

# Fas Ligand-mediated Killing by Intestinal Intraepithelial Lymphocytes

## Participation in Intestinal Graft-versus-Host Disease

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### Abstract

In vitro studies have demonstrated that intestinal intraepithelial lymphocytes (IEL) are constitutively cytotoxic; however, the mechanism and target of their cytotoxicity are unknown. Apoptosis of intestinal epithelial cells (IEC) and an increase in IEL numbers are classical signs of intestinal graft-versus-host disease (GVHD), although whether IEL can mediate IEC apoptosis directly in GVHD is unclear. Recent evidence suggests that target epithelial organ injury observed in GVHD is predominantly Fas-mediated; therefore, we investigated the possibility that IEL induce apoptosis of IEC through a Fas-mediated mechanism. Here, we demonstrate that the IEL isolated from normal mice readily display potent Fas ligand (FasL)-mediated killing activity after CD3 stimulation, and that IEC express Fas, suggesting that IEC are potential targets for FasL-mediated killing by IEL. In vitro, IEL isolated from GVHD mice have markedly increased FasL-mediated killing potential and are spontaneously cytolytic toward host-derived tumor cells predominantly through a Fas-mediated pathway. In vivo transfer of IEL isolated from GVHD mice induced significantly more IEC apoptosis in F1 wild-type mice than in Fas-defective *F1lpr* mice. Thus, these results demonstrate that FasL-mediated death of IEC by IEL is a major mechanism of IEC apoptosis seen in GVHD. (*J. Clin. Invest.* 1998. 101:570–577.) Key words:  $\gamma\delta$  T cells • graft-versus-host disease • Fas • Fas ligand • intestinal intraepithelial lymphocytes

### Introduction

Murine intestinal intraepithelial lymphocytes (IEL)<sup>1</sup> are a phenotypically diverse and complex T cell population which differs markedly from the T cell population found in the periphery (1, 2). Unlike peripheral T cells, a large percentage of murine IEL use the  $\gamma\delta$  T cell receptor (TCR). Furthermore,

the vast majority of murine IEL, although clearly T cells, lack conventional T cell markers such as Thy 1, CD2, and CD5 (1, 3–5). These results have led several investigators to conclude that the majority of murine IEL are derived from a separate lineage, possibly through an extrathymic pathway (6–8).

Despite their phenotypic diversity, the majority of murine IEL express the cytotoxic CD8<sup>+</sup> phenotype. Why IEL are skewed towards a CD8<sup>+</sup> phenotype is unclear, but this characteristic appears to be evolutionarily conserved among several species (9–11). Functional in vitro studies using redirected cytolytic killing assays have revealed that murine IEL possess potent cytotoxicity. However, the biological significance of IEL cytotoxicity is unclear. The localization of IEL to areas constantly exposed to high microbiological content suggests that they may play a role in host defense; however, evidence supporting this hypothesis has not been conclusive (12–14).

In graft-versus-host disease (GVHD), donor cells recognize and eliminate host cells. In the intestine, an increase in the total number of IEL and apoptosis of intestinal epithelial cells (IEC) have been observed in GVHD (15, 16). Interestingly, direct evidence demonstrating that IEL can induce IEC apoptosis in GVHD has not been described. Several studies have suggested that the vast majority of IEL are anergic (3, 17, 18). In addition, studies in transgenic (Tg) mice which express a TCR that recognizes self antigen have revealed that in the presence of self antigen, Tg T cells are deleted in the thymus (negative selection), but for unclear reasons the IEL population contains an abundant number of these Tg T cells (19, 20). The lack of an obvious deleterious effect to the intestinal epithelium, despite the abundant presence of potentially autoreactive T cells, supports the argument that most IEL are anergic.

GVHD is mediated by the activity of two different mechanisms of cytotoxic T cell function, one dependent on perforin and one dependent on Fas (21). Recent studies suggest that both perforin- and Fas-mediated pathways are involved in the systemic signs of GVHD; however, target organ epithelial injury to the liver and skin appears to be especially restricted to Fas-mediated injury (22). These observations led us to investigate the role of Fas-mediated death in intestinal GVHD, specifically whether IEL participate directly in IEC apoptosis during GVHD through a Fas-mediated pathway.

### Methods

*Mice and induction of GVHD.* C57BL/6 (H-2<sup>b</sup>), B6D2F1/J (C57BL/6 × DBA/2, H-2<sup>b×d</sup>), B6C3F1 (C57BL/6 × C3H/He, H-2<sup>b×k</sup>), C57BL/

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1. *Abbreviations used in this paper:* FasL, Fas ligand; FCM, flow cytometry analysis; GVHD, graft-versus-host disease; IEC, intestinal epithelial cell(s); IEL, intestinal intraepithelial lymphocyte(s); PE, phycoerythrin; TCR, T cell receptor; Tg, transgenic; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling.

6lpr, and C3Hlpr mice were obtained from The Jackson Laboratory (Bar Harbor, ME). B6C3F1lpr mice were obtained by mating C57BL/6lpr with C3Hlpr mice. All mice were raised under specific-pathogen-free conditions in the animal care facility at the La Jolla Institute of Allergy and Immunology. Acute GVHD was induced by the injection of  $10^8$  C57BL/6 spleen cells into the tail vein of either B6D2F1/J or B6C3HF1 mice. Unless stated otherwise, all mice were killed 3 wk after the induction of GVHD.

**Reagents and cells.** Jurkat E6 cells were obtained from American Type Culture Collection (Rockville, MD). L1210 cells, which express low levels of Fas, and the Fas-transfected L1210-Fas were kindly provided by Pierre Golstein (INSERM-CNRS, Marseilles, France) (23). Fas-Fc was produced through baculovirus expression as described previously (24). Concanamycin A was obtained from Sigma Chemical Co. (St. Louis, MO).

