Fibronectin-Associated Fas Ligand Rapidly Induces Opposing and Time-Dependent Effects on the Activation and Apoptosis of T Cells

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J Immunol 2003; 171:5882-5889; doi: 10.4049/jimmunol.171.11.5882
http://www.jimmunol.org/content/171/11/5882
Fibronectin-Associated Fas Ligand Rapidly Induces Opposing and Time-Dependent Effects on the Activation and Apoptosis of T Cells

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Recently, it has been shown that Fas ligand (FasL) interacts with the extracellular matrix (ECM) protein fibronectin (FN), and that the bound FasL retains its cytotoxic efficacy. Herein, we examined the ramifications of FasL-ECM protein interactions throughout a specific time period, in the absence or presence of additional activating molecules, assuming that these complexed interactions occur during inflammation. We found that exposure of purified human T cells to FN-associated recombinant FasL for as brief as 5–10 min at 0.1–100 ng/ml induced their adhesion in β1 integrin- and FasR-dependent manners while activating the intracellular protein kinase, Pyk-2. The FN-associated FasL stops the CXCL12 (stromal cell-derived factor 1α)-induced chemotaxis of T cells by inhibiting the chemokine-induced extracellular signal-regulated kinase signaling and cytoskeletal rearrangement. This short term exposure of T cells to the FN-bound FasL (1 ng/ml), which was followed by T cell activation via the CD3 complex, resulted in 1) increased secretion of IFN-γ (measured after 24 h), and 2) enhanced T cell apoptosis (measured after 72 h).

Thus, in the context of inflamed ECM and depending on the time after FasL activation, its concentration, and the nature of other contextual mediators, FasL initially retains effector T cells at sites of inflammation and, later, induces T cell apoptosis and return to homeostasis.

T he functioning of immune cells, such as T cells, during inflammation must be tightly controlled and time-limited. These T cell functions depend on the existence of diverse inflammatory-modulating molecules, the cell’s state of activation, and the recognition of other collaborating cells and tissue components, including those of the extracellular matrix (ECM). One such mechanism that controls cell activation, function, and viability may be associated with the local interaction of T cells with Fas ligand (FasL; CD95L). The engagement of stimulated T cells with cross-linked FasL leads to the activation of a caspase signaling pathway that culminates in activation-induced apoptosis (1, 2). However, in addition to its proapoptotic effects, it is now becoming evident that FasL can function as an immune cell-activating factor and the recognition of other collaborating cells and tissue components, in addition to cell adhesion, migration, and cytokine and enzyme secretion (3–6). Fas activation leads to T cell and neutrophil proliferation (7), cytokine secretion (8, 9), angiogenesis and endothelial cell proliferation (9), and an increase in integrin expression on epithelial cells (10). Although soluble FasL do not signal cell death (1, 2), it induces the chemotaxis of neutrophils (11, 12) and reduces neutrophil adhesion to endothelial cells (13). FasL can function in the context of the ECM; it has been found that FasL binds fibronectin (FN) and vitronectin, two prototypic cell-adhesive glycoproteins of the ECM, and that such ECM-anchored FasL retains its cytotoxic potential (14).

We and other researchers have found that the ECM and, in particular, FN, can bind various growth factors, cytokines, and chemokines and thereby affect immune cell activation and function in a site-restrictive manner (15–19). The ECM-anchored or associated mediators affect monocyte and T cell activation, as well as β1 integrin functions associated with recognition of the surrounding tissue components, in addition to cell adhesion, migration, and cytokine and enzyme secretion (17, 20, 21). We have assumed that although the ECM-associated FasL can ultimately signal T cell death upon activation, in the short term it can also affect the proinflammatory functions of these cells. Based on our results, we postulate that within the ECM, and depending on the time kinetics of the interactions and the presence of other activatory moieties, FasL may differentially affect the activatory behavior of T cells and their death and, consequently, the duration and intensity of the inflammatory reaction.

