Inducible Nonlymphoid Expression of Fas Ligand Is Responsible for Superantigen-Induced Peripheral Deletion of T Cells

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Summary

Fas (CD95) and Fas ligand (FasL) play major roles in staphylococcal enterotoxin B (SEB)–induced peripheral deletion of $V\beta 8^+$ T cells. We found that peripheral deletion was defective in radiation chimeras with nonfunctional tissue FasL, regardless of the FasL status of the bone marrow–derived cells. SEB induced a dramatic upregulation of FasL expression and function in nonlymphoid cells of liver and small intestine. This effect was resistant to inhibition by cyclosporin A, which also failed to inhibit peripheral deletion. In SCID animals nonlymphoid tissues did not express FasL in response to SEB unless transplanted lymphocytes were present. Thus, some immune responses induce FasL in nonlymphoid tissues, which in turn kills activated lymphocytes, leading to peripheral T cell deletion.

Introduction

The apoptotic death of effector T cells following stimulation by a strong antigenic challenge (peripheral deletion) is one of the major regulatory mechanisms maintaining immune homeostasis. T cells respond to antigen by expanding in numbers (as predicted by clonal selection), and after a period of days these numbers decline, often to below the original levels via an apoptotic process (Kawabe and Ochi, 1991). One mechanism by which this apoptotic deletion occurs is via expression of Fas (CD95) and Fas ligand (FasL, CD95L), as peripheral deletion is partially defective in animals lacking Fas (Singer and Abbas, 1994; Mogil et al., 1995). In vitro, T cell activation results in coexpression of both Fas and its ligand, resulting in "suicide" of the cells (Alderson et al., 1995;

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Brunner et al., 1995; Dhein et al., 1995; Ju et al., 1995), which is believed to be one mechanism underlying peripheral deletion. Mice or humans lacking functional Fas or FasL (Watanabe-Fukunaga et al., 1992; Fisher et al., 1995; Rieux-Laucat et al., 1995) display profound lymphoaccumulative disorders with accelerated autoimmune dysfunction, possibly as a consequence of the defect in peripheral deletion.

Although FasL is functional on activated T cells, it is also expressed in several nonlymphoid cells and tissues, including epithelial cells, macrophages, dendritic cells, and other cell types (Bellgrau et al., 1995; Griffith et al., 1995; French et al., 1996, 1997; Suss and Shortman, 1996; Lu et al., 1997). FasL present in nonlymphoid tissue has been shown to delete reactive lymphoid cells during viral infections and is responsible for protecting immune privileged sites from cellular immune-mediated damage (Griffith et al., 1995, 1996). FasL expression on corneal grafts and tumor cells also protects these tissues from the immune response (Hahne et al., 1996; Stuart et al., 1997). These studies prompted us to examine the role of nonlymphoid FasL in the phenomenon of peripheral deletion with the idea that FasL expressed outside the lymphoid compartment might have a more general role in controlling immune reactions.

Results and Discussion

We employed a well-characterized system wherein $V\beta 8^+$ T cells are deleted in response to a superantigen, SEB (Kawabe and Ochi, 1991). SEB induces expansion and peripheral deletion by binding to MHC class II molecules of antigen-presenting cells where it is presented to most $V\beta 8^+$ T cells without a processing requirement (White et al., 1989). To further examine the role of FasL in peripheral deletion, we used mice defective in expression or function of Fas (lpr) and FasL (gld) (Takahashi et al., 1994). In the experiment shown in Figure 1, SEB induced the expansion of $V\beta 8^+$ T cells in wild-type (wt) animals, followed by deletion. This $V\beta 8^+$ T cell deletion was not observed in the FasL-defective gld mice where $V\beta 8^+$ T cells continued to expand throughout the course of the experiment. These observations confirm that Fas/FasL interactions participate in peripheral deletion of $V\beta 8^+$ T cells induced by SEB.

We then examined this phenomenon in bone marrow chimeric animals, in which lethally irradiated wt or gld mice were reconstituted with bone marrow from wt or gld donors. Six to eight weeks after reconstitution, mice were challenged with SEB, and the pattern of T cell expansion or deletion in the peripheral blood was analyzed. In the experiment shown in Figure 1, irradiated wt mice reconstituted with gld bone marrow (gld→wt) displayed an expansion of $V\beta 8^+$ T cells followed by the peripheral deletion characteristic of wt mice. Peripheral deletion was not prevented by the defect in FasL on bone marrow–derived cells. In striking contrast, wt→gld mice showed an absence of peripheral deletion, displaying a pattern similar to that of gld mice (Figure 1A).

In some groups the percentage of Vβ6⁺ T cells was also determined during the course of the experiment (Figure 1B). The results showed that the expansion and deletion in response to SEB was specific to the Vβ8⁺ subset. Similar results were obtained in three additional experiments. Thus, while functional FasL appears to participate in peripheral deletion, its functional expression in peripheral nonlymphoid tissues rather than the T cell compartment appears to be the predominant factor in this process. To confirm these results, chimeras were constructed in which gld bone marrow was transplanted into irradiated lpr mice (gld→lpr). In these animals normal FasL is expressed in the parenchymal tissues, but without Fas. The bone marrow–derived cells have defective FasL but normal Fas. Thus, while neither animal is capable of normal peripheral deletion (Figure 1A; Singer and Abbas, 1994; Mogil et al., 1995), the SEB-induced deletion of Vβ8⁺ T cells in the chimera was indistinguishable from that of wt mice.

Although some tissues are known to constitutively express FasL (Bellgrau et al., 1995; Griffith et al., 1995; French et al., 1996, 1997; Lu et al., 1997), it was possible that some might only express it following superantigen administration. Therefore, to determine which nonlymphoid tissues might be involved in T cell deletion, we screened several tissues for FasL mRNA expression using RT–PCR at 3, 6, and 9 days after SEB administration. We found that within 3 days of SEB injection, FasL was inducibly expressed in spleen (as expected), as well as lung, liver, and small intestine (Figure 2). Large intestine and brain were negative.