Antibodies were obtained from the following suppliers: FITC-conjugated anti-TCR  $\delta$  and nonconjugated anti-TCR  $\delta$  (GL-3; PharMingen, San Diego, CA), FITC-conjugated and nonconjugated anti-TCR  $\beta$  (H57-597; PharMingen), phycoerythrin (PE)-conjugated anti-Thy 1.2 (PharMingen), biotin-conjugated anti-CD8 $\beta$  (Caltag Laboratories, Inc., Burlingame CA), biotin-conjugated anti-H-2K<sup>d</sup> (PharMingen), FITC-conjugated anti-CD4 (PharMingen), anti-Fas (Jo-2; PharMingen), hamster IgG (PharMingen), PE-conjugated anti-hamster IgG (Caltag Laboratories, Inc.).

**Cell isolation and flow cytometry analysis (FCM).** Isolation of IEC was performed following the protocol described by Komano et al. (25). Isolation of IEL has been described previously (5). Spleen and lymph node cells were isolated by gently meshing the respective organs between two slides, followed by filtering through a cotton gauze. The cells were first stained with hamster and goat serum to block nonspecific staining and subsequently stained with the appropriate antibodies. Two- or three-color FCM was performed with a FACScan<sup>®</sup> flow cytometer (Becton Dickinson, Mountain View, CA). The data were analyzed with the Macintosh Cell Quest program.

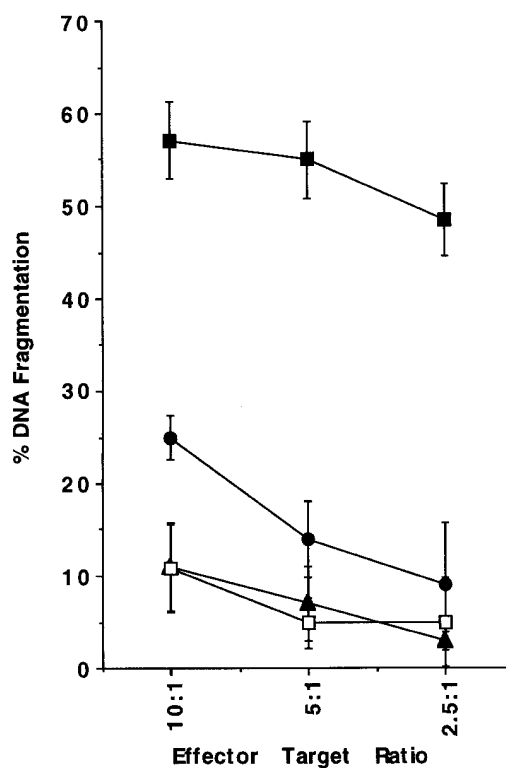
**DNA fragmentation assay.** DNA fragmentation in L1210, L1210-Fas, and Fas-positive Jurkat cells as targets has been described previously (26, 27). Briefly, target cells ( $10^6$ /ml) were labeled with 5  $\mu$ Ci/ml [<sup>3</sup>H]thymidine for 2 h. Unincorporated [<sup>3</sup>H]thymidine was removed by two washes with HBSS. Target cells ( $2 \times 10^4$ ) were incubated with effector cells at various concentrations in flat-bottomed 96-well plates, coated previously with 3  $\mu$ g/ml of anti-CD3 $\epsilon$ , anti-TCR  $\beta$ , anti-TCR  $\delta$ , or no antibody. After 12 h, cells were harvested using a Skatron Instruments cell harvester (Sterling, VA) and [<sup>3</sup>H]thymidine-labeled unfragmented DNA was calculated as follows: % DNA fragmentation =  $100 (1 - \text{cpm experimental group}/\text{cpm control group}) \pm \text{SD}$ . Assays were done in triplicate.

**Isolation of Thy 1<sup>+</sup> and Thy 1<sup>-</sup> IEL.** Thy 1<sup>+</sup> IEL and Thy 1<sup>-</sup> IEL were isolated by flow cytometry sorting (FACStar<sup>®</sup>, Becton Dickinson). Purity was determined to be > 97% by flow cytometry (data not shown).

**Histologic evaluation of IEC apoptosis.** IEC apoptosis was detected in formalin-fixed sections of murine small intestine using the TUNEL assay (for terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling) to detect DNA fragmentation. Briefly, the small intestine was removed and incised longitudinally. The intestine was washed briefly in HBSS to remove fecal material and fixed overnight in 10% buffered formalin solution (Fisher Scientific Co., Pittsburgh, PA). Paraffin sections were deparaffinized with xylene and ethanol gradient. Tissue sections were then stained for TUNEL-positive cells following the manufacturer's suggested instructions (In Situ Death Detection kit, Boehringer Mannheim Biochemicals, Indianapolis, IN).

## Results

**IEL are potent inducers of Fas-mediated cell death.** T cell cytotoxicity involves two major mechanisms, one dependent on



**Figure 1.** Anti-CD3-stimulated IEL are potent killers of Jurkat cells. <sup>3</sup>H-labeled Jurkat cells were cultured with IEL (squares), spleen cells (circles), and lymph node cells (triangles) in the absence (open symbols) or presence (closed symbols) of plate-bound anti-CD3 cells at various E/T ratios. DNA fragmentation of Jurkat cells was assessed after 12 h. Minimal spontaneous killing (< 15%) was observed for nonstimulated lymph node cells and spleen cells (data not shown). Error bars represent SD of triplicate cultures. Cells were pooled from two mice, and results shown are representative of three independent experiments.

perforin and the other on Fas ligand (FasL) (21). Using an assay that specifically quantifies FasL-mediated killing by activated murine T cells (26, 27), we analyzed the ability of IEL to kill Jurkat cells. Fig. 2 demonstrates that freshly isolated IEL do not spontaneously kill Jurkat cells, but when stimulated with plate-bound anti-CD3, IEL readily became very potent killers (Fig. 1). Significant killing of Jurkat cells was observed with E/T ratios as low as 0.3:1 (data not shown). Furthermore, CD3-stimulated IEL were five- to sixfold better at killing Jurkat cells than CD3-stimulated either spleen or lymph node cells. This ability of anti-CD3-stimulated IEL to kill Jurkat cells more efficiently than spleen or lymph node cells cannot be explained entirely by the higher percentage of CD3<sup>+</sup> T cells found in the IEL population (80–90%), as lymph node cells have a comparable percentage of CD3<sup>+</sup> T cells (70–75%; data not shown). Furthermore, when the E/T ratio for spleen and lymph node cells was corrected for the higher percentage of CD3<sup>+</sup> cells found in the IEL population, IEL still induced significantly more Jurkat cell killing (data not shown).