Materials and Methods

Reagents

The following reagents and chemicals were purchased from the sources indicated: RPMI 1640 (Life Technologies, Paisley, U.K.); FCS, HEPES buffer, antibiotics, and sodium pyruvate (Kibbutz Beit-Haemek, Israel); collagen type I (CO-I; Cellagen, ICN Pharmaceuticals, Costa Mesa, CA); collagen type VI (CO-IV; Sigma-Aldrich, Rehovot, Israel); FN (Chemicon, Temecula, CA); laminin (LN); PMA, and the FN peptides, RGDS, RGES, and Leu-Asp-Val (LV; Sigma-Aldrich). Recombinant human stromal cell-derived factor 1α (SDF-1α), IL-2, soluble human extracellular FasL (a monomeric 19.9-kDa protein, 175 aa residues), and TNF-α were purchased from PeproTech Asia (Rehovot, Israel). The ED50 of the cytotoxic effect of this FasL is <10 ng/ml, corresponding to a sp. act. of >106 U/mg. mAbs directed against human β1 integrins (CD29, clone 3SS) and CD26 were obtained from Serotec (Oxford, U.K.). Neutralizing and activating mAb against FasL and FasR, clones ZB4 and CH-11, respectively, were purchased from MBL (Nagoya, Japan). Capturing mAb anti-FasL (clone 4A5) and biotin-labeled mAb anti-FasL (clone 4H9) were purchased from MBL (Nagoya, Japan). Polyclonal Ab against phosphorylated Pyk2 (clone py881) was obtained from BioSource (Camarillo, CA), anti-total Pyk2...
T cells were purified from healthy human donor peripheral blood. The whole blood of a healthy human T cell enrichment cocktail (StemCell Technologies, Vancouver, Canada) was added to microtiter plates that were previously coated with FN (10 μg/ml) (21, 22). 51 Cr-labeled T cells (2 × 10^6 cells/ml) were resuspended in 24-well plates in RPMI medium containing 10% FCS. The cells were then washed and replated (2 × 10^5 cells/ml) on 24-well plates that were precoated with mAb anti-CD3 (1 μg/ml; non-tissue culture grade plates) and cultured for 24 h. The supernatants were collected, and the cytokine content (TNF-α and IFN-γ) was determined by ELISA, using the appropriate mAb, according to the manufacturer’s instructions.

T cell apoptosis

Human T cells (2 × 10^6 cells/ml) were activated (10 min, 37°C) with soluble, FN- or plastic-bound FasL in 24-well plates in RPMI medium containing 10% FCS. The cells were then washed and replated (2 × 10^5 cells/ml) on 24-well plates that were precoated with mAb anti-CD3 (1 μg/ml; non-tissue culture grade plates) and cultured for 72 h in tissue culture conditions. Then, the percentage of cells undergoing apoptosis was determined using the annexin V detection assay. The cells were incubated (10 min in the dark) at room temperature in 200 μl of buffer containing FITC-conjugated human annexin V (5 μl; Bender MedSystem, San Bruno, CA). Finally, 10 μl of propidium iodide was added to each sample, and the percentage of cells undergoing apoptosis was analyzed by FACSscan at 525 nm using CellQuest Software. The annexin V+ propidium iodide+ cells corresponded to the apoptotic cells. The data are expressed as the percentage of increase above untreated controls.

Results

FasL induces T cell adhesion to FN and LN

First, we analyzed, using a specific mAb, the proportional quantity of FasL binding to immobilized FN. The results, shown in Fig. 1A, indicate that ~1% of the added protein actually bound FN. Hence, in the experiments specified below, the amount of FN-bound FasL was calculated according to the results of this molecular binding assay. Next, we examined the possible proadhesive effects of soluble and FN-bound FasL on human T cells. Briefly, T cells were seeded onto FN-coated wells. Some wells were pretreated with different amounts of FasL, which was washed away before cell seeding (bound FasL); alternatively, the T cells were added to the FN-coated wells together with soluble FasL. The amount of adherent T cells, measured after different time periods, is shown in Fig. 1, B and C. After a 40-min incubation, both soluble and FN-bound FasL induced T cell adhesion to FN; the maximal adhesion of T cells to FN was achieved with 0.001–1 ng/ml of soluble FasL and 0.1–10 ng/ml FN-bound FasL (Fig. 1B). Next, we measured the ability of FasL to induce T cell adhesion to FN after a 10-min exposure of the cells to the FN-FasL complex, either alone (soluble FasL) or with FN-bound FasL. The results demonstrate that such a short exposure of the T cells to both bound and soluble FasL induced appreciable levels of cell adhesion, a response that is manifested in a repeated pattern of bell-shaped dose-response curve; the maximal T cell adhesion induced by the mediator was also in the range of 0.01 ng/ml (Fig. 1B). Note that a considerable number of FN-bound FasL was then added, and after boiling, the samples, containing equal amounts of proteins, were separated on 10% SDS-PAGE gel and transferred to nitrocellulose membranes. The nitrocellulose membranes were blocked with TBST (20 mM Tris, 135 mM NaCl, and 0.1 Tween 20, pH 7.5) containing 5% low fat milk and probed with the following mAbs in blocking buffer: anti-phosphorylated Pyk2 (1.5 μg/ml), anti-total Pyk2 (0.2 μg/ml), anti-phosphorylated ERK (0.2 μg/ml), or anti-total ERK (1/20,000 from stock). Immunoreactive protein bands were visualized using an HRP-conjugated secondary Ab and the ECL system (20, 21).

