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### Research Article

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# Functional Expression of Fas and Fas Ligand on Human Gut Lamina Propria T Lymphocytes

## A Potential Role for the Acidic Sphingomyelinase Pathway in Normal Immunoregulation

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### Abstract

The expression and function of Fas (CD95/APO-1), a cell surface receptor directly responsible for triggering cell death by apoptosis, was investigated on human T lymphocytes resident within the intestinal lamina propria, a major site of antigen challenge and persistent lymphocyte activation. Three color immunofluorescence and FACS analysis indicated that virtually all freshly isolated human gut lamina propria T lymphocytes (T-LPL) express Fas, together with the marker of pregress activation CD45R0. A discrete fraction of freshly isolated T-LPL also constitutively expressed Fas ligand (FasL), perhaps as a result of recent in vivo activation. Importantly, whereas Fas cross-linking did not result in apoptosis induction in peripheral blood T lymphocytes (T-PBL), Fas was found to be fully effective in generating the apoptotic signal in T-LPL. This was associated with the activation of an acidic sphingomyelinase and with ceramide generation, early events known to be involved in Fas-mediated apoptotic signaling. By contrast, acidic sphingomyelinase activation and ceramide production were not detectable in T-PBL after Fas cross-linking. However C<sub>2</sub>-ceramide, a cell permeant synthetic analog of ceramide, could efficiently induce apoptosis in T-LPL and T-PBL when added exogenously. These data indicate that T-LPL constitutively express both Fas and FasL and that Fas cross-linking generates signals resulting in sphingomyelin hydrolysis and apoptosis, outlining a potential mechanism involved in intestinal tolerance. Moreover, they provide the first evidence of a role for ceramide-mediated pathways in normal immunoregulation. (*J. Clin. Invest.* 1996, 97:316–322.) Key words: Fas/APO-1 • CD95 • acidic sphingomyelinase • T-LPL • gut mucosa

### Introduction

Intestinal lamina propria-associated lymphocytes constitute a major lymphoid compartment, continuously exposed to heavy antigen challenge in vivo (1, 2). Persistent antigen-induced lymphocyte activation does not normally result in inflammatory tissue damage, suggesting the existence of mechanisms responsible for regulating local immune responses. These may include activation-induced hyporesponsiveness to T cell receptor/CD3 stimulation in T cells (3, 4), and the release of still unidentified soluble suppressive factors (5). Cell death by apoptosis may also effectively contribute to the regulation of T cell activation in vivo, by limiting clonal expansion during secondary immune responses and preventing the activation of clones potentially crossreactive with self determinants (6). Indeed, the generation of apoptotic signals within the intestinal environment is strongly suggested by the observation that a discrete proportion of freshly isolated intestinal T lymphocytes spontaneously die in vitro with an apoptotic phenotype (7).

A central pathway leading to cellular apoptosis in lymphocytes is initiated by Fas, a ~ 50 kD widely distributed surface receptor belonging to the TNF receptor family (8, 9). In T lymphocytes, Fas is preferentially expressed late following cellular activation (10) and its cross-linking results in rapid triggering of apoptotic programs (11). Fas cross-linking at the cell surface is mediated by Fas ligand (FasL)<sup>1</sup> (12) expressed transiently soon after activation by T lymphocytes (13). Fas/FasL interactions are therefore intimately related to the process of lymphocyte activation, and perhaps crucial for its control and termination by mediating apoptotic cell death of activated lymphocytes (14, 15).

The biochemical events which lead the signal from Fas to the nucleus are still largely unknown. It has been shown that Fas cross-linking activates an acidic sphingomyelinase (aSMase) (16) and that a ceramide-mediated pathway may be responsible for Fas-dependent apoptosis induction in tumor cell lines (17). Fas expression and function appears crucial for preventing abnormal proliferation of lymphocytes and activation of autoreactive T cell clones in the periphery, as suggested by the evidence that mice genetically defective for Fas, develop generalized lymphoaccumulation and autoimmune diseases, due to impaired peripheral clonal deletion (18, 19). Since control of lymphocyte activation and proliferation is especially crucial at

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1. *Abbreviations used in this paper:* FasL, Fas ligand; LPL, lamina propria lymphocytes; PBL, peripheral blood lymphocytes; T-LPL, lamina propria T-lymphocytes; T-PBL, peripheral blood T-lymphocytes.

sites of continuous antigen challenge, we investigated the expression and possible function of Fas in mediating apoptosis through the ceramide-mediated pathway, at the level of intestinal lamina propria, a primary site of antigen exposure and persistent T lymphocyte activation.