To investigate the mechanism by which CD3-stimulated IEL mediated the killing of Jurkat cells, we added Fas-Fc, which blocks Fas and FasL interactions (27), to our assay. Fig. 2 demonstrates that Fas-Fc blocked completely the killing of Jurkat cells, whereas concanamycin A, which inhibits perforin-

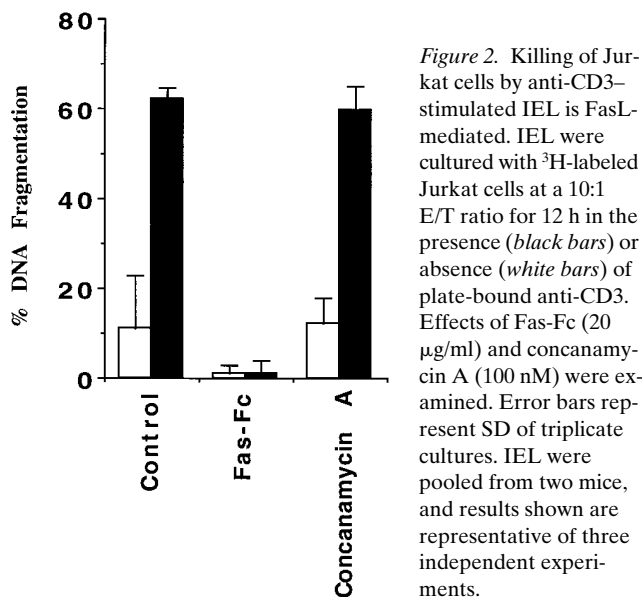


Figure 2. Killing of Jurkat cells by anti-CD3-stimulated IEL is FasL-mediated. IEL were cultured with  $^3\text{H}$ -labeled Jurkat cells at a 10:1 E/T ratio for 12 h in the presence (black bars) or absence (white bars) of plate-bound anti-CD3. Effects of Fas-Fc (20  $\mu\text{g/ml}$ ) and concanamycin A (100 nM) were examined. Error bars represent SD of triplicate cultures. IEL were pooled from two mice, and results shown are representative of three independent experiments.

mediated killing (28), had no apparent effect. Furthermore, IEL from *gld* mice, which have nonfunctional FasL, were unable to kill Jurkat cells (data not shown). Thus, in this assay, IEL appear to function effectively as killers via a FasL-Fas interaction.

*FasL-mediated killing by IEL is primarily by Thy 1<sup>+</sup> TCR  $\alpha\beta$  and TCR  $\gamma\delta$  IEL.* Because of the phenotypic complexity of the murine IEL population (1, 2), we attempted to determine which of the multiple IEL subsets were responsible for the Fas-mediated death of Jurkat cells. We sorted freshly isolated IEL into Thy 1<sup>+</sup> or Thy 1<sup>-</sup> population, because Thy 1 appears to be an accurate marker for delineating IEL which have

developed from two separate pathways (1, 7, 29). Thy 1<sup>+</sup> IEL phenotypically resemble peripheral T cells, hence are most likely derived from the same pathway as the vast majority of T cells found in the spleen and lymph node population. In contrast, the phenotype of Thy 1<sup>-</sup> IEL differs markedly from peripheral T cells, and thus Thy 1<sup>-</sup> are believed to be derived from a separate lineage which develops through both an extrathymic and a thymic pathway (1, 7, 29). Fig. 3 A demonstrates that the Thy 1<sup>+</sup> IEL population consists primarily of TCR  $\alpha\beta$  IEL, but a substantial number of TCR  $\gamma\delta$  IEL is also seen. In contrast, Thy 1<sup>-</sup> IEL consist predominantly of TCR  $\gamma\delta$  IEL, but a significant percentage of TCR  $\alpha\beta$  IEL is also present.

Stimulation of Thy 1-sorted IEL with anti-CD3 demonstrates that Thy 1<sup>+</sup> IEL are significantly more potent at FasL-mediated killing than Thy 1<sup>-</sup> IEL. Stimulation with the pan anti-TCR  $\beta$  (H57-597) and pan anti-TCR  $\delta$  (GL-3) suggests that both TCR  $\gamma\delta$  and TCR  $\alpha\beta$  IEL are capable of FasL-mediated killing. These results are consistent with the observation by Barrett et al. that Thy 1<sup>+</sup> IEL proliferate well upon TCR stimulation, but Thy 1<sup>-</sup> IEL proliferate poorly (3).

*IEC express Fas.* If IEL are such potent FasL-mediated killers, what is their natural target? Because IEL are essentially surrounded by IEC, we rationalized that the latter are the most likely targets for FasL-mediated killing by IEL. FCM supports this hypothesis. IEL express very low levels of Fas, but IEC express moderate levels (Fig. 4). These results are consistent with recent observations that Fas is expressed on colon cancer epithelial cell lines (30, 31).

*GVHD results in an increase in FasL-mediated killing by IEL which correlates with an infiltration of donor-derived Thy 1<sup>+</sup> TCR  $\alpha\beta$  CD4<sup>-</sup> CD8 $\beta$  IEL.* The expression of Fas on IEC suggests that FasL-mediated killing by IEL, if unregulated, may play a role in the destruction of IEC in certain intestinal diseases. In this regard, we chose to examine the role of FasL-