Since this approach did not discriminate between lymphoid and nonlymphoid FasL, we then employed in situ hybridization to examine the pattern of expression following SEB injection. As shown in Figure 3, small intestine and liver expressed a significant amount of FasL mRNA 3 days after exposure to the superantigen. This was especially dramatic in the small intestine, where its location on the epithelia and lamina propria was evident (Figure 3E). Lung was not examined by in situ hybridization for technical reasons.

We further characterized the upregulation of FasL in

these tissues by performing the experiments shown in Figures 4A and 4B. Liver cells were dissociated and stained with antibodies to FasL. As expected, some of the FasL⁺ cells seen following administration of SEB were CD3⁺ T cells. Notably, however, at least half of the FasL⁺ cells were negative for CD3 (CD3⁻) (Figure 4A, left panel). Using MAC-1 (CD11b) as a marker for macrophages (e.g., Kupffer cells) and fibroblasts (Springer et
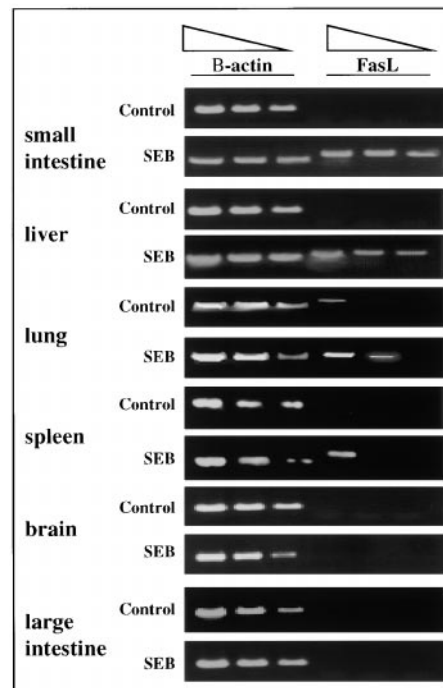

Figure 2. RT–PCR Analysis of FasL mRNA Expression in Organs of SEB-Treated and Untreated Mice

Liver, small intestine, large intestine, lung, brain, and spleen were assayed for FasL. Assessment of β-actin mRNA was used as a control and to titrate the amount of cDNA loaded. Three different dilutions were used (1:10, 1:100, 1:1000). Results are representative of three independent experiments.
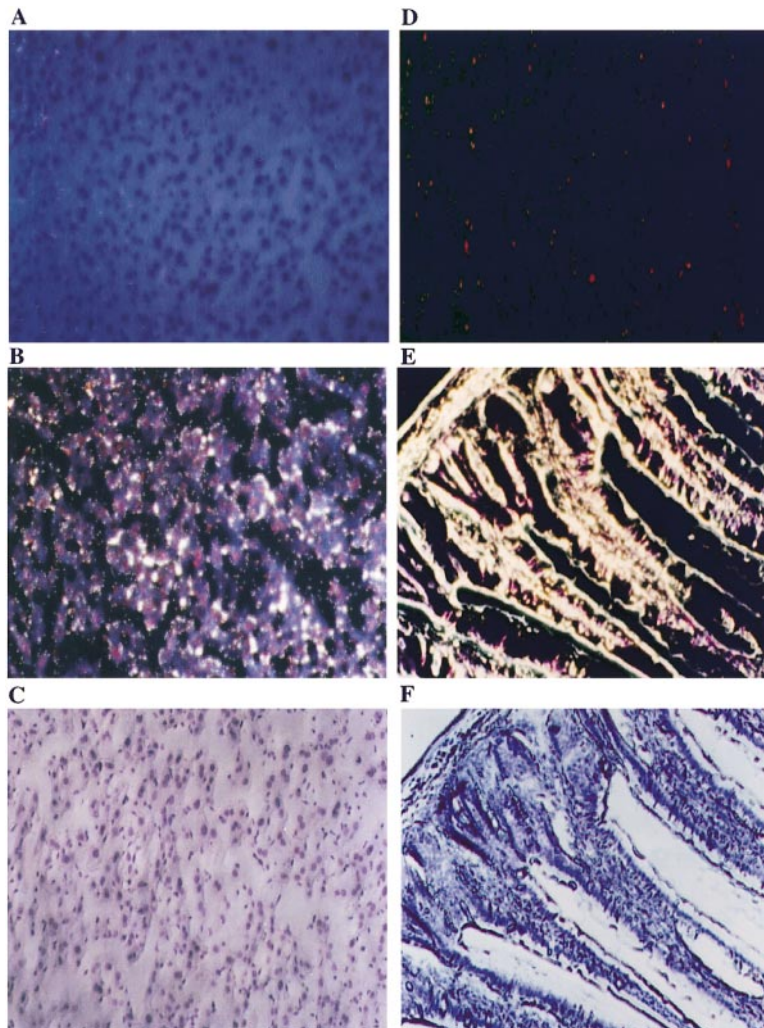
Figure 3. Detection of FasL mRNA in Non-lymphoid Tissues after SEB Challenge

In situ hybridization of FasL mRNA expression in the liver (A and B) and small intestine (D and E). Animals were untreated (A and D) or used 3 days after SEB challenge (B and E). Hematoxylin (H) staining of the corresponding antisense probe for FasL mRNA detection from the SEB-treated animals is also shown (C and F). A FasL sense probe for four specimens showed negligible endogenous hybridization (data not shown).

al., 1979; Beller et al., 1982), we found that most Mac-1$^+$ cells in the liver became FasL$^+$ following SEB administration (Figure 4A, center). Interestingly, in the experiment shown in the right panel of Figure 4A, we found that the induced expression of FasL was not inhibited by treatment of the animals with cyclosporin A (CsA) (see below). These observations of inducible FasL in the liver are consistent with an earlier study showing that the liver is an important site for peripheral deletion of exogenous antigen-responsive T lymphocytes (Huang et al., 1994). Our findings suggest that this may be a manifestation of inducible FasL expression and its interaction with activated Fas$^+$ T cells in the liver.