## Methods

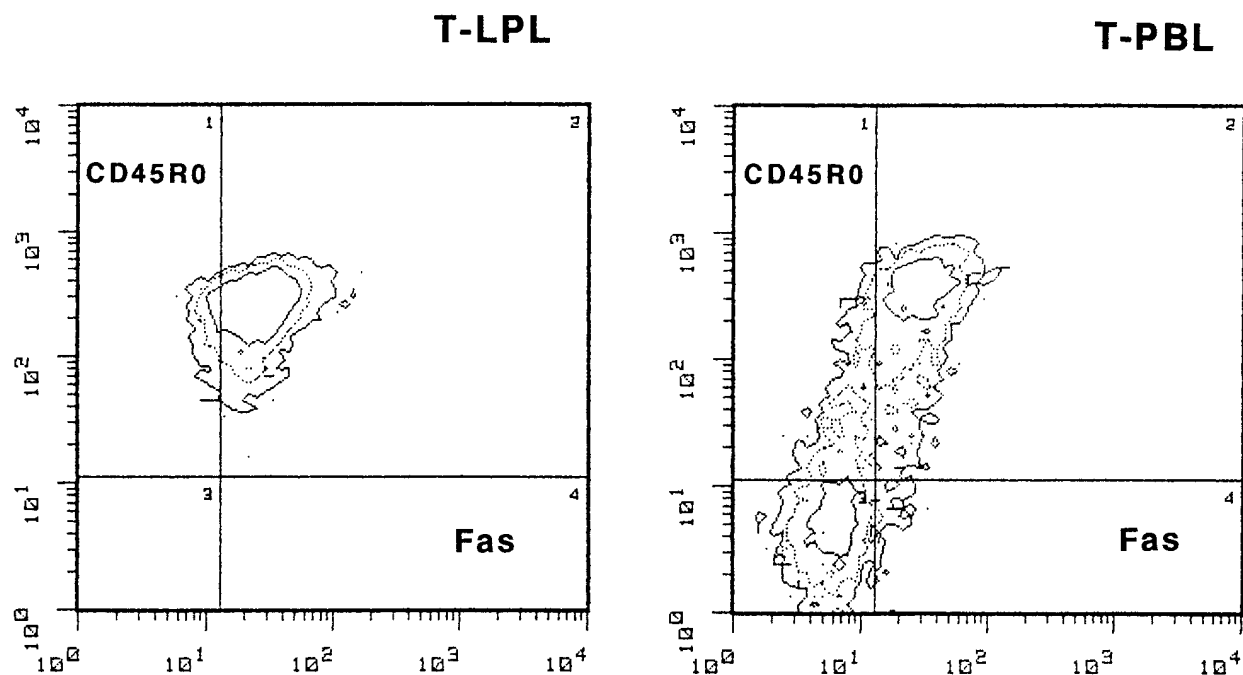
**Cells.** Mucosal samples, obtained from the macroscopically and microscopically uninvolved areas of resected carcinoma specimens, were used as a source of lamina propria lymphocytes (LPL). None of these samples showed evidence of histological abnormalities. The colonic mucosa was dissected within one hour of resection. LPL were isolated as previously described (20). Peripheral blood lymphocytes (PBL) were isolated by standard Ficoll-Hypaque density gradient centrifugation and passed through nylon wool columns to remove B lymphocytes and a portion of monocytes. Purified CD3<sup>+</sup>Fas<sup>+</sup> lymphocytes were obtained by negative immunomagnetic selection. PBL or LPL were labeled with saturating concentrations of anti-Leu18 (anti-CD45RA; Becton Dickinson, San Jose, CA), washed, and incubated twice with an appropriate number of sheep anti-mouse IgG-coupled beads (Dynal, Warral Merseyside, UK). CD45RA<sup>+</sup> cells bound to Ab-coated beads were then recovered by a magnetic device and discarded. Unbound cells were routinely 93–97% CD3<sup>+</sup>Fas<sup>+</sup>. For functional studies cells were cultured in RPMI 1640 medium (GIBCO, Chagrin Falls, OH), supplemented with 5% fetal calf serum, 1 mM glutamine and antibiotics in a humidified 5% CO<sub>2</sub> atmosphere.

**Immunofluorescence and flow cytometry analysis.** Freshly isolated LPL and PBL were washed twice in PBS containing 0.1% sodium azide and incubated at 4°C for 30 min with optimal amounts of purified DX2 mAb (anti-Fas IgG1; kindly provided by Dr. Lewis L. Lanier, DNAX Research Institute, Palo Alto CA), washed, and treated with FITC-conjugated goat anti-mouse IgG Abs (Becton Dickinson). After a further washing, cells were incubated for 10 min with 6% normal mouse serum. Alternatively, cells were incubated at 4°C for 30 min with 10 µg/ml of anti-Fas Ligand purified rabbit anti-

serum PE62 (21), or with a similar amount of anti-Sos purified rabbit antiserum as a control, washed and treated with FITC-conjugated goat anti-rabbit Ig Abs (Zymed, South San Francisco, CA). After a further washing, cells were incubated for 10 min with 6% normal mouse serum. Cells were then treated at a density of 10<sup>7</sup> cells/ml in a total volume of 100 µl with saturating concentrations of directly conjugated phycoerythrin (PE) anti-Leu45R0 (anti-CD45R0, IgG1), or PE anti-Leu5 (anti-CD2, IgG1), and peridinin chlorophyll protein (Per-CP) anti-Leu4 (anti-CD3, IgG1), or isotype matched normal, Per-CP, and PE Ab (Becton Dickinson). Cells were then washed twice in cold PBS/azide and resuspended at a density of 10<sup>6</sup> cells/ml for three-color cytofluorimetric analysis. Relative fluorescent intensities of individual cells were analyzed using a FACScan flow cytometer (Becton Dickinson). Correlated five parameters data were collected into list mode data files by using a FACScan research software and converted for analysis in Consort 30 data files.

