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Fas Ligand-Mediated Cytotoxicity Is Directly Responsible for Apoptosis of Normal CD4+ T Cells Responding to a Bacterial Superantigen


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Exposure of naive CD4+ T lymphocytes to superantigens such as staphylococcal enterotoxin B (SEB) induces a strong proliferative response. Prolonged exposure or subsequent restimulation of the responding T cell population with SEB leads to the apoptotic events of activation-induced cell death (AICD). However, T cells derived from either Fas-deficient lpr or Fas ligand-deficient gld autoimmune mouse strains, fail to undergo AICD under these conditions. Instead, these autoimmune T cells mount a vigorous proliferative response, suggesting a critical role for Fas/FasL interactions in this form of autoapoptosis. In the current study, we found that SEB-induced AICD was tied to the rapid induction of FasL expression in cells constitutively expressing high levels of Fas. Furthermore, the addition of soluble Fas-lgG fusion protein to the SEB-restimulated cultures blocked AICD and resulted in a 2° proliferative response that was comparable in magnitude and kinetics to that of the lpr and gld T cells. The rapid onset of apoptosis in normal T cells subsequent to restimulation with SEB was in direct contrast to the proliferative response of the initial cultures, even though comparable levels of Fas and Fasl RNA were found in T cells after 1° and 2° challenge. The clonal expansion of the normal T cells responding to the initial SEB stimulation was, however, dramatically compromised when the normal cells were cocultured with an MRL-lpr responder population; addition of soluble Fas-lgG rescued the normal component of the response. Together, these data demonstrate first, that Fas/FasL interactions are intimately tied to superantigen-induced AICD, a form of autocrine cell death, and second, that Fasl-mediated cytotoxicity is responsible for the disappearance of normal CD4+ T cells in lpr cocultures. The Journal of Immunology, 1995, 154: 4302–4308.

Recent studies have demonstrated that cells expressing the cell surface molecule Fas/APO-1 can be triggered to undergo apoptosis with mAbs specific for Fas (1, 2), or by Fas ligand (Fasl) expressing effector populations (3). A significant component of CD8+ CTL cytotoxic activity and all CD4+ Th1 CTL cytotoxic activity can be attributed to Fasl function (4–6). Studies on mouse strains expressing the lpr or gld mutation (defective in functional Fas or Fasl expression, respectively) have indicated that Fas/Fasl interactions are also involved in the induction of peripheral T cell tolerance. For example, when wild-type and mutant mice transgenic for the 2B4 TCR are injected with cytochrome c peptide, there is a rapid loss of peripheral 2B4+ T cells in +/- mice, but little disappearance of 2B4+ cells in lpr/lpr mice (7). Moreover, when normal mice are injected with staphylococcal enterotoxin B (SEB), SEB-reactive cells rapidly become anergic and disappear from the peripheral circulation for an extended period of time; when SEB is injected into lpr mice, anergy induction is delayed and the kinetics of SEB nonreactivity are significantly shorter overall (8, 9). As an in vitro correlate to these in vivo events, T cells
Lymph node cell (LNC) suspensions were depleted of CD8+ T cells by treatment with the mAb 3.1.55 (kindly provided by Dr. H. Wortis, Tufts University School of Medicine, Boston, MA) and rabbit C'. Complete removal of the CD8+ population was confirmed by flow cytometry. All starting populations also lacked detectable numbers of CD4+ CD8- T cells. CD8-depleted LNC were cultured in RPMI 1640 supplemented with 10% FCS at a final density of 2 X 10^6 cells/ml in either 96-well or 24-well plates with 5 μg/ml of SEB (Sigma Chemical Co., St. Louis, MO). On day 5, cells were collected from the wells, washed, and rested in medium containing 5 U/ml of murine rIL-2. When these cells had reverted to a resting phenotype (days 8 to 10) as determined by cell morphology and confirmed by forward- vs side-scatter parameters on a flow cytometer, >90% of the cells were CD4+. Viable cells were isolated by Ficoll density gradient centrifugation and restimulated in 96-well plates at a density of 5 X 10^5 cells/ml with 10 to 50 U/ml rIL-2, 5 to 10 μg/ml SEB, or plate-bound (20 μg/ml for 2 h) anti-CD3 mAb 2C11, (kindly provided by Dr. K. Bottomly, Yale University, New Haven, CT). For the coculture experiments, equal numbers of MRL and B10.A CD8-depleted LNC were seeded at a final density of 2 X 10^5 total cells/ml and stimulated with 5 μg/ml SEB. Soluble Fas-IgG fusion protein (see below), TNF-R60-IgG, or control human IgG (IgG3) were added to some of these cultures at concentrations ranging from 1.5 to 15 μg/ml. Proliferation was assayed by a 6-h pulse with [3H]thymidine incorporation at the times indicated in the text.