To evaluate the function of nonlymphoid FasL, we isolated intestinal epithelial cells (IEC) from SEB-treated and untreated animals and tested their ability to kill Fas$^+$ target cells (Brunner et al., 1996). These epithelial preparations contained less than 1% T cells, as detected using antibodies to CD3, TCRβ, and TCRδ (data not shown). As shown in Figure 4B, intestinal epithelial cells from SEB-treated but not untreated animals effectively killed targets bearing Fas, but not the Fas$^-$ parental line (Figure 4B). Thus, the FasL induced on intestinal epithelium by in vivo administration of SEB is functional. Interestingly, this effect of SEB administration was not inhibited

by treatment of the mice with CsA (Figure 4B), similar to the lack of effect on inducible FasL expression in Mac-1$^+$ liver cells (Figure 4A).

In contrast, FasL expression on SEB-activated spleen cells was sensitive to inhibition by CsA in vivo. Spleen cells from SEB-treated mice were potent killers of Fas$^+$ target cells either ex vivo (data not shown) or following restimulation with anti-CD3 (Figure 4C). At an effector to target cell ratio of 6.5 to 1, in vivo activated and restimulated spleen cells induced greater than 50% DNA fragmentation in Fas$^+$ target cells. However, FasL function on restimulated SEB-induced splenic T cells was suppressed by in vivo administration of CsA (Figure 4C). Thus, CsA inhibits the priming of splenocytes for FasL expression by SEB, but not SEB-induced expression of functional FasL on other, nonlymphoid cell types.

The failure of CsA to suppress functional FasL on intestinal epithelium is consistent with a role of non-lymphoid FasL in peripheral deletion, as CsA does not inhibit either Fas-mediated death or peripheral deletion (Gonzalo et al., 1992; Vanier and Proud'Homme, 1992; Kuschnaroff et al., 1997). We confirmed these results in the experiment shown in Figure 4D. Administration of CsA failed to interfere with peripheral deletion of Vβ8$^+$ T cells in response to SEB. Recently Latinis et al. (1997)
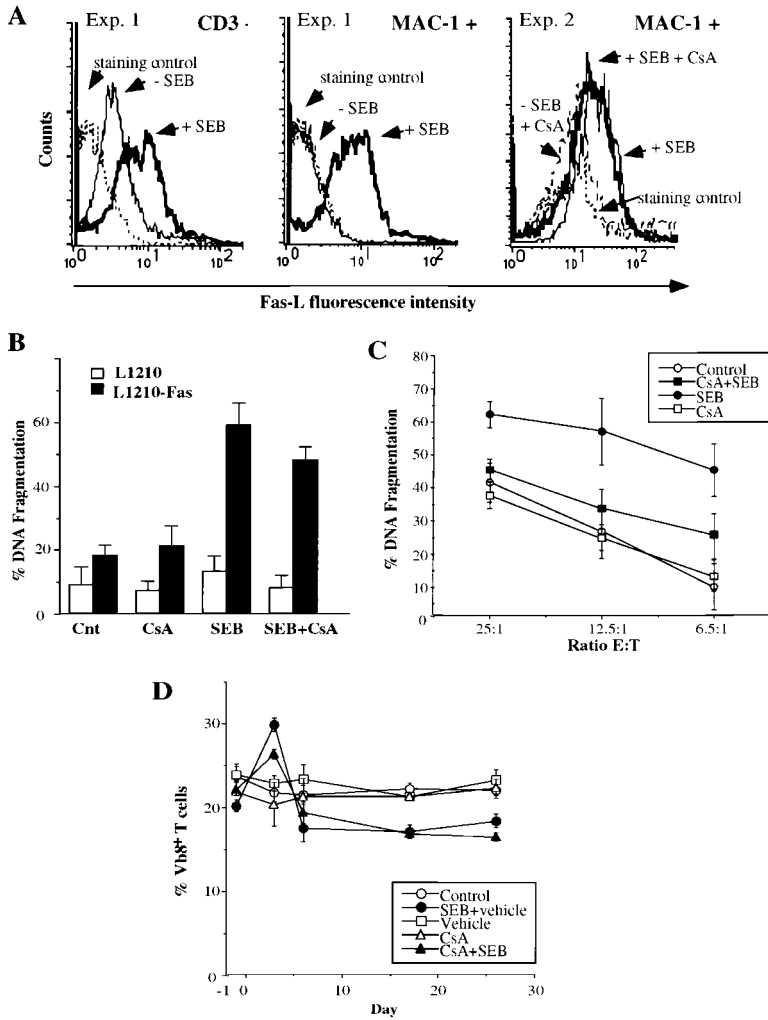
Figure 4. Detection of FasL Upregulation on Nonlymphoid and Lymphoid Cells

(A) FACS analysis of freshly dissociated liver cells double stained with anti-CD3-FITC or anti-MAC-1-FITC and anti-FasL-PE. FasL expression on CD3$^-$ cells (left), MAC-1$^+$ cells (center and right) in liver from untreated mice (thin black line, left and center panels), three days after SEB treatment (solid black line), or three days after injection of CsA plus SEB (thin black line, right panel) are shown, as well as isotype matched PE-conjugated antibody (dotted black line). Twenty thousand cells/sample were assessed. The results are representative of three independent experiments.