Actin polymerization

T cells (3 × 10^5 cells/ml) were preincubated with FasL (1 h, 37°C, 7% CO_2 humidified atmosphere) and treated with 500 μl of SDF-1α for 15 s at 37°C and then fixed by the addition of a 3-fold volume of 3.7% PFA for 10 min at 22°C. Next, the cells were extensively washed, and the membranes were permeabilized; the T cells were stained with FITC-phalloidin (2 μg/ml), washed, and analyzed by FACSscan at 525 nm, as we previously described (20).

Cytokine secretion

T cells (2 × 10^5 cells/ml) were activated (10 min, 37°C) with the indicated concentration of either soluble or FN-bound Fasl, in 24-well plates in a serum-free medium containing 0.1% BSA. The cells were then washed and replated in the same concentration on anti-CD3 mAb precoated 24-well plates (1 μg/ml; non-tissue culture grade plates; 4°C, 24 h). The supernatants were collected, and the cytokine content (TNF-α and IFN-γ) was determined by ELISA, using the appropriate mAb, according to the manufacturer’s instructions.

T cell adhesion and migration assays

Analysis of T cell adhesion to ECM components was determined as previously described (19–21). Briefly, 3Cr-labeled T cells were resuspended in RPMI medium supplemented with 1% HEPES buffer and 0.1% BSA (adhesion medium). Then, the indicated activators were added to microtiter wells (which were precoated with ECM proteins (10 μg/ml)) together with the cells (10^5 cells/well). The plates containing the test materials were further incubated (37°C, 7% CO_2 humidified atmosphere) and then gently washed. The adherent T cells were lysed, removed, and counted using a gamma counter (Packard, Downers Grove, IL). The results are expressed as the mean ± SD of the percentage of bound T cells from quadruplicate wells. The percentage of FasL binding to FN was determined as FN-bound FasL/soluble FasL using a binding curve of FasL to the mAb, clone 4A5. The percentage of binding was analyzed using diluted HRP-labeled streptavidin and anti-human FasL detection mAb (clone 4H9) was added to the washed wells, and binding was analyzed using diluted HRP-labeled streptavidin and anti-human FasL detection mAb (clone 4H9). Then, biotin-labeled anti-human FasL detection mAb (clone 4H9) was added to the washed wells, and binding was analyzed using diluted HRP-labeled streptavidin and anti-human FasL detection mAb (clone 4H9). The amounts of FN-bound FasL were calculated as FN-bound FasL/soluble FasL using the detection Abs against FasL (clone 4H9) as described above. The amounts of FN-bound FasL were calculated using a binding curve of FasL to the mAb, clone 4A5. The percentage of FasL binding to FN was determined as FN-bound FasL/soluble FasL × 100%.

Phosphorylation of Pyk-2 and ERK

T cells were incubated in starvation medium (RPMI medium without serum) for 48 h. Before testing, 5 × 10^5 cells/sample were activated with ICN Biomedicals, Irvine, CA), polyclonal Ab against phosphorylated extracellular signal-regulated kinase2 (ERK) (2 × 10^5 cells) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), polyclonal Ab against phosphorylated extracellular signal-regulated kinase2 (ERK2) was obtained from BioSource, anti-total ERK2 was obtained from Sigma-Aldrich, and anti-human CCR7 (clone 6B3) were purchased from R&D Systems (Minneapolis, MN) and MBL (Nagoya, Japan), respectively. Inhibitors of intracellular signal transduction pathways, namely wortmannin and LY294002 (phosphoinositol 3-kinase (PI-3 K) inhibitors), GFI0203X (protein kinase C (PKC) inhibitor), and pertussis toxin (G protein inhibitor) were obtained from Calbiochem (San Diego, CA).