Fusion proteins

Soluble Fas-IgG was obtained as described previously (18) by stable transfection of 3T3 cells with a construct consisting of the extracellular domain of Fas attached to the constant region of human IgG. 5 X 10^6 transfected cells were incubated s.c. into SCID mice and serum containing the Fas-IgG protein was collected from these mice from days 7 to 14. Fas-IgG was then purified on Protein A columns by standard procedures. Purified TNF-R60-IgG (19) was kindly provided by Dr. J. Browning (Biogen, Boston, MA).

Flow cytometric analyses

Cell surface expression of CD4 and CD8 on the starting and experimental populations was assessed with the mAbs GK1.5 and 53.6.72 (17). Cells recovered from the coculture experiments were stained with reagents specific for Dn (15.5.S4) or Dn (35.2.12) as described previously (20). Surface expression of Fas was detected with the monoclonal anti-Fas Ab, Jo2, coupled to phycoerythrin (PE) (PharMingen, San Diego, CA). All populations were analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). The extent of apoptosis in the SEB and anti-CD3 restimulated cultures was assessed by propidium iodide (PI) staining according to the method of Nicoletti (21). Briefly, cells were gently resuspended in 0.5 ml of a hypotonic fluorescence solution consisting of 50 μg/ml PI in 0.1% sodium citrate and 0.1% Triton X-100. After remaining in the PI solution overnight at 4°C, the samples were analyzed by flow cytometry.

Expression of Fas and FasL

Total RNA was isolated from 1 X 10^6 treated and untreated cells and reverse transcribed to cDNA. Aliquots from each cDNA preparation were amplified by PCR specific for Fas (forward: 5'(ATCGAGACTCT GAGAGGGGCGTGCATGAAAC3'); reverse: 5'(GGAGGTTCTA GATTCAAGTCA TCTCGATCCG3') (22) or FasL (forward: 5'(CAGACT TCCACCTGCAGAAGG3'); reverse: 5'(GAATTCCTCAAAAATGGAT CAGAAGAGG3') (13) in the presence of 1 μg of Taq polymerase. The reaction mixtures were placed in a DNA thermal cycler and the reaction was started by the addition of 1 unit of thermus aquaticus DNA polymerase (Promega, Madison, WI). The conditions for the PCR, 1 cycle of 94°C for 2 min followed by 25 cycles of 94°C for 1.5 min, 60°C for 30 s, and 72°C for 2 min, were optimized to yield products that were quantitatively representative of the initial RNA levels. The products were resolved on a 4% nondenaturing polyacrylamide gel followed by autoradiography and densitometry using a Molecular Dynamics (Sunnyvale, CA) personal densitometer.

Materials and Methods

Mice

MRL/MpJ-lpr (MRL-lpr, H-2KkDd), MRL/MpJ-+/+ (MRL-+/+), and B10.A (H-2KkDd) mice were obtained from The Jackson Laboratories (Bar Harbor, ME). MRL/MpJ-gld (MRL-gld) mice were kindly provided by Dr. C. Sidman (University of Cincinnati, Cincinnati, OH). All mice were maintained by the Division of Laboratory Animal Maintenance at Boston University School of Medicine. Mice used in this study were 4 to 6 wk of age.

from both lpr and gld mice fail to undergo AICD following exposure to superantigens or anti-CD3 mAb under conditions that result in the death of T cells derived from the wild-type congenic strains (10-12). The in vitro AICD process is most dramatic in T cells that have previously been exposed to Ag. MRL-+/+, MRL-lpr, and MRL-gld T cells can all proliferate comparably in control SEB cultures; it is only when these T cells are allowed to revert to a resting phenotype and are then restimulated with SEB, that the dramatic difference between the +/+ and the two autoimmune genotypes becomes apparent.