(B) Epithelial cells from the small intestine of SEB-treated mice have functional FasL. BALB/cBy mice were challenged in vivo with SEB, and epithelial cells were prepared. These were cocultured with previously ^{3}H-TdR-labeled L1210 or L1210-Fas target cells for 18 hr (E:T ratio, 20:1). Induction of apoptosis in the targets was assessed as percent DNA fragmentation.

(C) FasL function on SEB-stimulated spleen cells. Splenocytes from animals treated in vivo with SEB and/or CsA were cocultured with L1210-Fas cells at the indicated ratios in anti-CD3-coated plates. Target cell DNA fragmentation was determined after 18 hr. The experiment shown is representative of three independent experiments.

(D) SEB-induced peripheral deletion of Vβ8$^+$ T cells in CsA-treated animals. The percentage of Vβ8$^+$ T cells was analyzed after CsA and/or SEB treatment in C3H mice (five mice/group). The results shown are representative of two independent experiments.

found that while NF-AT activation (which can be blocked by CsA) is required for FasL promoter activation in T cells, it is not required for activity of this promoter in a nonlymphoid tissue, testicular sertoli cells. Similarly, inducible nonlymphoid expression of FasL appears to be resistant to the effects of CsA.

Our results suggest that peripheral deletion of Vβ8$^+$ T cells in response to SEB is predominantly dependent upon inducible expression of FasL on nonlymphoid cells. However, we and others (Brunner et al., 1995; Dhein et al., 1995) have previously shown that activation-induced cell death (AICD) in transformed T cells can proceed via cell autonomous Fas/FasL interactions. That is, activated T cells can commit suicide. Why, then, do T cells in vivo fail to undergo this suicide (e.g., in wt→gld chimeras; Figure 1A)?

To address this question, we reexamined the phenomenon of activation-induced apoptosis in transformed and nontransformed T cells. In the experiments shown in Figure 5A, we activated T cells at different densities and measured AICD. As we had previously observed, A1.1 T cells underwent AICD in a density-independent manner (Brunner et al., 1995). In contrast, another T cell hybridoma, 2B4, failed to undergo AICD at low density,

suggesting a requirement for cell–cell interactions (i.e., "fratricide"). Similarly, activation of murine T cell blasts only led to apoptosis when the cells were cultured at higher densities, permitting such fratricide (Figure 5A). As a control, both 2B4 and murine T cell blasts underwent apoptosis in response to other agents (dexamethasone, etoposide) in a density-independent fashion (data not shown). These results are representative of five independent experiments.

In vivo, contact between activated T lymphocytes may be limited. As another approach toward evaluating the requirement for fratricide during AICD, we cultured T cells in increasing concentrations of dextran to reduce cell contact as the media became more viscous. This approach has been described previously (Martz, 1975). AICD in A1.1 cells proceeded independently of dextran concentration (Figure 5B), as did apoptosis induced by other agents (dexamethasone, etoposide) in 2B4 and murine T cell blasts (data not shown). However, AICD in the latter cells was effectively inhibited at higher dextran concentrations. These results are representative of three independent experiments. Therefore, unlike A1.1 T cells, nontransformed T cells are more efficiently killed by FasL on another cell than on their own surface.
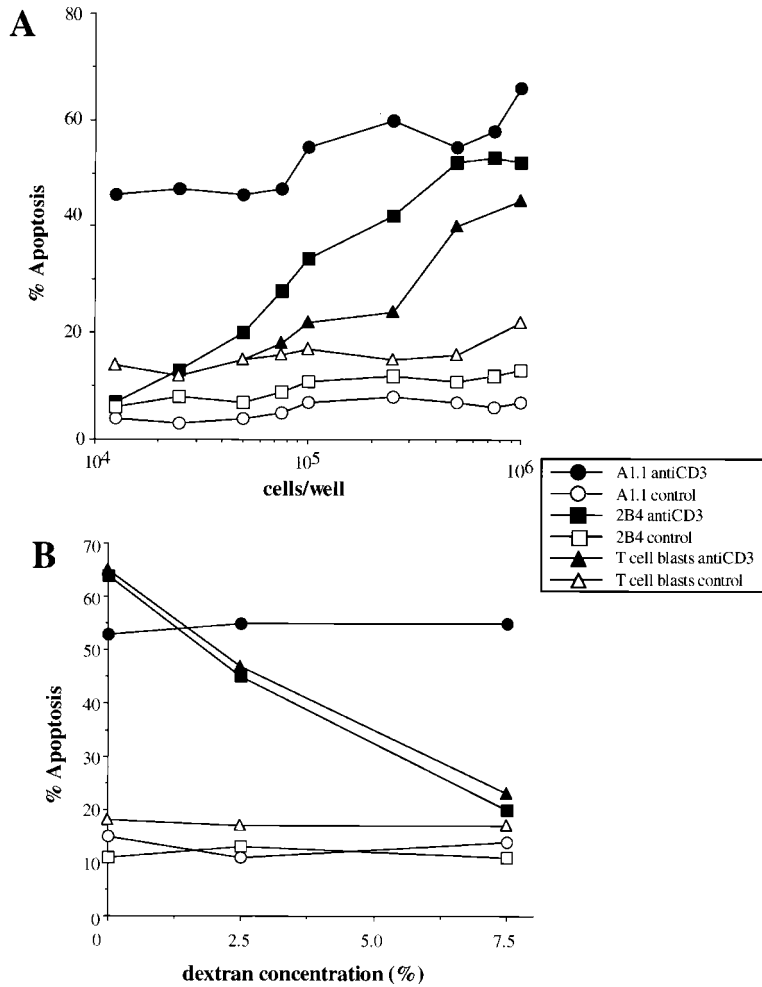
A

B

Figure 5. Cell Contact Dependence of Activation-Induced Cell Death in Mouse T Cell Hybridomas and Blasts

Cell death was assessed by propidium iodide staining after 18 hr.
(A) Cell density dependence of death induced by anti-CD3 in the T cell hybridomas A1.1 (filled circles) and 2B4 (filled squares) and in mouse T cell blasts (filled triangles).
(B) Effects on activation-induced cell death of viscous dextran in the cell culture medium of A1.1 (filled circles), 2B4 (filled squares), and mouse T cell blasts (filled triangles) in anti-CD3-coated plates. Control cells were treated with hamster IgG.