The flat-bottom microtiter plates were precoated with FN, LN, CO-1, or CO-VI (1 μg/ml), and the remaining binding sites were blocked with 0.1% BSA. rFasl, diluted in PBS containing 0.1% BSA, was incubated on the immobilized ECM protein (1 h at 37°C, 7% CO_2 in a humidified atmosphere) and then gently washed. The remaining binding sites were blocked with 0.1% BSA. Analysis of recombinant human FasL binding to FN was conducted as follows. Various concentrations of soluble Fasl were incubated with 18 h, 37°C, 96-well plates, which were previously coated with capture mAb anti-human Fasl (clone 4A5). Then, biotin-labeled anti-human FasL detection mAb (clone 4H9) was added to the washed wells, and binding was analyzed using diluted HRP-labeled streptavidin and anti-human FasL detection mAb (clone 4H9). The reaction was stopped with 50 μl of 1 M H_2 SO_4 , and the OD of the reaction was measured (450/570 nm) using a spectrophotometer. In parallel, various concentrations of soluble Fasl were incubated for 1 h at 37°C in 96-well plates that were previously coated with FN (10 μg/ml, 1 h, 37°C). The binding to FN was measured using the detection Abs against Fasl (clone 4A5) as described above. The amounts of FN-bound FasL were calculated using a binding curve of FasL to the mAb, clone 4A5. The percentage of FasL binding to FN was determined as FN-bound FasL/soluble FasL × 100%.

Analysis of T cell adhesion to ECM components was determined as previously described (19–21). Briefly, 3Cr-labeled T cells were resuspended in RPMI medium supplemented with 1% HEPES buffer and 0.1% BSA (adhesion medium). Then, the indicated activators were added to microtiter wells (which were precoated with ECM proteins (10 μg/ml)) together with the cells (10^5 cells/well). The plates containing the test materials were further incubated (37°C, 7% CO_2 humidified atmosphere) and then gently washed. The adherent T cells were lysed, removed, and counted using a gamma counter (Packard, Downers Grove, IL). The results are expressed as the mean ± SD of the percentage of bound T cells from quadruplicate wells. The migration of 3Cr-labeled T cells was examined in a Transwell chemotaxis apparatus (6.5-mm diameter; Corning, Corning, NY), consisting of two compartments separated by polycarbonate filters (5-μm pore size) pretreated (1 h, 37°C) with FN (25 μg/ml) (21, 22). 3Cr-labeled T cells (2 × 10^5 in 100 μl of adhesion medium) were added to the upper chambers with or without activators. The bottom chambers contained 0.6 ml of the same medium, with or without SDF-1α (250 ng/ml). After incubation, the cells that had transmigrated into the lower wells were collected, centrifuged, and lysed, and the radioactivity in the resulting supernatants was determined. The percentage ± SD of cell migration was calculated as the radioactivity counts in the lysates of the lower chambers (representative of the migrating cells) divided by the total counts (representative of 2 × 10^5 cells).
of T cells adhered to FN even if the cells were briefly (10 min) exposed to soluble FasL and washed before their seeding on the FN-coated wells, and that under these conditions the FasL-treated T cells remained viable (data not shown). Thus, similar to its intact soluble form, FN-anchored FasL can induce T cell adhesion.

We then examined the ability of FasL to induce T cell adhesion to ECM glycoproteins other than FN. T cell adhesion was examined following a 10-min exposure of the cells to the soluble form of the mediator. We found that FasL, induced an appreciable level of T cell adhesion to LN, but not to CO-I and CO-IV (Fig. 1D), whereas a T cell adhesion-inducing chemokine, SDF-1α (125 ng/ml; 30-min pretreatment plus a 30-min adhesion assay), as well as PMA induced a significant level of T cell adhesion to the four substrates (data not shown).