Cells derived from lpr mice have been found to exert unanticipated negative effects on normal lymphocytes. Reconstitution of lethally irradiated normal mice with T-depleted stem cells from histocompatible lpr mice results in a wasting syndrome that is triggered independently of detectable alloantigenic differences (13, 14). Tetraparental mice produced by embryo fusion of lpr and non-lpr parental blastocyst populations develop immune systems in which most of the T and B lymphocytes and all the circulating Ab are derived from the lpr parent, even in tetraparental mice where up to 95% of the bone marrow population is derived from the normal parent (15, 16). We have recently demonstrated that the in vivo suppression of normal lymphocyte development, or the “lpr anti-normal effect,” apparent in these chimeric mice can be recapitulated in vitro. In primary cocultures of (B10.A + MRL-lpr) T cells stimulated either with alloantigen or mitogen, the normally vigorous response of the B10.A T cells was significantly depressed. This outcome was not simply a result of the failure of the lpr T cells to undergo AICD, because the B10.A response was not suppressed in primary (B10.A + MRL-gld) cocultures, even though lpr and gld T cells are comparably resistant to AICD (17).

The current study was undertaken to further define the role of Fas/FasL interactions in both AICD and the lpr anti-normal effect, by analyzing 1° and 2° in vitro SEB responses. We found that both responses were associated with increased FasL expression and could be blocked with a soluble Fas-IgG fusion protein. In the case of AICD, the activated population expresses both Fas and FasL and undergoes auto-apoptosis. In the lpr anti-normal situation, FasL produced by lpr T cells induces premature apoptosis of the normal responder population.
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Table I. Fas-lgG blocks lpr-mediated suppression of normal 1° T cell responses to SEB

<table>
<thead>
<tr>
<th>LNC</th>
<th>Proliferation a (cpm X 10^-3)</th>
<th>hlgG b</th>
<th>% B10.A</th>
<th>% MRL</th>
<th>Fas-lgG c</th>
<th>% B10.A</th>
<th>% MRL</th>
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<tr>
<td>B10.A</td>
<td>301 ± 23</td>
<td>99</td>
<td>2</td>
<td></td>
<td></td>
<td>45 ± 1</td>
<td>55 ± 1</td>
</tr>
<tr>
<td>MRL-+/+</td>
<td>371 ± 11</td>
<td>1</td>
<td>99</td>
<td></td>
<td></td>
<td>43 ± 2</td>
<td>57 ± 2</td>
</tr>
<tr>
<td>MRL-lpr</td>
<td>244 ± 25</td>
<td>2</td>
<td>98</td>
<td></td>
<td></td>
<td>58 ± 6</td>
<td>42 ± 6</td>
</tr>
<tr>
<td>MRL-gld</td>
<td>331 ± 16</td>
<td>1</td>
<td>99</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B10.A + MRL-+/+</td>
<td>335 ± 7</td>
<td>41 ± 3</td>
<td>59 ± 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B10.A + MRL-lpr</td>
<td>293 ± 4</td>
<td>17 ± 7</td>
<td>83 ± 6</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>B10.A + MRL-gld</td>
<td>278 ± 13</td>
<td>52 ± 8</td>
<td>48 ± 8</td>
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</table>

CD8-depleted LNC were stimulated with 5 μg/ml SEB in the presence of 10 μg/ml control human (hlgG) or Fas-lgG. After 4 days of culture, proliferation was determined by [3H]thymidine incorporation and the parental origin of the activated cells was determined by flow cytometry using mAb specific for either H-2D^d (B10.A; 35.2.12) or H-2D^k (MRL; 15.5.5).

