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Stored Fas Ligand (FasL), a Mediator of Rapid CTL-Mediated Killing, Has a Lower Threshold for Response Than Degranulation or Newly Synthesized FasL

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CTL lyse target cells through the release of cytolytic granule mediators and expression of the death receptor ligand Fas ligand (FasL). We previously demonstrated that FasL is stored in vesicles distinct from cytolytic granules and is translocated to the cell surface within 15 min of TCR stimulation, followed by a later wave of newly synthesized FasL cell surface expression at 2 h post-stimulation. Initial studies suggested that the two FasL responses had different signaling thresholds. To test this possibility directly, we titrated Ag presented to murine CTL to measure FasL and degranulation response thresholds. Stored FasL translocation to the cell surface required substantially lower concentrations of peptide than was required for de novo expression of FasL and degranulation. Furthermore, a low-affinity agonist peptide stimulated strong stored FasL translocation but only limited de novo FasL expression and degranulation. These data imply that the two FasL populations may have distinct functions. We examined bystander killing and found that the rapidly expressed FasL triggered highly specific lysis of target cells, as did degranulation. In contrast, the newly synthesized later wave of FasL mediated extensive Fas-dependent bystander killing. Our data indicate that stored FasL is mobilized in response to low concentrations of Ag to mediate rapid, highly specific lysis of target cells, whereas the later, newly synthesized FasL requires higher concentrations of Ag and mediates indiscriminate lysis. These findings suggest that early and late FasL and degranulation represent nonredundant lytic mechanisms that have been selected for distinct situations, possibly for optimal pathogen clearance. The Journal of Immunology, 2010, 184: 000–000.
Materials and Methods

Mice and cells

The murine alloreactive CD8+ CTL clone AB.1 (H-2d anti-H-2b) and the murine peptide-specific CTL clone 3/4 that recognizes H-2D^b–restricted NP\textsubscript{366-374} peptide (ASNNMETM) derived from nucleoprotein of the A/PR/8/34 (H1N1) influenza virus were described previously (16, 17). CTL clones were maintained by weekly stimulation with irradiated (2500 rad) C57BL/6 spleenocytes alone (for AB.1) or pulsed with 200 μg/ml NP\textsubscript{366-374} peptide (for clone 3/4) and 10 U/ml rIL-2 and were used 5–6 d later. CTL clones were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM t-glutamine, 100 μg/ml penicillin/streptomycin, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 53 mM 2-ME. All animal studies have been approved by the University Animal Policy and Welfare Committee at the University of Alberta.

The L1210 lymphoma cell lines expressing chimeric class I MHC (L1210/K\textsuperscript{K}) (18) or Fas (L.Fas) were gifts from Dr. K. P. Kane (University of Alberta). EL4 cells were obtained from the American Type Culture Collection (Manassas, VA). Cell lines L1210, L1210\textsuperscript{K}, L.Fas, and EL4 were grown in DMEM supplemented with 8% defined bovine calf serum.

Abs, peptide, and reagents

PE-conjugated anti-FasL mAb (MFL3), FITC-conjugated anti-CD107a mAb (1D4B), and PE-Cy5–conjugated anti-CD8\textsuperscript{a} (Ly-2) mAb (53-6.7) were purchased from BD Pharmingen (San Diego, CA). NP\textsubscript{366-374} peptide (ASNNMETM) was synthesized by Sigma-Genosys (Canada). OVAp (SIINFKEK) and its low-affinity variant G4 (SIGFKEK) were synthesized by GenScript (Piscataway, NJ). Cycloheximide (CHX) and EGTA were purchased from Sigma-Aldrich (St. Louis, MO). BAPTA-AM was purchased from Calbiochem (San Diego, CA). Surfactant-free, 5.2-μm white sulfate latex beads were purchased from Interfacial Dynamics (Eugene, OR). Na\textsubscript{2}CrO\textsubscript{4} (1Cr) was purchased from PerkinElmer (Wellesley, MA).

In vitro generation of bone marrow–derived dendritic cells

Bone marrow–derived DCs (BMDCs) were generated by culture of C57BL/6-derived BM in RPMI 1640 supplemented with 10% FCS, 2 mM t-glutamine, 100 μg/ml penicillin/streptomycin, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 53 mM 2-ME, and 5 μg/ml rGM-CSF. After 9 d, DC maturation was induced by overnight incubation with 0.5 μg/ml LPS. Mature DCs were pulsed at 10\textsuperscript{6} cells/ml in FCS for 60 min at 37˚C with 10 μM SIINFKEK peptide, then washed three times with 2% FCS/ RPMI 1640 and used for primary OT-I CD8\textsuperscript{+} T cell coculture.

In vitro generation of OT-I CTLs

OT-I CTLs, which are specific for H-2K\textsuperscript{b}-restricted OVA peptide (SIINFKEK), was generated by in vitro stimulation and culture. OT-I T cells were purified from spleens of female OT-I (RAG1\textsuperscript{-/-}) transgenic mice (C57BL/6 background) (a gift from Dr. K. P. Kane) with EasySep mouse CD8\textsuperscript{+} T cells enrichment kit (StemCell Technologies, Vancouver, British Columbia, Canada). Purified OT-I T cells (10\textsuperscript{5}) and 2 × 10\textsuperscript{5} 10 μM SIINFKEK–pulsed mature BMDCs were cocultured in 200 μl of CTL clone medium in 96-well tissue culture–treated plates. On day 4, cultures were supplemented with 10 U/ml IL-2. On day 7, the cells were split and supplemented with fresh media containing 10 U/ml IL-2. Cells were used on day 11 after initiation of the culture.

