag-initiated up-regulation of surface FasL expression on $CD8^+$ and $CD4^+$ T cells. In addition, the factors controlling susceptibility to Fas-mediated death may influence this division of function by FasL. Each of these levels of control influences how $CD8^+$ and $CD4^+$ T cells interpret the interaction of FasL with its partner molecule Fas. Through these interconnecting networks, FasL can differentially regulate $CD8^+$ and $CD4^+$ mature peripheral T cells.

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