Proliferation is reported as the mean ± SE of triplicate wells ± SE from 1 of 4 comparable experiments for cells cultured in the presence of hlgG.

Data represents the mean ± SE of four separate experiments.

Results and Discussion

Inhibition of AICD by the addition of soluble Fas-lgG fusion protein to 2° SEB or anti-CD3 stimulated cultures

To definitively prove that SEB-mediated AICD requires Fas/FasL interactions, we tested the ability of soluble Fas-lgG to reverse the AICD process in normal T cell populations. CD8-depleted LNC from young B10.A, MRL-+/+, MRL-lpr, and MRL-gld mice were initially stimulated with SEB as described in Materials and Methods. Eight to ten days later, cells recovered from the initial cultures were restimulated with either 50 U/ml IL-2 (A), 5 μg/ml SEB (B), or plate-bound anti-CD3 (B). 5 μg/ml hlgG (open bars) or Fas-lgG (cross-hatched bars) were added to the SEB and anti-CD3 stimulated cultures (B). Proliferation was determined 16 to 20 h later by [3H]thymidine incorporation. The data represents the mean ± SE of three separate experiments in total cpm (A). In (B) the relative levels of proliferation are presented as % IL-2 response, calculated according to the formula: (total cpm in response to SEB (or anti-CD3)/total cpm in response to IL-2) × 100. The data represents the mean ± SE from three individual experiments.

FIGURE 1. Proliferative response of previously stimulated normal and autoimmune CD4+ T cells to IL-2, SEB, or anti-CD3, in the presence or absence of soluble Fas-lgG. CD8-depleted LNC from young B10.A, MRL-+/+, MRL-lpr, and MRL-gld mice were initially stimulated with SEB as described in Materials and Methods. Eight to ten days later, cells recovered from the initial cultures were restimulated with either 50 U/ml IL-2 (A), 5 μg/ml SEB (B), or plate-bound anti-CD3 (B). 5 μg/ml hlgG (open bars) or Fas-lgG (cross-hatched bars) were added to the SEB and anti-CD3 stimulated cultures (B). Proliferation was determined 16 to 20 h later by [3H]thymidine incorporation. The data represents the mean ± SE of three separate experiments in total cpm (A). In (B) the relative levels of proliferation are presented as % IL-2 response, calculated according to the formula: (total cpm in response to SEB (or anti-CD3)/total cpm in response to IL-2) × 100. The data represents the mean ± SE from three individual experiments.

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The addition of soluble Fas-lgG to the 2° cultures at the time of restimulation dramatically improved the proliferative response of the normal T cells, whereas control hlgG had no effect (Fig. 1B). Increasing the concentration of the Fas-lgG to 15 μg/ml brought the SEB and
The journal various time points after 1° and 2° restimulation with SEB.

AICD involve changes in the level of Fas and/or FasL. Both Fas and FasL were up-regulated by day 3 of the 1° response (Fig. 4A and data not shown). When the cells subsequently reverted to a resting phenotype, FasL levels returned to baseline, but Fas expression remained high. Restimulation with SEB resulted in a rapid and dramatic increase in FasL mRNA expression but only a minimal increase in the level of Fas mRNA (Fig. 4B). The resting and reactivated populations were also found to express comparable levels of Fas protein on their cell surface, as detected by flow cytometry subsequent to staining with an anti-Fas mAb (Fig. 5). These results indicate that AICD is intimately associated with the rapid up-regulation of FasL, and more moderate increases in Fas expression. We have also detected dramatic increases of FasL expression in T cell hybridomas and T cell lines undergoing AICD following stimulation with anti-CD3 (18). Together, these data indicate that FasL expression is the pivotal regulatory component in the general phenomenon of AICD. Because AICD can be induced in T cell hybridomas stimulated with anti-CD3 under limiting dilution conditions, it is likely that co-expression of sufficient levels of Fas and FasL by individual cells can lead to self-induced or auto-apoptosis (18).