**DNA labeling and evaluation of apoptotic cells.** Purified CD3<sup>+</sup>Fas<sup>+</sup> lamina propria and peripheral T cells were incubated in 24-well cell culture plates (Costar, Cambridge, MA) at 10<sup>6</sup>/ml in complete medium in the presence of different stimuli. Anti-Fas mAb (clone CH-11), was from UBI (Lake Placid, NY). C<sub>2</sub>-ceramide (N-acetyl-D-dihydrospingosine) were from Biomol (Plymouth Meeting, PA), and were used at a range of 3–100 µM. After different times of incubation, cells were recovered and washed in PBS, and processed for apoptotic cell detection as previously shown (16). Briefly, cells were resuspended in hypotonic fluorochrome solution (Propidium iodide 50 µg/ml (Sigma Chemical Co., St. Louis, MO) in 0.1% sodium citrate plus 0.1% Triton X-100), kept 4–8 h at 4°C in the dark, and analyzed by a FACScan cytofluorimeter. Percentage of apoptotic cells was determined by evaluating hypodiploid nuclei.

**Ceramide mass measurement (diacylglycerol kinase assay).** After stimulation, lipids were extracted and then incubated with *Escherichia coli* diacylglycerol kinase (22). Ceramide phosphate was then isolated by TLC using CHCl<sub>3</sub>/CH<sub>3</sub>OH/CH<sub>3</sub>COOH (65/15/5, vol/vol/vol) as solvent. Authentic ceramide-1-phosphate was identified by autoradiography at Rf 0.25. Quantitative results for ceramide production are expressed as pmoles ceramide-1-phosphate/10<sup>6</sup> cells.



**Figure 1.** Expression of Fas. Simultaneous expression of CD45R0 and Fas in electronically gated CD3<sup>+</sup> T-LPL and CD3<sup>+</sup> T-PBL by three-color immunofluorescence and FACS analysis is shown. > 98% isotype-matched control mAb treated cells (not shown) were included in the lower left quadrants. Data from one representative donor out of nine studied are shown.

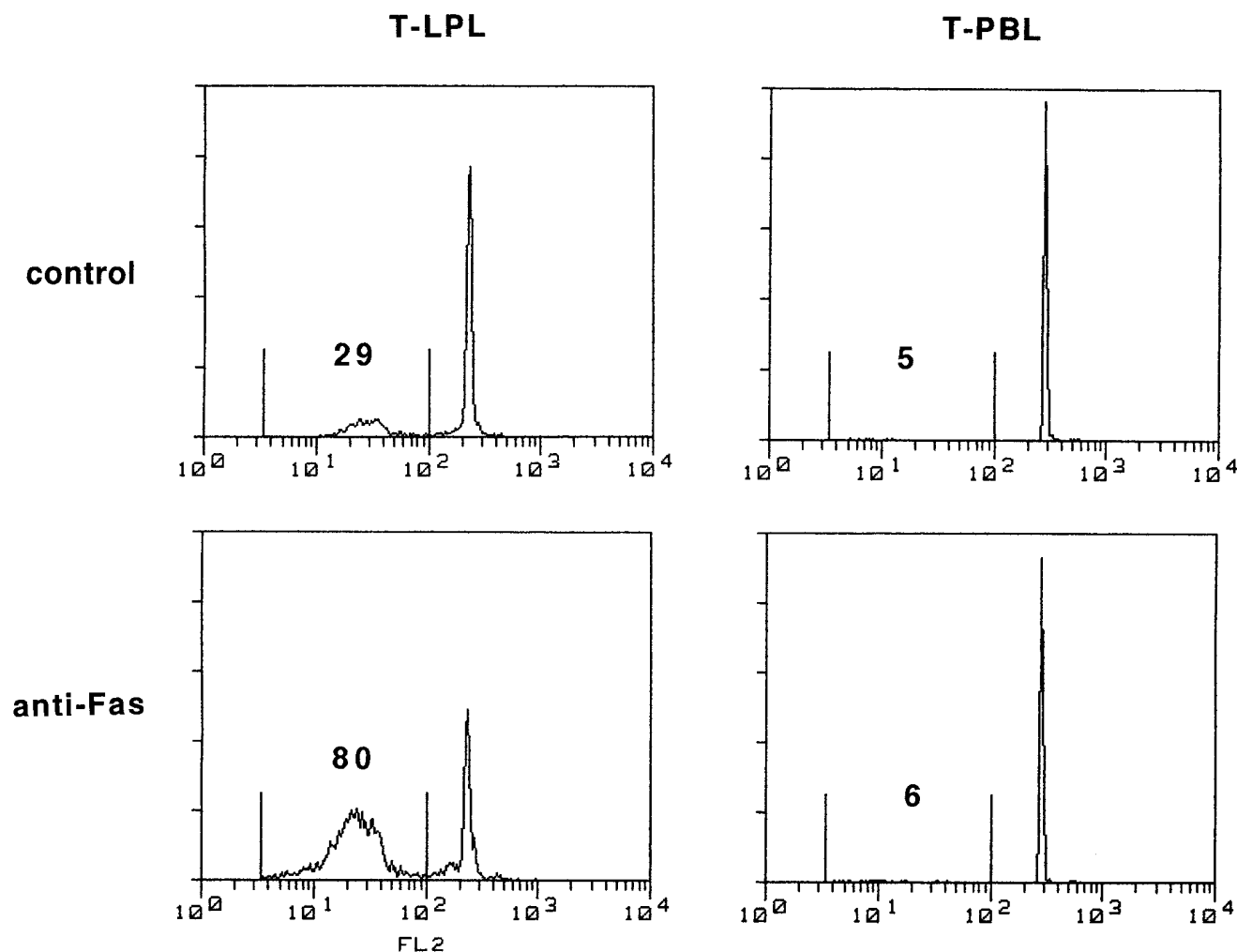
**Acidic sphingomyelinase assay.** For in vitro aSMase assay the cells were treated with anti-Fas mAb at 37°C for the indicated times, washed and then resuspended in Triton X-100 0.2% (23). Cells were lysed by sonication with a cell sonifier (Vibracell; Sonic & Materials Inc., Danbury, CT). Protein concentrations were determined using a Bio-Rad Protein Assay (Bio-Rad Co., Richmond, CA). 100  $\mu$ l of the whole cell lysate were added to 200  $\mu$ l reaction buffer containing the substrate [*N*-methyl-<sup>14</sup>C]SM (0.2  $\mu$ Ci/ml, specific activity 56.6 mCi/mmol), 250 mM sodium acetate (pH 5.0) and 1 mM EDTA. After incubation for 2 h at 37°C, the reaction was stopped by the addition of 250  $\mu$ l of CHCl<sub>3</sub>/CH<sub>3</sub>OH/CH<sub>3</sub>COOH (4:2:1). Phospholipids were extracted, analyzed on TLC plate (16) and [<sup>14</sup>C]SM hydrolysis quantitated by autoradiography and liquid scintillation. aSMase activation was expressed as pmoles of SM hydrolyzed/10<sup>6</sup> cells.