It is unclear why AICD in A1.1 (Brunner et al., 1995) and in Jurkat T cells (Dhein et al., 1995) proceeds in a cell autonomous manner, while AICD in nontransformed murine T cells (and transformed 2B4 cells) does not. It is possible, though, that in the transformed cells in vitro, folding or blebbing of the membrane allows presentation of functional FasL to Fas on the same cell, and that this effect does not efficiently proceed in normal T cell blasts.

If FasL responsible for peripheral deletion comes from cells other than the activated T cell (as our data suggest), then specificity must come from another aspect of T cell activation. One role for T cell activation during peripheral deletion might therefore be the generation of susceptibility to Fas-mediated apoptosis, which would ensure that only activated T cells die upon contact with nonlymphoid FasL. Susceptibility to Fas-mediated apoptosis is induced by T cell activation (Klas et al., 1993; Brunner et al., 1996; Refaeli et al., 1998).

We suspected that T cell activation also plays another role in the process of peripheral deletion, in that activated T cells may induce nonlymphoid FasL expression. The ability of SEB to induce FasL expression in peripheral tissues might either be a direct effect of the enterotoxin or might occur as a consequence of the immune response (e.g., via an effect of SEB-induced T cells). Therefore, we examined the effects of SEB administration on FasL expression in mice carrying the SCID mutation. As shown in Figure 6, in situ hybridization failed to detect significant levels of FasL mRNA expression in the livers or intestines of SEB-treated SCID animals. This absence of FasL expression was confirmed by RT–PCR of liver and small intestine in these mice (data not shown). However, when SCID mice were reconstituted with splenic lymphocytes (4×10^7) from wt donors, SEB effectively induced FasL expression in the peripheral tissues. In the latter case, the expression of FasL on nonlymphoid cells (e.g., epithelia) was readily apparent. Therefore, the expression of FasL on peripheral tissues in response to superantigen is dependent on the presence of responsive lymphocytes.

A more formal demonstration that SEB-activated T cells induce FasL expression in SCID mice is provided by the experiment shown in Figure 7A. SCID mice were reconstituted with wt or gld splenocytes and then injected with SEB. Three days later, IEC were isolated and tested for functional FasL expression. IEC from SEB-injected SCID mice failed to kill Fas^+ target cells, unless the animals were reconstituted with splenocytes. However, the ability of the splenocytes to express function
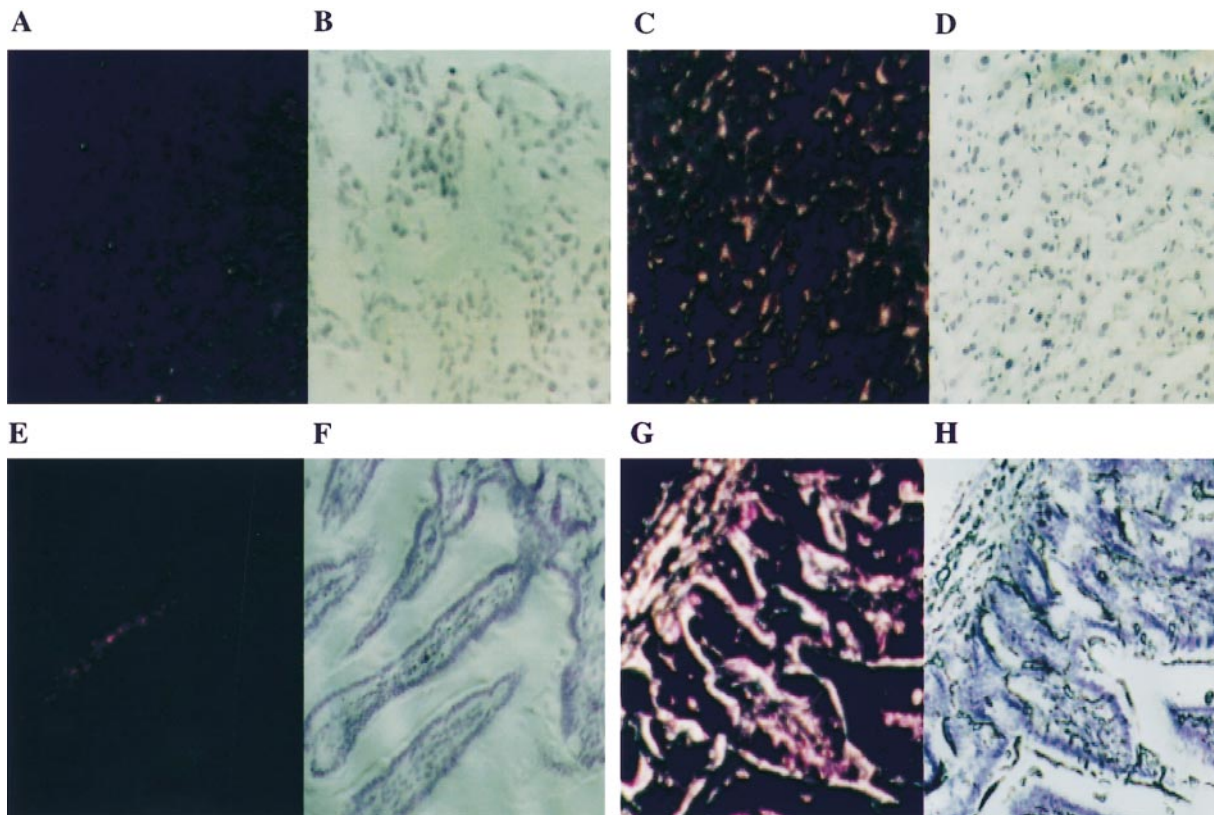
Figure 6. Functional Lymphocytes Are Required for SEB-Induced FasL mRNA Expression in Nonlymphoid Tissues