**FIGURE 2.** Soluble Fas-IgG can restore the ability of normal CD4⁺ T cells to proliferate in response to 2° challenge with SEB. CD8-depleted LNC from young MRL-+/+, MRL—lpr, or MRL—+/+ mice were restimulated with SEB as described in Figure 1, in the presence or absence of increasing concentrations of control hlgG (open symbols) or Fas-IgG (closed symbols, solid line). Fas-IgG was also added to the MRL—+/+ cells in the absence of SEB (dotted line). Proliferative responses were determined 20 h later by [³H]thymidine incorporation. The data represent one of three similar experiments. All points were assayed in triplicate and SEM were less than 10%.

anti-CD3 response of the B10.A and MRL—+/+ cells to the same level as the mutant strains (Fig. 2, and data not shown). Fas-IgG did not enhance the SEB or anti-CD3 response of the MRL—lpr and MRL—gld cells, or by itself stimulate the B10.A or MRL—+/+ cells, demonstrating that the effect of the Fas-IgG was not simply a result of mitogenic or costimulatory activity. The failure of the B10.A and MRL—+/+ cells to incorporate [³H]thymidine following stimulation with SEB or anti-CD3 reflected the fact that these cells were actively induced to undergo apoptosis as shown by PI staining (Fig. 3). The addition of Fas-IgG, but not TNFRp60-IgG, to the SEB or anti-CD3 restimulated cultures dramatically reduced the extent of apoptosis. These results confirm and extend in vitro studies from this lab and others (10—12), demonstrating that the inability to effectively express Fas or FasL results in the breakdown of the normal AICD pathway.

**AICD is associated with rapid up-regulation of FasL expression**

To better understand whether the controlling events in AICD involve changes in the level of Fas and/or FasL expression, RNA was isolated from CD8-depleted cells at various time points after 1° and 2° restimulation with SEB. Both Fas and FasL were up-regulated by day 3 of the 1° response (Fig. 4A and data not shown). When the cells subsequently reverted to a resting phenotype, FasL levels returned to baseline, but Fas expression remained high. Restimulation with SEB resulted in a rapid and dramatic increase in FasL mRNA expression but only a minimal increase in the level of Fas mRNA (Fig. 4B). The resting and reactivated populations were also found to express comparable levels of Fas protein on their cell surface, as detected by flow cytometry subsequent to staining with an anti-Fas mAb (Fig. 5). These results indicate that AICD is intimately associated with the rapid up-regulation of FasL, and more moderate increases in Fas expression. We have also detected dramatic increases of FasL expression in T cell hybridomas and T cell lines undergoing AICD following stimulation with anti-CD3 (18). Together, these data indicate that FasL expression is the pivotal regulatory component in the general phenomenon of AICD. Because AICD can be induced in T cell hybridomas stimulated with anti-CD3 under limiting dilution conditions, it is likely that co-expression of sufficient levels of Fas and FasL by individual cells can lead to self-induced or auto-apoptosis (18).

Reconstitution of irradiated normal mice with T cell-depleted stem cells from an lpr strain results in the induction of an unusual GVHD-like wasting syndrome (13). In mice reconstituted with a mixture of lpr and normal bone marrow cells, we have found that both stem populations initially seed the peripheral immune system. However, before overt clinical signs such as weight loss and general malaise became apparent, Ab and lymphocytes derived from the normal stem cells (not the lpr stem cells) selectively disappeared from the circulation (14). The dominant presence of lpr-derived Ab and cells has been observed in vivo in several other (lpr + normal) chimeric models (14—16, 20, 23).