## Results

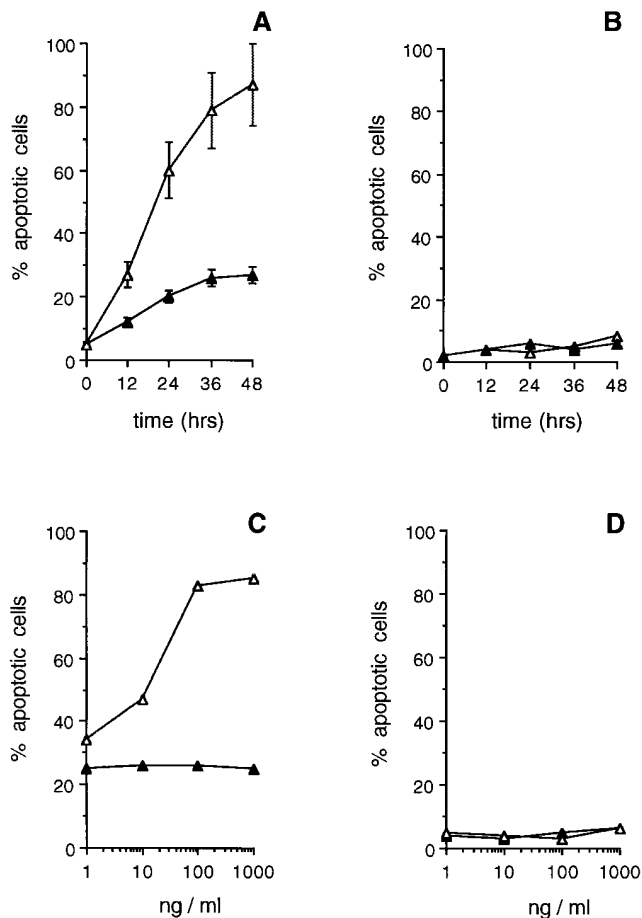
**T-LPL express Fas.** The expression of Fas was analyzed on T-LPL together with the expression of CD45R0, the low molecular weight isoform of CD45 preferentially expressed by lymphocytes which have undergone previous antigen-induced cell proliferation. Essentially all T-LPL express CD45R0 (24), as they have undergone cell division in specialized gut-associ-

ated areas, following antigen exposure, before setting within the lamina propria (25, 26). Freshly isolated LPL therefore were reacted with a combination of anti-CD3, anti-CD45R0 and anti-Fas mAbs, then analyzed by three color FACS analysis. A similar procedure was performed on peripheral blood lymphocytes (PBL), for comparison. CD3<sup>+</sup> lymphocytes were electronically gated to isolate T-LPL and T-PBL, and the correlated expression of CD45R0 and Fas analyzed. Fig. 1 shows that most T-LPL coexpressed Fas together with CD45R0. In contrast, only 40–50% of T-PBL expressed Fas, those coexpressing also CD45R0 (10).