Finally, we compared the time-dependent pattern of FasL-induced T cell adhesion to FN with the proadhesive effects of PMA and IL-2. IL-2 was chosen based on its ability to induce T cell adhesion to FN (22). The results, shown in Fig. 2, indicate that only FasL can induce T cell adhesion following as brief as a 5-min exposure of human T cells (p < 0.05 compared with PMA and IL-2). After a 10-min exposure, the ability of FasL to induce cell adhesion was equal to that of PMA, but significantly superior to that of IL-2 (p < 0.05). The three T cell activators reached their maximal proadhesive effect upon a 30-min incubation, an effect that gradually and moderately decreased with time, although FasL and PMA exerted a profound effect even after 180 min. Thus, in comparison with IL-2, FasL is capable of inducing rapid and prolonged T cell-FN interactions.

FasL-induced T cell adhesion to FN involves FasR recognition and FN-specific β1 integrins

T cell recognition of and adhesion to FN are mediated primarily by the LDV- and Arg-Gly-Asp (RGD)-recognizing α5β1 and α6β1 integrins (CD29), respectively. To test whether these receptors are indeed involved in the apparent FasL-induced adhesion of T cells to immobilized FN, we pretreated the responsive T cells with the indicated peptides and mAb as well as control molecules; their adhesion, induced by the soluble form of FasL, was measured 30 min after their seeding onto the FN-coated wells. The results, shown in Fig. 3A, demonstrate that the RGD and LDV peptides as well as mAb anti-CD29, but not the control peptide or mAb anti-CD26, inhibited T cell adhesion.

Next we investigated whether the FasR can signal T cell adhesion. The T cells were treated with either the FasL inhibitory mAb...
ZB4 (Fig. 3B) or the FasR activatory mAb CH-11 (Fig. 3C), and their adhesion to FN was measured after a 30-min incubation. The results indicate that the CH-11 mAb indeed induced T cell adhesion FasL, especially at 100-1000 ng/ml, and that mAb ZB4 inhibited FasL-induced, but not PMA-induced adhesion. The relatively moderate proadhesive effect of mAb CH-11, compared with those of FasL, could be explained by its relatively high m.w. and complexed form, which probably affect its binding to CD95 and its signaling. Thus, FasL exerts its αβ3 and αβ4 integrin-mediated rapid proadhesive effect via the FasR.

**FIGURE 3.** FasL-induced T cell adhesion to FN is very late Ag 4- and 5-dependent and is mediated by Fas signaling pathway. A, Purified human T cells were radiolabeled, then pretreated with the indicated mAb or FN peptides. The cells were then treated with FasL (1 ng/ml) and seeded onto FN-coated microtiter wells, and the amount of cell adhesion was determined 30 min later. The mean ± SD of three different experiments are shown. B, Purified human T cells were labeled and pretreated with the indicated mAb (10 μg/ml). The cells were treated with soluble FasL (1 ng/ml) or PMA (50 ng/ml) and seeded onto FN-coated wells. The amount of cell adhesion was determined 30 min later. C, The labeled T cells were treated with different concentrations of FasL or activatory anti-Fas Abs (CH-11) and seeded onto FN-coated wells, and the amount of cell adhesion was determined 30 min later. The mean ± SD of four different experiments are presented in B and C. *, p < 0.05.

**FIGURE 4.** Signaling mechanisms involved in FasL-induced T cell adhesion to FN. A, T cells were pretreated with the indicated intracellular signal transduction inhibitors, wortmannin (2 μg/ml), GF109203X (20 nM), pertussis toxin (2 μg/ml), and LY294002 (20 nM). The cells were then treated with FasL (1 ng/ml) and seeded onto FN-coated microtiter wells, and the amount of cell adhesion was determined 30 min later. The mean ± SD of three different experiments are presented in A. T cells were exposed to different concentrations of sFasL for 2 min (B) or 10 min (C) and then lysed. The lysates were run on SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with Ab anti-phospho-Pyk-2 (pPYK2) and anti-total Pyk-2 (tPYK2). The densitometric histograms of the experiment are expressed as phosphorylated Pyk-2/total Pyk-2 × 100%, and are from one experiment representative of three. These histograms are the average calculated from the results of three different experiments ± SD. *, p < 0.05.