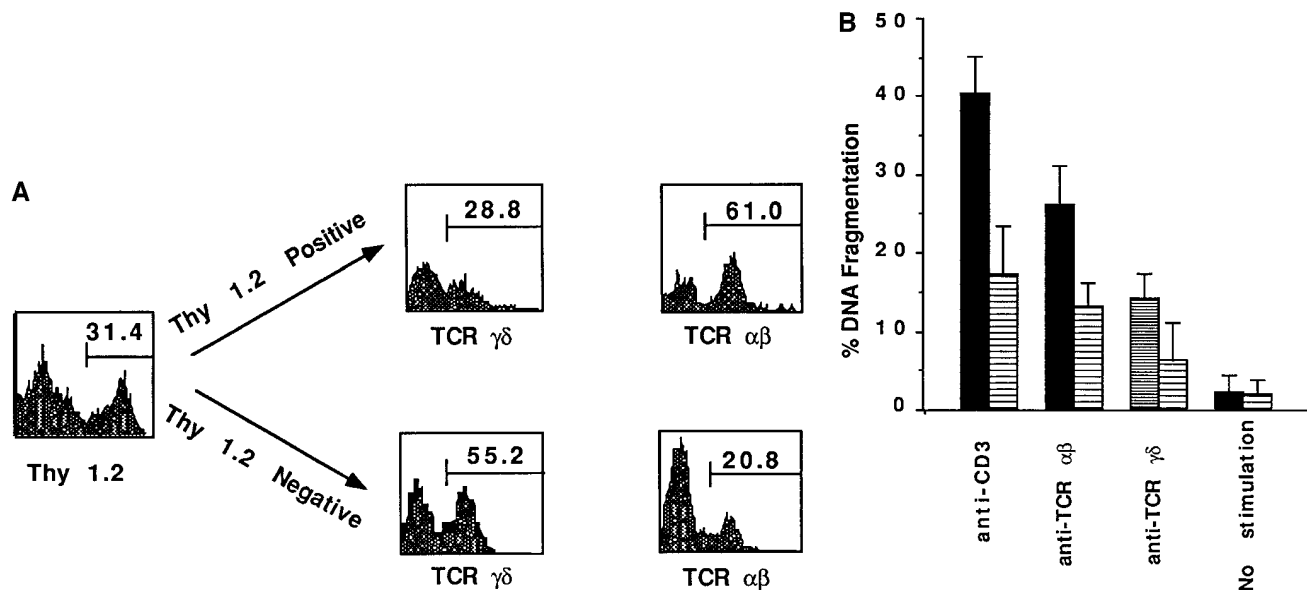


Figure 3. Thy 1<sup>+</sup> TCR  $\alpha\beta$  and Thy 1<sup>+</sup> TCR  $\gamma\delta$  IEL are the most potent FasL-mediated killers. (A) Two-color FCM and histogram analysis of IEL before and after separation by FACS<sup>®</sup> sorting into Thy 1<sup>+</sup> and Thy 1<sup>-</sup> IEL. (B) FACS<sup>®</sup>-sorted Thy 1<sup>+</sup> (black bars) and Thy 1<sup>-</sup> (striped bars) IEL were cultured with  $^3\text{H}$ -labeled Jurkat cells at an E/T ratio of 5:1 in the presence of plate-bound anti-CD3, anti-TCR  $\delta$  (GL-3), or anti-TCR  $\beta$  (H597-597). Results shown are from IEL pooled from 10 mice.

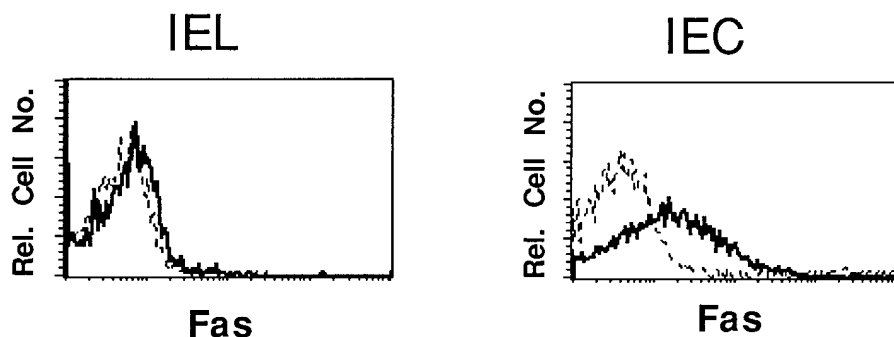


Figure 4. FCM histogram analysis of Fas expression by IEL and IEC. *Dashed lines*, Control staining (hamster IgG followed by PE-conjugated anti-hamster IgG). *Solid lines*, Fas staining (anti-Fas followed by PE-conjugated anti-hamster IgG).

mediated killing by IEL in GVHD, a disease characterized by an increase in the total number of IEL, as well as apoptosis of IEC. We used a model of murine acute GVHD in which parental spleen cells (C57BL/6, H-2<sup>b</sup>) are injected into F1 hosts (H-2<sup>b</sup> × H-2<sup>d</sup>) (15, 16, 32). In this model, donor-derived cells can be distinguished from host-derived cells by the lack of expression of host MHC H-2<sup>d</sup>. Consistent with previous studies (15), this model of GVHD resulted in a two- to threefold increase in total IEL number as well as IEC apoptosis (data not shown and Fig. 5). Similar findings were observed when C57BL/6 spleen cells were injected into B6C3HF1 (H-2<sup>b</sup> × k) mice (data not shown).

We examined the FasL-mediated cytotoxicity of IEL at weekly intervals after the induction of GVHD. Beginning at week 2 of GVHD, FasL-mediated killing by the IEL of GVHD mice rose dramatically (Fig. 6). This increase in FasL-mediated killing by the IEL of GVHD mice was not due to a difference in the percentage of CD3<sup>+</sup> IEL, because the percentages were similar in both control and GVHD mice (data not shown). Instead, the increased killing activity was most likely due to a 10-fold increase in the percentage of Thy 1<sup>+</sup> IEL in GVHD mice (Fig. 7A).

Phenotypic analysis by flow cytometry revealed that the vast majority of these Thy 1<sup>+</sup> IEL were donor-derived (H-2<sup>d</sup>-), TCR αβ<sup>+</sup>, CD4<sup>-</sup>, and CD8β<sup>+</sup> (Fig. 7, A and B). Thus, the elevated FasL-mediated killing by IEL observed in GVHD mice was most likely due to the infiltration of TCR αβ CD4<sup>-</sup> CD8β<sup>+</sup>

Thy 1<sup>+</sup> donor-derived IEL. These results are consistent with our finding that Thy 1<sup>+</sup> IEL are more potent at FasL-mediated killing than Thy 1<sup>-</sup> IEL.