**Fas is functional on T-LPL.** We therefore investigated whether Fas is effective in inducing apoptosis of T-LPL. Purified populations of CD3<sup>+</sup> Fas<sup>+</sup> LPL and PBL were prepared by immunomagnetic cell separation, and Fas cross-linked by mAbs. Apoptotic cells were quantitated by propidium iodide staining of nuclei at different time points. As shown in Fig. 2, stimulation of Fas fully triggered apoptosis of T-LPL, but not of T-PBL. Figs. 3, A and C show the kinetics and dose-response of anti-Fas-induced apoptosis in T-LPL. A discrete percentage of T-LPL underwent “spontaneous” apoptosis within 36–48 h after isolation and seeding in control cultures (Fig. 3 A). These



**Figure 2.** Apoptosis induction in T-LPL and T-PBL after Fas cross-linking. Immunomagnetically purified CD3<sup>+</sup> Fas<sup>+</sup> T-LPL and T-PBL were cultured in the presence of 200 ng/ml anti-Fas mAb, or control mAb, then assayed for apoptotic nuclei after 48 h. Percentage of apoptotic nuclei, contained between markers, is shown by numbers. Data from one representative donor out of seven are shown.



**Figure 3.** Kinetics and dose response of anti-Fas-induced apoptosis. Immunomagnetically purified CD3<sup>+</sup>Fas<sup>+</sup> T-LPL (A) and T-PBL (B) were cultured in the presence of 200 ng/ml anti-Fas mAb ( $\Delta$ ) or control mAb ( $\blacktriangle$ ), then assayed for apoptotic nuclei at different time points. Means and SD from five different donors are shown. Immunomagnetically purified CD3<sup>+</sup>Fas<sup>+</sup> T-LPL (C) and T-PBL (D) were treated with different concentrations of anti-Fas mAb ( $\Delta$ ) or control mAb ( $\blacktriangle$ ), then assayed for apoptotic nuclei at 24 h. One representative experiment out of three performed is shown.

“spontaneous” apoptotic cells never exceeded 25–30% even after longer culture periods (not shown). On the contrary, Fas cross-linking never resulted in apoptosis of T-PBL over a wide range of antibody concentrations (Fig. 3 D), nor T-PBL underwent “spontaneous” apoptosis (Fig. 3 B). Spontaneous apoptosis of T-LPL is not the result of the isolation procedure, as similar treatments do not induce apoptosis of T-PBL (not shown). These data indicated that, although expressed on both cell populations, Fas is functional in inducing apoptosis on T-LPL, but not on T-PBL.

**T-LPL express FasL.** Spontaneous apoptosis *in vitro* suggestive of a commitment to undergo apoptosis following a signal received *in vivo*, perhaps through Fas/FasL interactions. The possibility that some T-LPL were also expressing FasL was therefore investigated. Simultaneous staining for CD3, CD2 and FasL, followed by three color FACS analysis of freshly isolated LPL revealed that a discrete proportion of T-LPL, ranging from 10 to 20%, constitutively express FasL (Fig. 4 A), likely as a result of a recent *in vivo* activation (4). By contrast, no FasL expression could be detected in resting

T-PBL (Fig. 4 B). The expression of FasL on T-LPL and their sensitivity to Fas crosslinking suggest that Fas/FasL interactions may be operative *in vivo* in mediating apoptosis of T-LPL.

**Fas cross-linking activates sphingomyelin breakdown in T-LPL.** Cross-linking of Fas triggers sphingomyelin hydrolysis, with generation of ceramides, in several tumor cell lines (16). When released intracellularly ceramide is directly responsible for activating a number of kinases and phosphatases, eventually leading to triggering of apoptotic programs (27, 28). Fas-induced sphingomyelin breakdown was therefore investigated in T-LPL and T-PBL. Ceramide generation *in vivo*, as detected by the diacylglycerol kinase assay, was detected following Fas cross-linking in T-LPL (Fig. 5 A), while substantial ceramide production was not observed in Fas<sup>+</sup> T-PBL after Fas stimulation (Fig. 5 B). Moreover, direct measurement of enzymatic activity *in vitro* showed strong activation of an acidic sphingomyelinase in T-LPL, but not in T-PBL, after Fas cross-linking (Fig. 6), suggesting that sphingomyelin breakdown with ceramide release could be responsible for mediating the apoptotic pathway in T-LPL.