More recently, we have found that this in vivo lpr anti-normal effect can be recapitulated in vitro by the coculture of LNC from young (4- to 6-wk-old) MRL—lpr and normal B10.A mice stimulated with alloantigens or the mitogen Con A (17). Class I-specific alloresponses were avoided by removing CD8+ cells from the cocultured responder populations before culture. As shown in Table I, lpr CD4⁺ T cells can also suppress normal CD4⁺ T cell responses to SEB, as evidenced by the low percentage of B10.A cells recovered from 1° SEB-stimulated (lpr + B10.A) cocultures on day 4. The skewed recovery of lpr cells is not simply a result of enhanced proliferation or survival of the lpr cells for at least two reasons. First, when all the parental populations were cultured individually, they incorporated comparable levels of [³H]thymidine. Second, the total number of B10.A cells recovered from the (lpr + B10.A) mixed cultures was significantly below the expected number based on recovery from the individual B10.A culture. Moreover, even though gld T cells exhibit...
FIGURE 3. Apoptosis of normal CD4+ T cells restimulated with SEB or anti-CD3 can be prevented by soluble Fas-IgG. CD8-depleted LNC from young MRL-+/+, MRL-lpr, and MRL-gld mice were initially stimulated with SEB as in Figure 1. Ten days later, viable cells were recovered and cultured with either medium, 10 μg/ml SEB, or plate-bound anti-CD3 in the presence of 10 U/ml rIL-2. 15 μg/ml of either Fas-IgG or TNFRp60-IgG were added to the cultures at the time of restimulation. Apoptosis of the anti-CD3-stimulated cells was assessed after 16 h in culture and apoptosis of the SEB-stimulated cells was assessed after 40 h in culture by incorporation of PI as described. The staining patterns represent the data obtained from one of three similar experiments.

FIGURE 4. Fas and FasL mRNA expression in SEB-stimulated cells. (A) MRL-+/+, B10.A, MRL-lpr, and MRL-gld CD8-depleted LNC were stimulated with SEB, and RNA samples were collected at 0, 3, and 4 days after initiation of the cultures and assayed for FasL expression. (B) Cells recovered from cultures initially stimulated with SEB were recultured in medium or restimulated with SEB as described in Figure 1; cultures were supplemented with the addition of low concentrations of IL-2 (5 U/ml) to maintain the viability of all the nonstimulated control populations. RNA samples were collected at 0, 6, and 12 h after restimulation of the cultures and Fas and FasL expression was assessed as described in Materials and Methods.
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In contrast to the individually cultured normal T cells, the normal cells present in the 1° SEB (lpr + normal) cocultures seem to be sensitive to FasL-mediated cell death; apoptosis of these cells can be prevented by the addition of soluble Fas-IgG to the initial cultures. In this protocol, the death of the normal cells may result from accelerated or excessive FasL production on the part of the lpr responder population, or the normal cells may be exposed to extra FasL signals that were not saturated by lpr (Fas-deficient) FasL-producing cells. Alternatively, the sensitivity of the lpr cocultured normal cells to FasL-induced cell death may be associated with the presence or absence of additional factors produced by the lpr population that influence the Fas signaling pathway. Experiments to explore these alternatives are in progress.

In summary, this study demonstrates that rapid up-regulation of FasL is a pivotal step in the induction of AICD after SEB or anti-CD3 mAb activation. As such, it serves as a critical control element to ensure appropriate down-regulation of T-dependent immune responses. Moreover, FasL-induced cytotoxicity is directly involved in the lpr-mediated suppression of normal T cell responses. It is apparent that both self-directed and intercellular immunosuppressive signals are intimately associated with up-regulation of FasL expression. The factors that control FasL expression are complex and warrant further investigation.

Acknowledgments

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FIGURE 5. Cell surface Fas expression of SEB-stimulated CD4+ T cells. CD8-depleted LNC were obtained from young MRL-+/+, MRL-lpr, and MRL-gld mice and Fas expression was measured on the freshly isolated LNC with PE-conjugated Jo2 mAb (starting column 1). These cells were then stimulated with SEB and Fas levels were determined at the time of peak proliferation (1° SEB; column 2). The cells were then rested in growth factor as described in the text and stained again (resting; column 3). Finally, these CD4+ T cells were restimulated with SEB or anti-CD3 and Fas expression was determined 16 h later (2° SEB and 2° anti-CD3; columns 4 and 5).

References