*IEL from GVHD mice can mediate the killing of Jurkat cells through recognition of host antigen, and can spontaneously kill host-derived target cells in vitro primarily through a Fas-mediated pathway.* In our model of GVHD, donor or parental C57BL/6 (B6) spleen cells were injected into host B6D2F1 (C57BL/6 × DBA/2) mice. In this model, C57BL/6 (donor)-derived spleen cells which infiltrate the host intestinal epithelium should recognize DBA/2 (host)-derived antigen. To test this hypothesis, we stimulated IEL from GVHD mice with DBA/2-derived L1210 cells (instead of anti-CD3). Fig. 8 demonstrates that L1210 cells readily stimulate IEL from GVHD (B6→B6D2F1) mice to mediate the killing of Jurkat cells. This stimulation appears to be specific, because L1210 cells did not stimulate IEL from non-GVHD (F1→F1) mice. Furthermore, in a model of GVHD where the host DBA/2 (H-2<sup>d</sup>) in the F1 mice is replaced with C3H (H-2<sup>k</sup>), L1210 cells were unable to stimulate IEL isolated from B6→B6C3F1 GVHD mice to mediate the killing of Jurkat cells. It is unlikely that IEL from B6→B6C3F1 GVHD mice were defective in FasL-mediated killing when compared with IEL from B6D2F1 mice, because in both combinations a similar level of infiltration by donor-derived Thy 1<sup>+</sup> CD8β IEL into the intestinal epithelium was observed (> 90%, data not shown). Furthermore, IEL isolated from either combination were equally potent in

### B6D2F1 --> B6D2F1 Intestine    C57BL/6 --> B6D2F1 Intestine

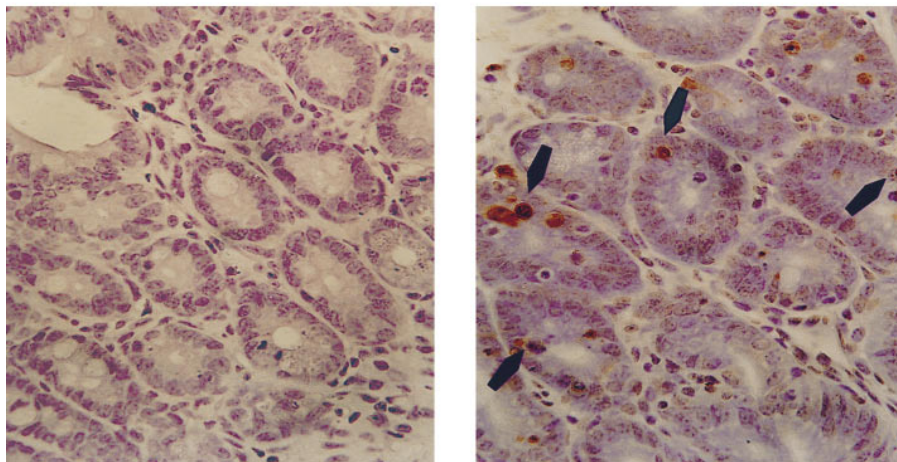
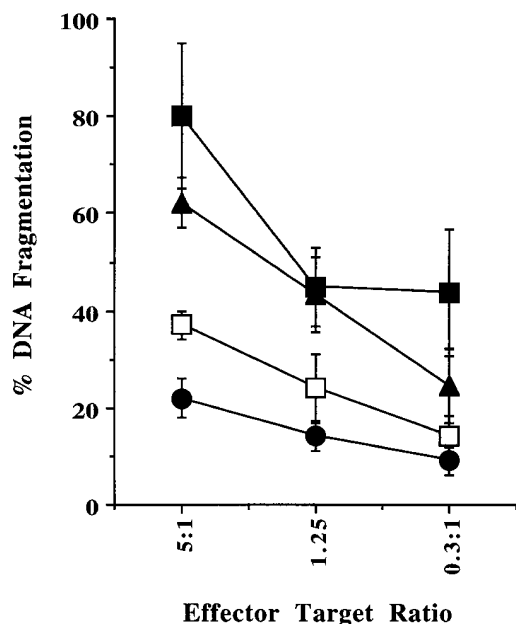


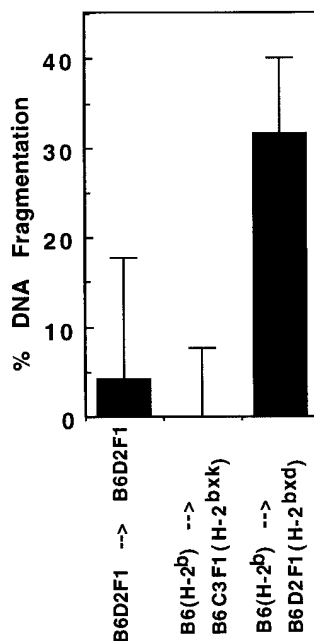
Figure 5. Injection of C57BL/6 spleen cells into B6D2F1 mice results in intestinal GVHD. Small intestine from B6→B6D2F1 GVHD mice was isolated 3 wk after injection and assessed for apoptosis by TUNEL staining. *Arrows*, Apoptotic cells found frequently in GVHD mice but infrequently in control (F1→F1) mice. Results shown are representative of three separate experiments.



**Figure 6.** IEL from GVHD mice have increased FasL-mediated killing activity. IEL isolated from control (F1→F1) mice (open squares) and mice (B6→F1) at week 1 (filled circles), week 2 (filled triangles), and week 3 (filled squares) after the induction of GVHD were cultured with plate-bound anti-CD3 and <sup>3</sup>H-labeled Jurkat cells. IEL were pooled from two mice, and results shown are representative of two independent experiments.

the CD3-stimulated killing of Jurkat cells (data not shown). Overall, these results suggest that donor-derived IEL infiltrating the intestinal epithelium recognize host-derived antigen and mediate the killing of bystander cells presumably through a Fas-mediated mechanism.