**T-LPL are sensitive to ceramide.** To assess the sensitivity of T-LPL to sphingomyelinase-generated ceramide in apoptosis induction, T-LPL were treated with different concentrations of C<sub>2</sub>-ceramide, a cell-permeant synthetic short acyl chain ceramide which mimics the apoptotic effects of natural ceramides (29). As shown in Fig. 7 A, C<sub>2</sub>-ceramide was able to induce massive apoptosis in T-LPL within 24 h, indicating that sphingomyelin hydrolysis can trigger an apoptotic program in T-LPL. On the contrary, C<sub>2</sub>-dihydroceramide, a very closely related synthetic analog, which lacks the double hydrogen bond between positions 4 and 5 in the sphingosine chain, was ineffective. Interestingly, C<sub>2</sub>-ceramide could trigger apoptosis in T-PBL as well (Fig. 7 B), suggesting that the apoptotic machinery downstream ceramide production is functional in T-PBL, and that the inability to activate the acidic sphingomyelinase pathway may account for resistance to Fas cross-linking.

## Discussion

Here we show that Fas (CD95/APO-1) is expressed on all intestinal T-LPL, and that its cross-linking triggers sphingomyelin breakdown with ceramide generation, through an acidic sphingomyelinase, eventually resulting in apoptotic cell death. As a discrete fraction of T-LPL was found to constitutively express FasL, Fas–FasL interactions could be relevant in the homeostasis of the gut mucosa-associated lymphoid compartment.

Accumulating evidence suggests that Fas may play an important role in regulating the lifespan of activated lymphocytes (30, 31). Fas is induced late during lymphocyte activation, and signaling through Fas triggers apoptosis in lymphocytes which have undergone recent antigen-induced activation and proliferation (11). Cell death by apoptosis is most likely involved in both the regulation of the lymphocyte population size and the termination of the immune response at sites of antigen exposure and inflammation, perhaps also preventing the prolonged activation and clonal expansion of potentially self-reactive lymphocytes (6, 30). Fas-mediated apoptosis induction is therefore expected to significantly contribute to limitation of clonal proliferation and to the regulation of lymphocyte number and turnover at specialized lymphoid areas, particularly those con-