**FasL-induced T cell adhesion involves PKC and PI3-K signaling, as well as Pyk-2 phosphorylation**

The adhesion of activated T cells to immobilized ECM glycoproteins requires specific intracellular signaling pathways that render nonresponding β1 integrins to their ECM recognition and binding states (23, 24). To verify that the FasL-induced T cell adhesion to FN involves cell activation and to discern which intracellular activation pathways are involved in this process, we exposed the T cells to FasL together with the inhibitory compounds. The results, showing that T cell adhesion was inhibited by GF109203X, wortmannin, and LY294002, but not by pertussis toxin, indicate that the adhesive effects of FasL require the activation of PKC and PI3-K, but not G protein-coupled receptor signaling (Fig. 4A).

The focal adhesion kinase, designated cytoplasmic tyrosine kinase 2 (Pyk-2), is phosphorylated upon PKC activation and links β1 integrins to multiple signaling pathways regulating adhesive and migratory processes in T cells (23, 24). Fig. 4, B and C, shows...
that brief treatments (10 and 2 min, respectively) of T cells with soluble FasL induced the phosphorylation of Pyk-2. In the 10-min exposure experiment, a concentration of >1 pg/ml FasL induced Pyk-2 phosphorylation, and in the 2-min exposure experiment, similar levels of phosphorylation occurred at >1 ng/ml. Note that in the 2-min assay, similar to T cell adhesion to FN, as shown in with Fig. 1C. Pyk-2 phosphorylation showed a bell-shaped dose-response curve with increasing amounts of soluble FasL. Thus, Pyk-2 phosphorylation accompanies the response of T cells to effective concentrations of FasL.

**FN-associated FasL stops SDF-1α-induced T cell chemotaxis, actin cytoskeleton rearrangement, and signaling**

We then investigated whether FasL-FN interactions affect T cell chemotaxis induced by the inflammatory chemokine SDF-1α (25). To this end, we analyzed T cell migration in the Transwell apparatus through FN-coated membranes, in which net leukocyte migration toward a chemokine over time (3 h) was measured. First, we measured T cell chemotaxis induced by the FasL present in the lower chambers in different amounts and found that this mediator did not induce T cell migration, even after 3 h (not shown). Next, we measured T cell response to SDF-1α present in the lower wells in the absence or the presence of soluble or FN-bound FasL, in the upper wells. We found that a substantial number of T cells migrated when only SDF-1α was present in the lower chambers. However, this migration of T cells was markedly reduced, in a dose-response manner, if either soluble or FN-bound FasL was present in the upper chambers. Maximal inhibition of T cell chemotaxis occurred with 100 ng/ml of soluble or bound FasL (Fig. 5A). A similar pattern of results were obtained when the chemokine ELC (CCL19) was used as the T cell chemotactic (data not shown).

These results were reminiscent of those previously reported by us where ECM-associated TNF-α was shown to stop the SDF-1α-induced chemotaxis of CD4+ human T cells (26). To determine whether FN-associated FasL stops T cell chemotaxis similarly to TNF-α, we compared the SDF-1α-induced chemotaxis-modulating abilities of FasL to those of TNF-α or IL-2 (as a control proinflammatory and T cell adhesion-inducing cytokine) (22) present in the upper chambers. The results, shown in Fig. 5B, demonstrate that both FasL and TNF-α, but not IL-2, induced a similar dose-dependent attenuated pattern of T cell chemotaxis; their maximal inhibitory effect occurred at 100 ng/ml (p < 0.01). Thus, FN-associated FasL can stop T cell chemotaxis and, therefore, probably restricts the presence of T cells to defined ECM sites.

T cell adhesion to and migration through immobilized ECM ligands require specific intracellular signaling and cellular polarization, a process associated with the polymerization of the actin cytoskeleton (27). To study the effect of FasL on SDF-1α-induced actin polymerization, T cells were left intact or were treated with sFasL (60 min) and then activated them by SDF-1α (100 ng/ml) for 15 s. Fig. 5C shows that soluble FasL at 10–100 ng/ml markedly inhibited actin polymerization induced by SDF-1α. Next, we examined the effect of FasL on SDF-1α-induced ERK phosphorylation. Activated ERK regulates cell adhesion and motility by enhancing the activity and phosphorylation of myosin and the polymerization of actin fibers (28). The results, shown in Fig. 5D, indicate that FasL (1–100 ng/ml; 60 min) inhibits the SDF-1α-induced ERK phosphorylation in the T cells in a dose-dependent manner, reaching its maximal inhibitory effect at 100 ng/ml. When used alone, FasL did not induce ERK phosphorylation (not shown). Note that the results of the densitometric analysis, as depicted in these figures, demonstrate the average of three different experiments. Thus, FasL delivers a stop signal to chemotactically migrating T cells by blocking their ability to react to the chemokine by undergoing polarization and intracellular ERK signaling.