Although we observed a heavy infiltration of donor-derived IEL into the host intestinal epithelium during GVHD (Fig. 6, A and B), whether these IEL were capable of inducing host IEC injury directly is uncertain. Because freshly isolated IEC undergo a very high rate of spontaneous death in culture (our unpublished observations), we tested the ability of IEL from B6 (H-2<sup>b</sup>)→B6D2F1 (H-2<sup>bxd</sup>) GVHD mice to recognize and spontaneously kill DBA/2 (host)-derived L1210 and L1210-Fas tumor cells in vitro. L1210 cells express very low levels of Fas, whereas L1210-Fas cells express higher levels and are more sensitive to Fas-induced cell death (23). Fig. 9, A and B, demonstrates that IEL isolated from GVHD mice have rela-



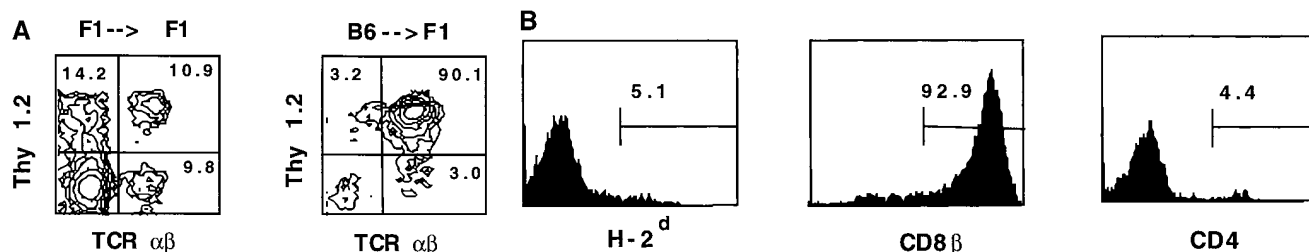
**Figure 8.** IEL from GVHD mice can mediate the killing of bystander Jurkat T cells after stimulation with host-derived L1210 cells. IEL from B6 (H-2<sup>b</sup>)→B6D2F1(H-2<sup>bxd</sup>) mice and B6 (H-2<sup>b</sup>)→B6C3F1(H-2<sup>b</sup>xk) mice were isolated 3 wk after induction of GVHD and cultured with irradiated (10,000 rads) L1210 cells (H-2<sup>d+</sup>) as stimulators and <sup>3</sup>H-labeled Jurkat T cells as target cells at an E/T ratio of 5:1 for 12 h. Bars represent SD.

tively low spontaneous cytotoxicity toward L1210 cells, but they were significantly more cytotoxic toward L1210-Fas cells, suggesting that the killing in vitro of host-derived target cells by IEL involves primarily a Fas-mediated mechanism.

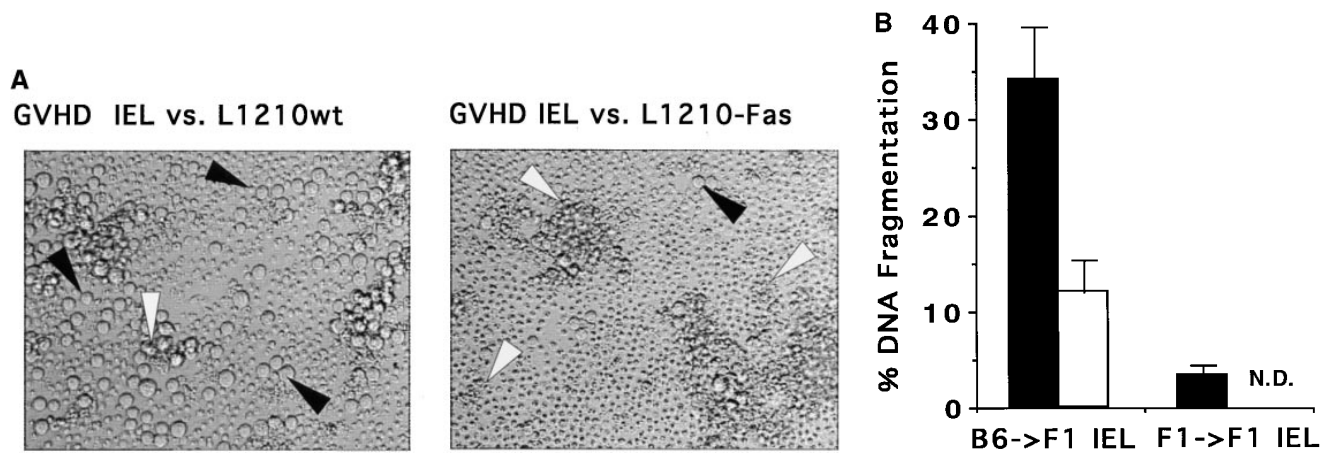
*IEL from GVHD mice induce IEC apoptosis in vivo primarily through a Fas-mediated mechanism.* Thus far, we have demonstrated that IEL isolated from GVHD mice have potent FasL-mediated cytotoxicity toward host-derived target cells in vitro. To test whether this also applies in vivo, we isolated GVHD IEL from B6→B6C3F1 mice 3 wk after induction of GVHD. The IEL were injected into wild-type B6C3F1 and B6C3F1<sup>lpr</sup> mice (the latter have defective Fas expression). Although IEC apoptosis was observed in both cases, a significant decrease in IEC apoptosis was observed in B6C3F1<sup>lpr</sup> mice compared with B6C3F1 mice (Figs. 10 and 11). These results suggest that IEL from GVHD mice induce IEC apoptosis primarily through a Fas-mediated pathway.

## Discussion

Using an assay which had been shown initially to specifically measure FasL-mediated killing of Jurkat cells by activated T



**Figure 7.** FCM of IEL isolated from GVHD mice at week 3 demonstrate that the vast majority of IEL are donor-derived Thy 1<sup>+</sup> TCR αβ<sup>+</sup> CD8β<sup>+</sup>. (A) Two-color FCM analysis demonstrating an increase in TCR αβ IEL in GVHD mice. (B) FCM histogram analysis reveals that the majority of GVHD IEL are of donor origin (H-2<sup>d+</sup>), CD8β<sup>+</sup>, and CD4<sup>+</sup>. Results shown are representative of three independent experiments.