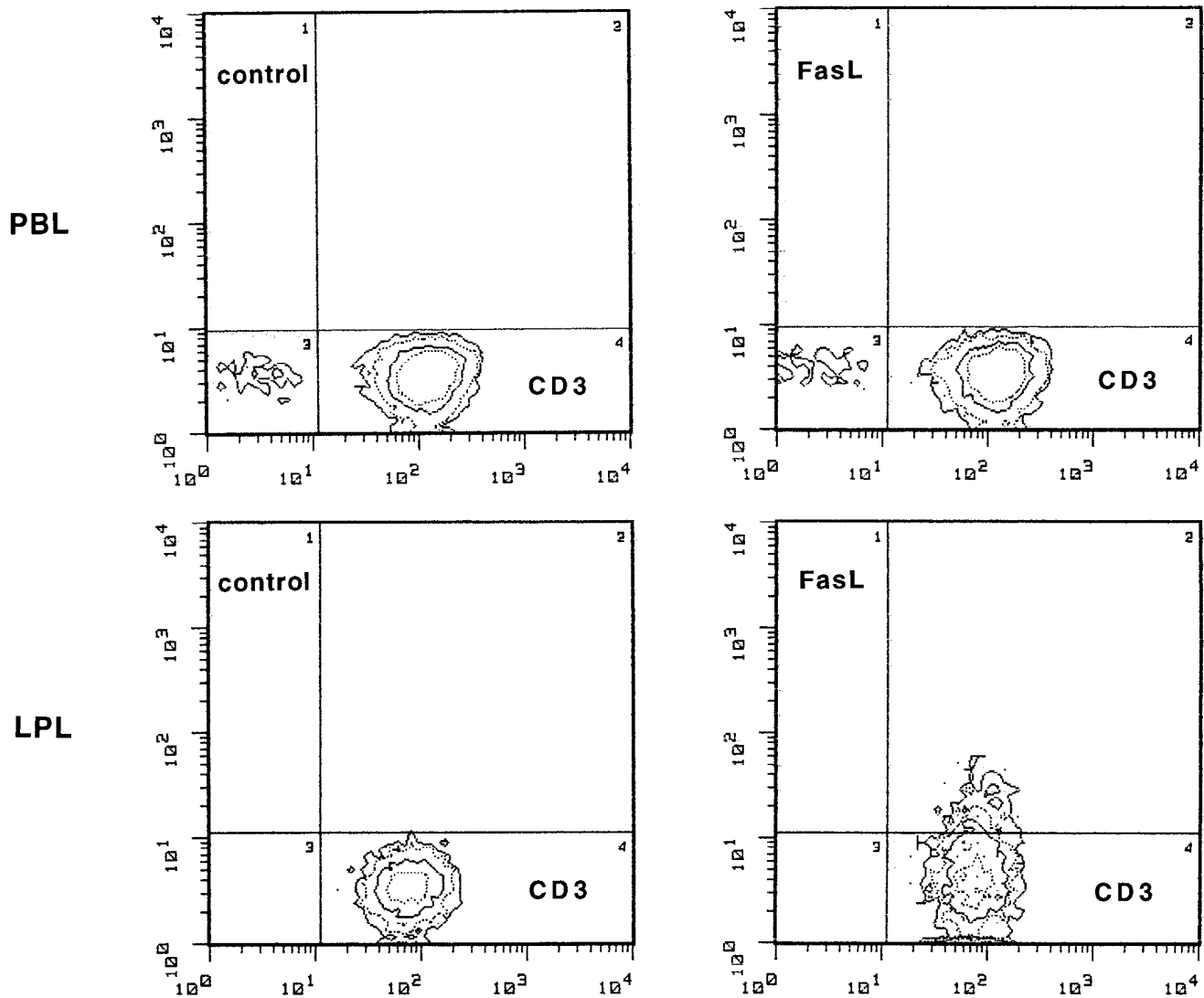


Figure 4. Expression of FasL. Simultaneous expression of CD3 and FasL in electronically gated CD2<sup>+</sup> T-LPL and CD2<sup>+</sup> T-PBL, by three-color immunofluorescence and FACS analysis is shown. Data from one representative donor out of four studied are shown.

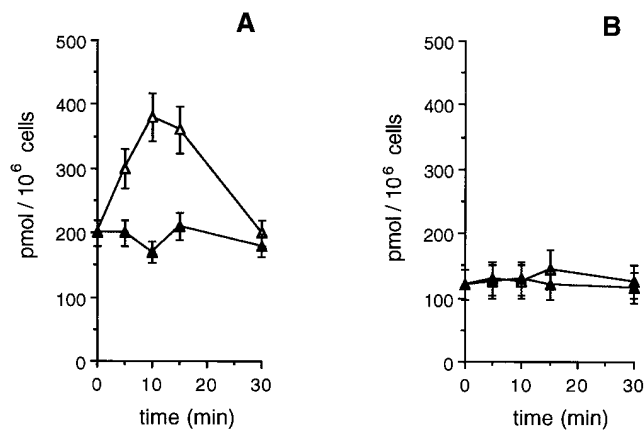
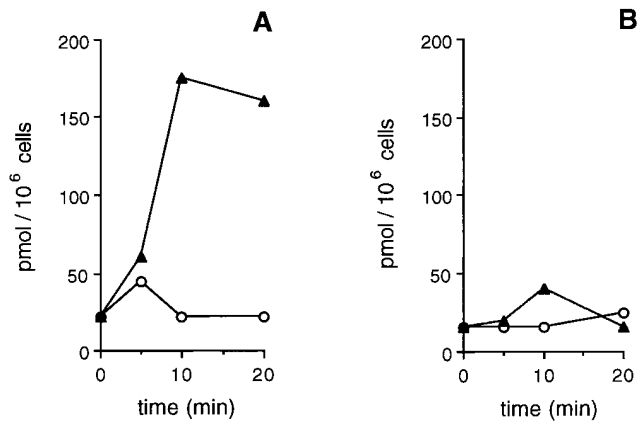


Figure 5. Ceramide generation after Fas cross-linking. Immunomagnetically purified CD3<sup>+</sup>Fas<sup>+</sup> T-LPL (A) and T-PBL (B) were cultured for the indicated times in the presence of 200 ng/ml anti-Fas mAb (△) or control mAb (▲), then assayed for endogenous ceramide generation at different time points. Means and SD from five different donors are shown.

tinuously exposed to antigen challenge (32). We examined the expression and function of Fas in T lymphocytes resident in the lamina propria (T-LPL), as T-LPL constitutively express receptors associated with both very early and late stages of cellular activation, like CD69 and CD45R0 respectively, suggesting that they are continuously responding to antigenic stimulation (4). As we have shown, virtually all T-LPL express Fas and a discrete fraction of them also express FasL. Importantly, unlike Fas<sup>+</sup> T-PBL, T-LPL undergo apoptosis after *in vitro* Fas cross-linking.

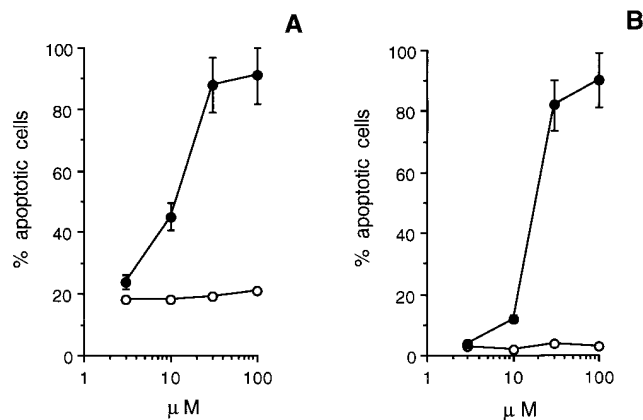
After antigen priming and cellular proliferation in mucosa-associated lymphoid follicles, T lymphocytes re-enter the intestinal mucosa from peripheral blood as CD45R0<sup>+</sup> cells (25, 26). As CD45R0 and Fas expressions appear associated, it is likely that T cells which gain access to the mucosa already express also Fas. Virtually all T-LPL are potentially susceptible to Fas-mediated cell death, as our *in vitro* evidence indicates. However, massive Fas-induced cell death is unlikely to happen *in vivo*, as *in situ* lifespan of T-LPL seems to be long and recruitment from peripheral blood, under normal conditions, minimal. Different factors may account for *in vivo* protection