Three-week-old C3HSmn.CPrkdeSCID mice were injected with 75 µg SEB, and tissues were analyzed by in situ hybridization with antisense FasL riboprobe as in Figure 3. Liver (A and C) and small intestine (E and G) of SCID mice (A and E) or SCID mice reconstituted with 4×10^7 spleen cells (C, D, G, and H) 3 days after SEB treatment are shown. Hematoxylin staining (B, D, F, and H) of the respective dark fields is also shown.

FasL was irrelevant; gld and wt splenocytes were equally effective for inducing functional FasL on epithelial cells. Consistent with this is the observation that Vβ8$^+$ T cells in SCID mice reconstituted with wt or gld splenocytes showed the same pattern of expansion and deletion following administration of SEB (Figure 7B). As a control, the percentage of Vβ6$^+$ T cells was also determined during the course of the experiment, and this population did not undergo SEB-induced deletion (data not shown).

FasL expressed constitutively on nonlymphoid tissues can play a role in immune regulation. Indeed, immune privilege in the anterior chamber of the eye is dependent on functional FasL in the parenchymal cells of this organ (Griffith et al., 1995, 1996). FasL on testicular sertoli cells has also been suggested to protect these cells upon transplantation (Bellgrau et al., 1995), although this is controversial (Allison et al., 1997). Moreover, FasL on cornea functions to prevent allogeneic rejection of this tissue (Stuart et al., 1997), and expression of the ligand on a murine melanoma line functions to prevent rejection of the tumor (Hahne et al., 1996). During viral infections in the eye, as well as during corneal allograft acceptance, inhibition of immune responses corresponds to induction of apoptosis in infiltrating cells (Griffith et al., 1996; Stuart et al., 1997). Thus, nonlymphoid FasL is capable of inducing apoptosis in infiltrating T cells, thereby minimizing potential tissue damage as a consequence of inflammation.

Here, we have demonstrated that other nonlymphoid tissues, which perhaps should not express constitutive FasL due to their high visibility to the lymphoid system, can do so in response to an intensive cellular immune reaction. Our observations lead to a model of lymphocyte–periphery interactions in which the extent of an immune response regulates FasL expression in some tissues, which in turn induces lymphocyte apoptosis and reduces the numbers of responding cells. This might function as a form of conditional immune privilege, in which a tissue effectively dictates, via inducible FasL expression, the degree of inflammation it will allow. Different tissues may form a continuum, from constitutively privileged tissues (the eye) where responses are almost never permitted, to inducible tissues (liver, intestine?), to perhaps some tissues that never express FasL and tolerate any amount of immunological damage. Thus, each tissue monitors the extent of local response and expresses FasL when a critical threshold is reached. This monitoring might involve cytokine release from responding T cells, most likely one that is not inhibited by CsA (based on the results shown in Figure 4). There are several cytokines for which production by T cells can be partially or completely resistant to CsA, including IL-5, IL-10, IL-13, and TGFβ (Rao et al., 1997). Whether these or other cytokines might be involved in the induction of nonlymphoid FasL expression by T cells in vivo has not yet been examined.
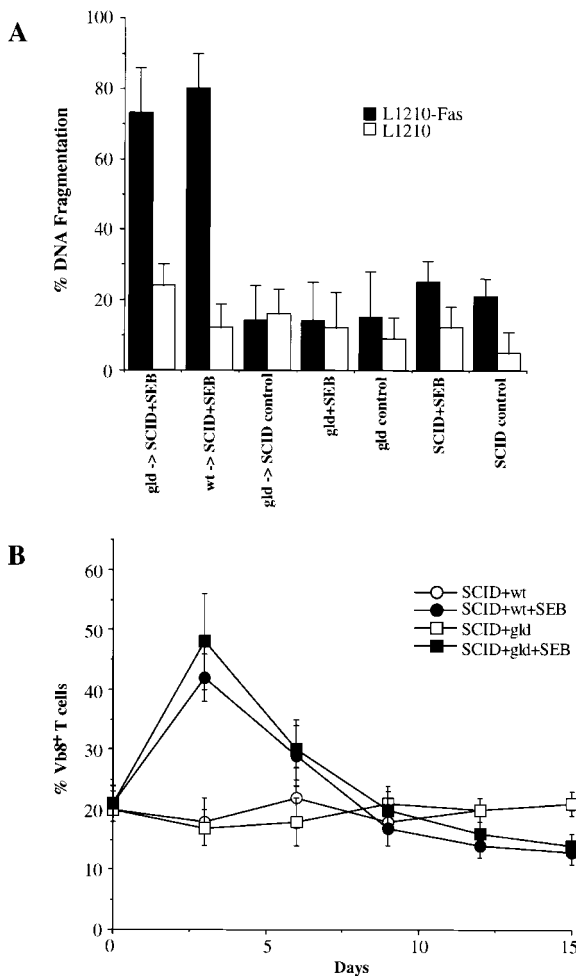
Figure 7. SEB-Activated T Cells Induce FasL Expression on Peripheral Nonlymphoid Tissues

(A) C3HSmn.CPrkdeSCID mice were transplanted with 4–5 × 10⁷ splenocytes from gld mice and 48 hr later were challenged in vivo with SEB. Epithelial cells (IEC) were prepared and cocultured with previously ³H-TdR-labeled L1210 or L1210-Fas target cells for 18 hr (ratio 20:1). As a control IEC from gld mice SEB-treated or not treated were collected. Induction of apoptosis in the target cells was assessed as percent DNA fragmentation.
(B) SEB induces peripheral Vβ8⁺ T cell deletion in SCID mice reconstituted with wt or gld splenocytes. SCID mice were transplanted with spleen cells from gld or wt mice of the same background strain and challenged with SEB (three mice/group). These results are representative of two independent experiments.