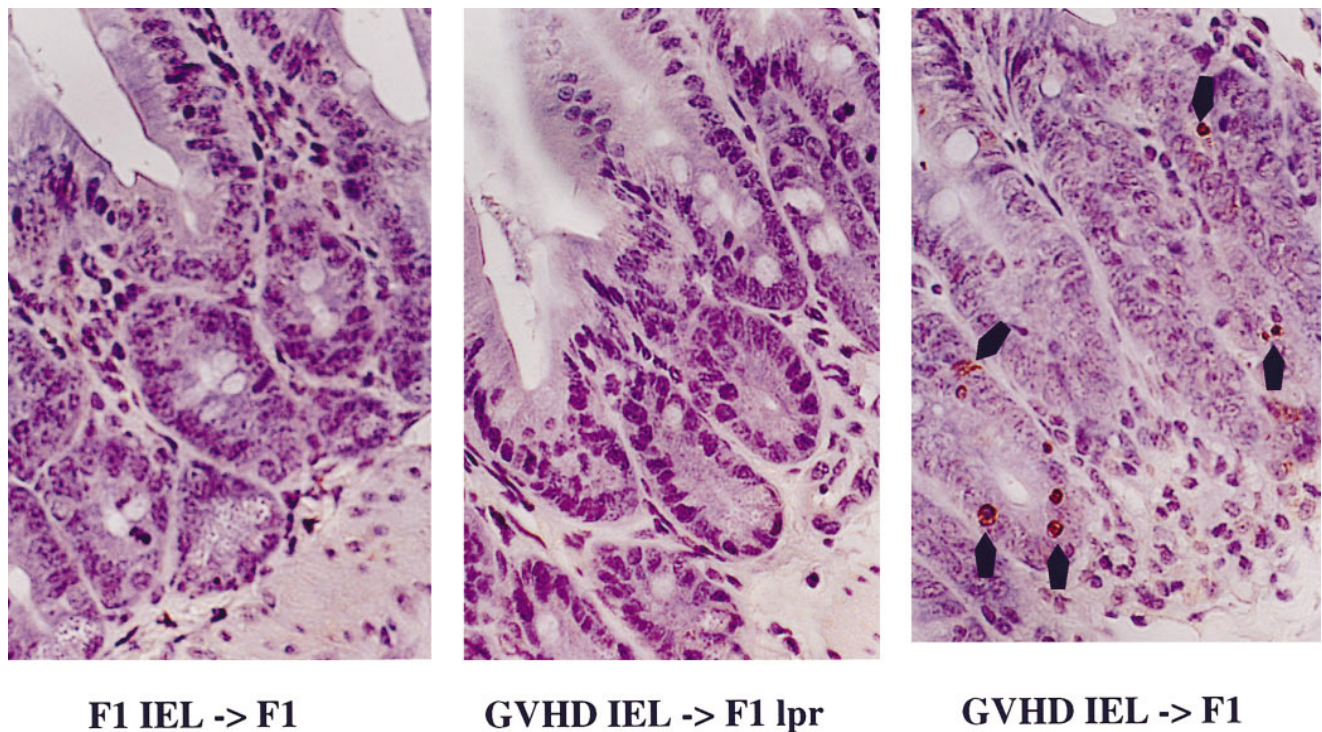


**Figure 9.** IEL from GVHD mice spontaneously kill host-derived target cells predominantly through a Fas-mediated mechanism. Microscopic and DNA fragmentation analysis of apoptosis of L1210 wild-type (*wt*) and L1210-Fas after 16 h of culture with IEL isolated from week 3 GVHD mice. (A) IEL from GVHD mice were cultured at a 10:1 E/T ratio. *Black arrowheads*, Healthy L1210/L1210-Fas cells. *White arrowheads*, Fragmented (apoptotic) cells. Minimal apoptosis of L1210wt and L1210-Fas cells was observed when cultured with IEL isolated from control mice (data not shown). (B) IEL from week 3 GVHD mice were cultured with <sup>3</sup>H-labeled L1210wt (*white bars*) and L1210-Fas target cells (*black bars*) at a 7:1 E/T ratio. In each experiment, IEL were pooled from two mice. *N.D.*, Not done.

cells (27), we have quantified the FasL-mediated killing potential of IEL. Our results demonstrate that unlike spleen and lymph node cells, IEL constitutively possess potent FasL-mediated cytotoxicity (Fig. 1). Furthermore, we have addressed directly the question of whether FasL-mediated cytotoxicity by IEL plays a role in the pathogenesis of intestinal GVHD.

Although several studies have demonstrated previously

that IEL have potent cytotoxic capability (33–35), all of these studies were performed using redirected cytolytic killing assays. The biological significance of this assay is unclear, because it is unlikely that such a mechanism occurs in vivo. In the redirected cytolytic killing assay, the Fc receptor present on the target cell presumably binds to the constant region of an antibody which recognizes a stimulatory molecule (usually



**Figure 10.** IEL from GVHD mice induce intestinal IEC apoptosis in vivo primarily through a Fas-mediated mechanism. IEL isolated from week 3 GVHD mice (B6→B6C3F1) were injected into B6C3F1*lpr* and B6C3F1 wild-type mice. *Arrows*, Apoptotic IEC as identified by TUNEL staining. Results shown are representative of three independent experiments.

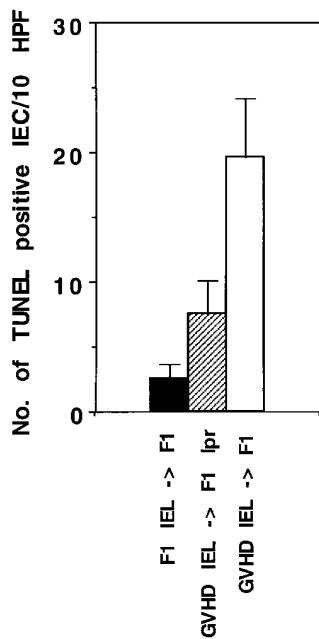


Figure 11. Quantification of IEC apoptosis after in vivo transfer of GVHD IEL (B6→B6C3F1) IEL into B6C3F1 wild-type and B6C3F1*lpr* mice. Mice were examined 2 wk after in vivo transfer. Results shown are the average number of TUNEL-positive cells (see Fig. 10) seen per 10 high power fields (HPF,  $\times 40$ ) from three separate experiments.

anti-TCR) present on cytotoxic T cells. Thus, the antibody-target cell complex serves as a template for the stimulation of cytotoxic effector cells, resulting in the killing of the target cell. The exact mechanism by which effector cells kill target cells in this assay is unclear. It is likely that both perforin- and Fas-mediated mechanisms are involved, because IEL isolated from either *gld* mice or perforin-deficient mice have both been shown to possess redirected cytotoxicity (34).