**A brief exposure of T cells to FN-associated FasL and mAb anti-CD3 results in enhanced IFN-γ secretion and T cell apoptosis**

We analyzed the effect of a short exposure of T cells to FN-bound FasL and compared it to the effect of an intact mediator. The T
cells were exposed to FasL for 10 min in its intact or FN-bound form and then collected, washed, and activated with mAb anti-CD3. T cell secretion of IFN-γ and TNF-α was analyzed 24 h later, and T cell apoptosis was measured after 72 h. The results indicate that FasL, either intact or FN-bound, induced the secretion of IFN-γ, but not TNF-α, with maximal and almost equal effects at 1 and 100 ng/ml for intact, soluble FasL or 1 ng/ml for the FN-bound mediator.

Next, we measured the effect of a brief exposure of T cells to FasL on T cell apoptosis induced by CD3 activation. The results indicate that FN-bound FasL indeed induced cell death. The maximal apoptotic effect occurred at 1–100 ng/ml for intact FasL and 1 ng/ml for FN-complexed FasL (Table I). Thus, FN-bound FasL appears to be an equal activator of T cells to the soluble mediator. Note that 10-min exposure of T cells to intact FN did not result in IFN-γ secretion or apoptosis. Hence, a brief exposure of human T cells to FN-associated FasL and a subsequent incubation period of 1–3 days with CD3-activating mAb definitively affects their proinflammatory capabilities (e.g., IFN-γ secretion) and later their cell death, respectively.

**Discussion**

In this study we have shown that as brief as a >10-min exposure of human T cells to FN (or LN)-associated recombinant FasL induces T cell activation and potentiates their proinflammatory capacities, such as adhesion to the ECM ligands, IFN-γ secretion,

<table>
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<th>Substrate/form of FasL</th>
<th>FasL (ng/ml)</th>
<th>IFN-γ (ng/ml)</th>
<th>TNF-α (ng/ml)</th>
<th>Increase in IFN-γ (%)</th>
<th>Increase in TNF-α (%)</th>
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<td>1.2 ± 0.1</td>
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<tr>
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<td>0.8 ± 0.1</td>
<td>49 ± 6</td>
<td>0</td>
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<tr>
<td>FN-bound FasL</td>
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<td>0.8 ± 0.1</td>
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</table>

* a T cells were seeded onto bare or FN-coated wells, treated (10-min) with the indicated concentrations of FN-bound FasL, collected, washed, and transferred to 24-well plates coated with mAb anti-CD3 (OKT3; 1 μg/ml) in serum-free medium (for cytokine secretion) or in medium containing 10% FCS (for apoptosis). The supernatants were collected after 24 h and analyzed for IFN-γ and TNF-α secretion. The percentage of cells undergoing apoptosis was determined, using the annexin V detection assay, after 72 h. The mean ± SD of five different experiments is shown.

* P < 0.05, comparing the activatory effects of FasL on IFN-γ secretion.

* P < 0.01, comparing the apoptotic effect of FN-bound vs. intact FasL at 100 ng/ml.

**FIGURE 6.** Schematic model of the time dependency of FasL-induced T cell activation and apoptosis at sites of inflammation.
retention in inflamed areas, and, later, T cell apoptosis (Fig. 6). In these reactions, FasL may affect T cell behavior when used alone on FN (adhesion), when the FN-associated FasL is presented to the cells before their exposure to mAb anti-CD3 (cytokine secretion and apoptosis), or when the T cells are first exposed to the matrix-associated mediator and then to a chemokine (chemotaxis).

The environmental conditions affect the mechanisms by which FasL confers immune privilege (tolerance) or induces inflammation (4, 5). For example, it has been shown that the exact site of inoculation of colon carcinoma cells determines their fate (6). It has also been reported that 1) depending on its form, e.g., membrane-expressed or soluble, FasL induces opposing effects on inflammation and tumor cell survival (9, 29); and 2) the opposing effects of FasL depend on the subpopulation of responding T cells (30). Also, the inflammatory or death-inducing capacities of FasL depend on the presence of other inflammatory-regulating cytokines; TGFβ synergizes with FasL in inducing tolerance (6). We postulate that the proinflammatory or apoptotic effects of FasL depend on its time of exposure, form (e.g., soluble or FN-associated), amount, copresence of migration-inducing chemokines, TCR-activating agents, and sequence of analysis of T cell behavior with time following recognition and ligation of the FasL-ECM complex.