A number of studies have shown that tumors and tumor lines from a variety of tissues express FasL (Hahne et al., 1996; O'Connell et al., 1996; Gratas et al., 1997; Niehans et al., 1997; Shiraki et al., 1997), and in some cases this may function to suppress anti-tumor immunity (Hahne et al., 1996). Chemotherapeutic drugs induce FasL expression in some lymphoid and nonlymphoid lines (Friesen et al., 1996; Kasibhatla et al., 1998), and this can be responsible for maximal apoptosis in response to these agents. Our results suggest that such nonlymphoid FasL expression may be a reflection of a normal physiological role for this molecule in regulating immune responses.

While our studies focus on the role of peripheral FasL

in deletion of superantigen-responsive T cells in vivo, they do not rule out the possibility that other molecules are similarly involved in this process. For example, tumor necrosis factor has been shown to play an important role, together with FasL, in T cell peripheral deletion (Sytwu et al., 1996). Other members of this ligand family might also function to induce T cell apoptosis (e.g., TRAIL/APO-2L) (Wiley et al., 1995); however, the exact role of these ligands awaits a more precise definition of their function in vivo.

In any case, FasL-mediated apoptosis is clearly complex. For example, one recent study has noted that macrophages expressing FasL intracellularly do not kill targets unless they receive a signal to induce the release of the FasL (Tsutsui et al., 1996). We have similarly noted that some FasL⁺ tumor lines fail to kill Fas⁺ targets (T. B., unpublished observations).

Our results support the idea that expression of FasL in response to lymphocyte reactivity plays an important role in regulating T cell expansion via peripheral deletion. If so, then those conditions under which inflammation is favored (rather than FasL-induced apoptosis) may contribute to direct bystander tissue damage by immune responses. This might result in disease states such as autoimmunity, graft rejection, and graft versus host responses. However, recent studies have shown that apoptosis of lymphoid cells can lead directly to the induction of immune tolerance (Griffith et al., 1996). This tolerance is not merely deletion but the result of a Th2-dominated immune deviation. Thus, immune responses that might lead to tissue damage and/or autoimmunity can be controlled by induction of apoptosis and by a subsequent regulatory immune reaction, triggered by a response to the apoptotic T cells. Therefore, lymphoid–parenchymal interactions centering on FasL (and perhaps related molecules) may play pivotal roles in the regulation of the immune response.

Experimental Procedures

Reagents, Animals, and Treatments

The following reagents were used for this study: CsA and SEB (Sigma Chemical Co.), [³H]thymidine, and ³²P (NEN).

BALB/cBy, C3H, C57BL/6J, and C3HSmn.CPrkdeSCID mice were purchased from Jackson Laboratories. The animals were treated in vivo with SEB and/or CsA injected intravenously and intraperitoneally, respectively. SEB was solubilized in distilled water, CsA in 90% light mineral oil and 10% ethanol. The dose of SEB producing optimal deletion of Vβ8⁺ T cells was determined in each mouse strain to be as follows: BALB/cBy (75 μg/20 g mouse), C3H and C57BL/6 (85 μg/20 g mouse), and bone marrow chimeras (115 μg/20 g mouse). CsA (15 mg/kg) pretreatment was done 1 day prior to SEB and then every day up to the final time point.

Functional Assay for FasL Expression

FasL function was detected by the ability of IEC or splenocytes to induce DNA fragmentation in Fas-positive target cells (Brunner et al., 1995). Briefly, parenteral L1210 and L1210-Fas cells (Rouvier et al., 1993) were labeled with 5 μCi/ml [³H]thymidine for 4 hr. After incubation cells were rinsed twice with HBSS to remove unincorporated thymidine. Target cells were then incubated in complete medium with dissociated epithelial cells from the small intestine of control or treated mice in 96-well plates or with splenocytes in anti-CD3-coated 96-well plates. After 18 hr DNA was harvested by MASH and counted in a β counter. DNA fragmentation was determined as (average counts per control target well − counts per experimental well)/(average counts per control target well) × 100. Assays were performed in triplicate.

Assessment of Peripheral Deletion in Vivo

Fresh blood was obtained from each mouse every second day from the intraorbital sinus. After rinsing the blood with PBS containing 50 U/ml heparin and 2% FCS, peripheral lymphocytes were isolated by centrifugation through ficoll Histopaque (density 1.007 g/ml). Peripheral blood mononuclear cells were incubated for 30 min with the following three antibodies: FITC-anti Vβ8+ and Vβ6+ (1:100, Pharmigen), PE-anti-CD4+ (1:100, Pharmigen), and RED-613-anti-CD8+ (1:100, GIBCO). After rinsing, surface expression of the corresponding proteins was assessed by FACScan flow cytometer (Becton Dickinson) as previously described (Mogil et al., 1995).

Bone Marrow Chimeras

Radiation bone marrow chimeras were prepared as described (Spangrude et al., 1994). Mice received 1100 R irradiation prior to receiving 1×10^7 bone marrow cells. The dose of 1100 R was used since we had previously determined that this dose permitted the establishment of fully allogeneic bone marrow chimeras in C57BL/6 mice. In control experiments using Thy-1 congenic animals, we found that >95% of T cells were derived from the gld or wt grafts (Griffith et al., 1995). Donor bone marrow cells were isolated on ficoll gradients, washed twice with Hanks' balanced salt solution (HBSS), and injected (5×10^6/mouse) into the irradiated host. Bone marrow chimeras were composed of FasL-defective *gld/gld* bone marrow in wt recipient mice (gld→wt) or wt bone marrow in gld recipient mice (wt→gld). FasL is functional on transplanted lymphoid cells but not on nonlymphoid organs in C57BL/6J→B6.gld chimeras and vice versa for C57BL/6J.gld→C57BL/6J.