Two previous studies have addressed the role of FasL-mediated killing by IEL (34, 36). Guy-Grand et al. demonstrated that IEL from both *gld* mice (defective FasL) and perforin-deficient mice are capable of redirected cytolytic killing (34). However, it is unclear from their study whether other cytotoxic mediators, such as TNF, can also play a role. Furthermore, from their results it is difficult to extrapolate the relative importance of FasL-mediated killing in the IEL of normal mice, because the lack of perforin in the perforin-knockout mice may result in a compensatory upregulation of FasL-mediated killing. In the second study, Gelfanov et al. concluded that both TCR  $\alpha\beta$  CD8 $\alpha\beta$  and TCR  $\alpha\beta$  CD8 $\alpha\alpha$  IEL have FasL-mediated killing activity (36). However, in their study, IEL were stimulated extensively in vitro for 9 d with anti-TCR  $\alpha\beta$ , anti-CD28, and IL-2, followed by a 3-h incubation with PMA and A23187. Hence, it is unclear whether FasL-mediated killing is a constitutive function present in most freshly isolated IEL or a function acquired by a small subset of IEL after extensive stimulation and expansion in vitro. In the same study, lymph node cells stimulated identically were also capable of FasL-mediated killing. We have also observed that spleen cells treated in vitro with PMA and ionomycin or concanamycin A over several days were also capable of FasL-mediated killing (our unpublished observations). Finally, Gelfanov et al. demonstrated that the killing of Fas-positive target cells by IEL was calcium independent and concluded that the killing that they observed was Fas-mediated (36). In contrast, with the use of Fas-Fc, we have demonstrated specifically that IEL are potent FasL-mediated killers.

Our results suggest that both TCR  $\gamma\delta$  and TCR  $\alpha\beta$  IEL are readily capable of FasL-mediated killing. To our knowledge, this is the first report demonstrating that TCR  $\gamma\delta$  IEL are capable of FasL-mediated killing. Although we cannot rule out the possibility that the in vitro stimulation of TCR  $\gamma\delta$  IEL with anti-TCR  $\delta$  mAb (GL-3) resulted in the indirect stimulation of TCR  $\alpha\beta$  IEL or vice versa, we feel that this is unlikely during the short culture period we used. The sum of FasL-mediated killing by IEL when stimulated separately with anti-TCR  $\delta$  and anti-TCR  $\beta$  was approximately equivalent to FasL-mediated killing by anti-CD3 alone (Fig. 3 B).

At week 3 of GVHD, host IEL are nearly entirely replaced by donor-derived Thy 1<sup>+</sup> TCR  $\alpha\beta$  CD8 $\alpha\beta$  IEL (Fig. 7). Thus, our results suggest that these infiltrating IEL are involved in the pathogenesis of intestinal GVHD. However, it remains possible that the small numbers of CD4<sup>+</sup> or Thy 1<sup>-</sup> donor-derived IEL or even host-derived IEL are also involved in the pathogenesis of GVHD. In the same GVHD model (B6→B6D2F1), Sakai et al. implicated a role for host TCR  $\gamma\delta$  IEL in the pathogenesis of intestinal GVHD (15). In their study, injection of depleting anti-TCR  $\gamma\delta$  mAb into host F1 mice before induction of GVHD with B6 spleen cells ameliorated significantly the severity of intestinal GVHD. Our observation that host TCR  $\gamma\delta$  IEL are capable of FasL-mediated cytotoxicity is consistent with their finding.

Our in vitro studies suggest that donor-derived IEL can induce Fas-mediated IEC death during GVHD through both a direct and an indirect mechanism. In the first mechanism, donor-derived IEL recognize host antigen present on IEC, and mediate directly the killing of the IEC. This is suggested by the spontaneous killing of L1210-Fas target cells by IEL from GVHD mice (Fig. 9). In the second mechanism, donor-derived IEL during GVHD recognize host antigen present on IEC, but instead induce Fas-mediated death upon neighboring or bystander Fas-positive IEC. This is suggested by our observation that L1210 cells can stimulate IEL from GVHD mice to mediate the killing of bystander Jurkat cells (Fig. 8).

Although our results suggest that FasL-mediated killing by IEL is involved in the pathogenesis of intestinal GVHD, we cannot rule out the possibility that IEL can also participate in the pathogenesis of intestinal GVHD through other mechanisms involving perforin or TNF. Compared to wild-type F1 mice, *lpr* F1 mice were relatively resistant to IEC apoptosis after in vivo transfer of IEL isolated from GVHD mice; however, this resistance was not complete (Fig. 11), suggesting that other mediators of IEC apoptosis are involved. IEL have been shown to be capable of perforin-mediated killing (34, 36). In addition, TNF has been implicated recently in early intestinal GVHD (37), but we are not aware of any studies demonstrating that IEL are capable of producing TNF.

Because of the proximity of IEL and IEC, our observations that IEL are potent FasL-mediated killers and that IEC normally express Fas suggest that interactions between FasL on IEL and Fas on IEC occur normally. Abreu-Martin et al. demonstrated that engagement of Fas on HT-29 colon cancer epithelial cells did not result in apoptosis, but instead resulted in IL-8 production (31). However, in the presence of IFN- $\gamma$ , HT-29 cells did become susceptible to Fas-mediated death. Thus, in some respects, intestinal GVHD can be perceived as an upregulation and a deviation from the natural interaction between FasL on IEL and Fas on IEC. These findings raise the question of whether FasL-mediated killing of IEC by IEL is a

mechanism in the pathogenesis of other intestinal diseases where total IEL numbers are also increased, such as celiac disease or milk and soy protein enteropathy (38, 39).

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