Our results indicate that when used alone, the ability of FasL to induce T cell adhesion to FN (or LN) involves the activation of specific βι integrins. This activation, which was blocked by specific inhibitors of PI3-K and PKC, was also associated with the phosphorylation of Pyk-2, a member of the focal adhesion kinase family of molecules. Pyk-2 is phosphorylated upon PKC activation; it links βι integrins to multiple signaling pathways that regulate T cell adhesion and migration. Such intracellular processes are also involved in the regulation of mitogen-activated protein kinases, such as ERK-2, and Jun-NH₂ kinase pathways (28, 31, 32). ERK regulates immune cell adhesion and motility by enhancing the activity of the myosin light chain kinase, myosin phosphorylation, and consequent polarization of the actin fibers (27, 28, 33). Our results also indicate that when the migrating T cells responded to both SDF-1α and FasL, the latter inhibited the chemokines’ ability to induce ERK signaling and the rearrangement of the actin cytoskeleton.

A shown here, a short exposure of T cells to FN-associated FasL, followed by activation via the CD3 pathway, was sufficient to induce the secretion of IFN-γ (24 h) and later (72 h) resulted in cell death. It is reasonable that these effects of FN-associated FasL involve NF-κB and ERK signaling (34–36). However, in contrast to its effect on IFN-γ secretion, a short exposure of T cells to FasL did not affect the secretion of TNF-α regardless of whether FasL was presented to the cells in the presence or the absence of FN. The reason for these diverse effects of FasL (and FN-specific integrins) on cytokine secretion is presently not clear. However, it has been documented that the ligation of TNF-α by TNF-R1 leads to protection against T cell death (37). We postulate that such an antiapoptotic, downstream effect of FasL, presented to the T cells within an inflamed site, is not desirable; T cell apoptosis only occurs following the prior functioning of T cells as modulators of inflammation.

An additional role of FasL, secreted and integrated into the ECM at sites of inflammation, may be to arrest the chemokine-induced migration of T cells at these sites, where their effector functions are mostly required. It has been shown that such an anchorage of T cells may also occur after ligation of TCR or TNF receptor (26, 38). As shown here, FasL-induced T cell adhesion to FN also involves PKC activation. This may explain the ability of FasL to inhibit T cell chemotaxis, as PKC activators were previously shown to desensitize chemokine receptor signaling (39, 40). The FasL-FN-induced retention of T cells within the inflamed spot probably enables their proinflammatory functioning, as it leads to their activation and the secretion of IFN-γ.

Several mediators, including TNF-α, TGFβ, and macrophage inflammatory protein-1β, can bind various components of the ECM (16–18). The ECM could thus serve as a transitory storage site for these inflammatory mediators, retaining them close to effector cells, where they ensure cellular responses even with minute amounts of the cytokines, because they are presented in a rather concentrated form to the attached leukocytes. These ECM-cytokine complexes may also restrict or modulate the cytokines’ activities at target sites and differentially regulate the distribution, availability, and activities of the matrix-associated mediators. FasL, which may be enzymatically removed from tissue-invading leukocytes and implanted into its ECM microenvironment (14), can therefore behave like one of these inflammatory-modulating cytokines. In their study on concentrated FasL-containing supernatants of CT26.CD95L cells, Aoki and co-workers found that the FN-bound FasL undergoes oligomerization, which probably accounts for its enhanced activity, even though it is present on the ECM protein and is active in low amounts.

Our findings indicate that the diverse effects of the ECM-associated FasL should be differentially examined on a time scale of minutes, hours, and days (Fig. 6). We suggest that the two seemingly opposing effects of FasL, i.e., induction of proinflammatory behavior (life) and apoptosis (death), which may occur in the context of the ECM in signal-rich extravascular sites of inflammation, should be regarded as a rather sophisticated mechanism by which the very same molecule that induces inflammation can later enable healing and ensure the return of tissue homeostasis.

References