RT–PCR

Total RNA was isolated from mouse organs using TRIzol reagent (GIBCO BRL). cDNA was first prepared by reverse transcription of 1 μg RNA in a 20 μl reaction volume containing 10× PCR buffer, 1 mM DTT, 200 μM dNTPs, 5 μM random primers (Superscript reverse transcriptase, GIBCO BRL). PCR amplifications were performed in 50 μl reaction volume containing 1 μg cDNA, with a master mix composed of 10× PCR buffer, 1 mM MgCl$_2$, 200 μM dNTPs, Taq polymerase, and 20 nM of each primer. Primers are as follows: mouse FasL sense, 5′-CAGCAGTGCCACTTCATCTTG-3′, antisense, 5′-TTCACTCCAGAGATCAGAGCGG-3′; β-actin sense, 5′-TGGATTC CTGTCGCATCCATGAAAG-3′, antisense, 5′-TTAAACGCAGCTCAG TAACAGTCCG-3′. The PCR products were separated by 1.2% agarose gel electrophoresis and stained with ethidium bromide.

In Situ Hybridization

In situ hybridization was performed as described (Simmons et al., 1989) with minor modifications. Organs were rinsed in PBS to remove blood contamination and quick-frozen in OCT embedding compound (Miles, Elkhart, IN) on dry ice. Seven-micrometer sections were cut and mounted on albumin-coated microscope slides and then air dried overnight. After fixation with 4% paraformaldehyde for 20 min, sections were rinsed in PBS and dehydrated in increasing ethanol concentrations, then incubated for 4 hr in prehybridization buffer (EDTA 0.5 mM, 5× SET, 0.2% SDS, 5× Denhart solution, 0.5 mg/ml sheared salmon sperm). Riboprobes were prepared by in vitro translation (Promega T4, T7) and ^{33}P labeling of the mouse FasL cDNA. Tissues were exposed to sense and antisense ^{33}P labeled riboprobes diluted into a prehybridization solution containing 10% dextran sulphate at 55°C overnight. Slides were rinsed with sodium saline citrate (SSC) for 4 hr and then embedded in NTB2 nuclear track emulsion (Kodak, Rochester, NY). Radiographic films were exposed for 4 weeks on tissue slides. The slides were developed and fixed with Kodak solutions (D19 and 3000A and B) and after hematoxylin staining mounted on glass coverslips. A Nikon microscope equipped with dark field and a 20× objective was used for microscopic analysis.

Liver Cell Dissociation and Surface Staining of FasL

Liver cells were isolated by collagenase IV (Sigma) perfusion as described (Seglen, 1973). After dissociation cells were washed twice with HBSS and then incubated in PBS, 1% calf serum, and 0.05% sodium azide with anti-MAC-1-FITC or anti-CD3-FITC and anti-mFasL-PE antibodies (Pharmingen, San Diego, CA) for 1 hr at 4°C.

After rinsing twice with PBS, cells were fixed in PBS and 1% formaldehyde, and staining was assessed by FACScan. An isotype-matched control antibody served to define background values.

Isolation of IEC

Mouse small intestine was filleted longitudinally and briefly washed in HBSS. The tissue was then cut into 1 cm pieces and placed into a conical tube containing 25 cc of RPMI 1640 medium with 10% fetal calf serum, penicillin (100 U/ml), streptomycin 100 μg/ml, and 1 mM DTT. The tube was shaken at 37°C in an orbital shaker for 30 min. The supernatant was filtered through cotton gauze, and cells were washed once with RPMI 1640 medium. Cells were then resuspended in 5 cc of 30% Percoll and centrifuged for 20 min at 400 × g. IEC were isolated from the top layer. Visual inspection by light microscopy revealed that cell viability by trypan blue exclusion was over 95%, and over 97% of live cells were IEC. Less than 1% of the isolated cells were CD3+ by flow cytometry.

Lymphocyte Reconstitution in SCID Mice

C3HSmn.CPrkdeSCID mice were injected i.p. with 4–5 × 10^7 splenocytes from C3H or C3H.gld mice. After 48 hr, SCID were challenged with SEB (75 μg/20 g mouse). Peripheral deletion, IEC induced killing, and in situ hybridization were assessed at the established time points as explained above.

T Cell Preparation and Assessment of Cell Death

Spleen cells were prepared from 6- to 10-week-old C57BL/6 mice by passage through a nylon wool column (Julius et al., 1973). Primary activation was carried out by treating these T cell preparations (1 × 10^5 cells/ml) in RPMI, 10% FCS, and penicillin/streptomycin with 2 ng/ml PMA and 1 μg/ml ionomycin for 2 days. Blast cells were then washed and resuspended in RPMI complete medium with 10 μg/ml IL-2.

Cell death was assessed by fluorescent microscopy, examining cell suspensions within a few minutes of resuspension in propidium iodide (5 μg/ml). A minimum of 200 cells were scored for nuclear fluorescence in each group.

Dextran Inhibition

A sterile stock solution of 10% (w/v) dextran (2 × 10^6 mw, Sigma) was prepared in RPMI complete medium to block CTL cytotoxicity by preventing effector–target contact (Martz, 1975). To assess the inhibitory effect on activation by immobilized antibodies, lymphocytes were allowed to sediment onto anti-CD3-coated wells in normal medium. Then after 1 hr the dextran-rich medium was added gently to displace the normal medium, which was removed from the top of the well.

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