**Figure 6.** Activation of acidic sphingomyelinase after Fas cross-linking. Immunomagnetically purified CD3<sup>+</sup>Fas<sup>+</sup> T-LPL (A) and T-PBL (B) were cultured for the indicated times in the presence of 200 ng/ml anti-Fas mAb (▲) or control mAb (○), then cell lysates were incubated with labeled sphingomyelin vesicles as indicated in Methods. After reaction, phospholipids were extracted, separated by TLC, and visualized by autoradiography. Relevant spots were scraped from the plate and counted by liquid scintillation. Two different donors gave similar results.

of T-LPL from Fas-induced cell death, including signals generated by cell-cell interactions through other receptors, or by soluble factors available in the mucosal microenvironment. It is in fact possible that the withdrawal of these protective signals from T-LPL previously stimulated in vivo through Fas accounts for their undergoing “spontaneous” apoptosis in vitro.

The other limiting factor in vivo is the expression of FasL (12). Little is known at the moment about FasL induction requirements and kinetics in human lymphocytes. FasL is induced very rapidly on the surface of TCR/CD3-stimulated T cells, and its cell membrane half-life is extremely short, being no more detectable a few hours after stimulation (13). This would result from both transient induction and proteolytic cleavage and shedding from the cellular membrane. Our finding that a relatively small fraction of T-LPL is found to express



**Figure 7.** Sensitivity to exogenous ceramide. Immunomagnetically purified CD3<sup>+</sup>Fas<sup>+</sup> T-LPL (A) and CD3<sup>+</sup>Fas<sup>+</sup> T-PBL (B) were cultured in the presence of different concentrations of C<sub>2</sub>-ceramide (○) or C<sub>2</sub>-dihydroceramide (●). After 24 h cells were collected and assayed for apoptosis induction. Means and SD from four different donors are shown.

FasL is consistent with both transient induction and the observation that the vast majority of T-LPL is actually refractory to TCR/CD3 stimulation at any given time (3, 4). Nevertheless, TCR/CD3-stimulated T cells transiently expressing the FasL could kill themselves and/or kill nearby Fas-expressing T cells (13, 14, 32, 33). Considering that FasL can potentially interact with several T-LPL in the close surroundings, all of which expressing Fas and susceptible to Fas-induced cell death, it is reasonable that a discrete fraction of T-LPL, receive an apoptotic signal in vivo. These cells can be then observed to “spontaneously” undergo apoptosis in vitro, where protective factors are absent and removal of apoptotic cells by macrophages does not occur. Fas/FasL interactions are therefore likely to play an important role in constraining both antigen-induced and bystander T cell activation in vivo.

Our data indicate, moreover, that the sphingomyelin breakdown pathway can play a role in normal immunoregulation. Sphingomyelin hydrolysis and ceramide release can be triggered in transformed cell lines by a number of soluble factors (34–38), and represents a major activation pathway involved in modulation of cell growth, differentiation and apoptosis in different cellular systems (27, 28, 39). A key role in this pathway seems to be played by ceramides, which have been shown to be able to activate different protein kinases (40, 41), as well as protein phosphatases (42), and proteases (43), resulting also in nuclear translocation and activation of the NFκB transcription factor (36, 44). Moreover, ceramides are responsible for inducing DNA fragmentation and apoptosis in different tumor cell lines (29, 45). We have recently shown that Fas cross-linking activates an acidic sphingomyelinase in promyelocytic leukemia U937 cells, resulting in ceramide production and apoptotic cell death (16). Here we show that, unlike in T-PBL, Fas cross-linking induces apoptosis in normal gut T-LPL, and this is associated with acidic sphingomyelinase activation, sphingomyelin hydrolysis, and ceramide release. Importantly, exogenous ceramide could trigger apoptosis in T-PBL as well as in T-LPL, suggesting that the apoptotic machinery downstream ceramide production is equally functional between the two populations. Critical differences may be confined in the early signal generation, as demonstrated by the inability to activate an acidic sphingomyelinase after Fas cross-linking in T-PBL. It is likely that differences in the activation status between T-LPL and T-PBL may account for differential coupling of receptors with relevant signal transducers, as shown for signaling originating at the TCR/CD3 complex (3, 4). It is noteworthy that long term in vitro activated T-PBL become susceptible to Fas cross-linking (11) and able to induce substantial amounts of FasL upon receptor stimulation, unlike resting T-PBL (46, 47) and data not shown. In this respect therefore, T-LPL share functional similarities with long term activated T cells.

In conclusion, our data provide evidence for a functional role of the sphingomyelin breakdown pathway in normal lymphocytes following Fas/FasL interactions, and they help define the molecular mechanisms underlying physiologic cell death in the immune system.

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