Peptide pulsing of target cells

EL4 cells were incubated in FCS at 1 × 10\textsuperscript{6} cells/ml with the indicated concentration of NP\textsubscript{366-374} or OVA peptides at 37˚C for 1 h. Cells were then washed with 2% calf serum in RPMI 1640, resuspended in ice-cold 4% calf serum in RPMI 1640, and used as target cells for stimulation of CTL clones.

CTL stimulation with target cells

CTL clones were mixed with target cells in ice-cold 4% calf serum in RPMI 1640 at a ratio of 1:1 and centrifuged at 200 × g for 2 min at 4˚C and then incubated at 37˚C for the indicated time. Where indicated, CTLs were preincubated with 10 μg/ml CHX or carrier control for 45 min and included in the assay or with BAPTA-AM at the indicated concentration for 30 min and then washed out before CTL and target cell conjugate formation. When indicated, 4 μM EGTA/3 mM MgCl\textsubscript{2} were added before mixing CTL and target cells. After stimulation, effector and target cell conjugates were separated with 5 mM EDTA in PBS and then stained for CD8\textsuperscript{a}, FasL, and CD107a. After gating on the CD8\textsuperscript{a} cells to examine only the CTL, the expression of FasL, and CD107a was assessed by flow cytometry.

Isolation of H-2K\textsuperscript{b}

H-2K\textsuperscript{b} was purified from EL4 cells by immunoaffinity chromatography as previously described (19), with modification. Briefly, Triton X-100 lysate from 1 × 10\textsuperscript{10} EL4 cell membrane was passed over a Y3 affinity column preceded by a Sepharose 4B precolumn. Elution was performed with elution buffer: 0.5% sodium deoxycholate, 0.05 M diethylamine, and 650 mM NaCl (pH 11.5). The H-2K\textsuperscript{b} column fractions were characterized by protein assay, ELISA, and alloreactive CTL degranulation response as described previously (17, 19).

Coating latex beads with ICAM-1/H-2-2K\textsuperscript{b} and stimulation of alloreactive CTLs

Latex beads were incubated at 1 × 10\textsuperscript{10} ml with 0.06 μg/ml ICAM-1 purified as previously described (20) (a gift from Dr. A. Kokaji, University of Alberta) and various concentrations of H-2K\textsuperscript{b} at 4˚C overnight with rotation. The concentration of ICAM-1 was selected by incubating latex beads with various concentrations of ICAM-1, then its level on the coated beads was compared by flow cytometry to EL4 cells. A concentration of 0.06 μg/ml ICAM-1 resulted in a similar level to staining as EL4. After incubation, equal volumes of 2% BSA in PBS was added to the latex beads, incubating at room temperature for 30 min with rotation. The latex beads were then washed with 0.1% BSA in PBS and resuspended in ice-cold 4% calf serum (CS) in RPMI 1640 for assay. CTL clones were conjugated with coated latex beads in ice-cold 4% CS in RPMI 1640 at a ratio of 1:2 for 5 min and centrifuged at 200 × g for 3 min at 4˚C and then incubated at 37˚C for the indicated times. After stimulation, beads and target cell conjugates were separated with 5 mM EDTA in PBS and then stained as described above.

Cell staining and flow cytometry

After stimulation, degranulation of CTLs was measured by cell surface expression of CD107a (21) by staining with FITC-conjugated anti-CD107a mAb or FITC-conjugated rat IgG isotype control (BD Pharmingen). In contrast to the previously described method (21), we did not add brefeldin A as a GolgiStop, because there was no difference in CD107a expression with or without the GolgiStop (data not shown). Cell surface FasL was revealed with PE-conjugated anti-FasL mAb or PE-conjugated hamster IgG isotype control (BD Pharmingen) diluted in 5 μM EDTA/4% CS/PBS. All cell surface staining was performed on ice for 30 min. For intracellular FasL detection, cells were fixed with 2% paraformaldehyde for 20 min at room temperature, then stained in permeabilization buffer (0.2% saponin and 4% calf serum in PBS) and analyzed. Data were acquired on a BD Biosciences FACSCalibur flow cytometer and analyzed with CellQuest software (BD Biosciences, San Jose, CA), or a Biosciences FACS\textsuperscript{Canto} flow cytometer and analyzed with FACS\textsuperscript{Divia} software (BD Biosciences) or FlowJo software (Tree Star, Ashland, OR).

35Cr release assay for Ag-specific killing and bystander killing

Target cells were labeled with 100–150 μCi of Na\textsubscript{35}CrO\textsubscript{4} (53Cr) at 37˚C for 1 h in FCS. After washing targets four times with 5% FCS in RPMI 1640, they were plated at 10\textsuperscript{4} 53Cr-labeled cells/well and mixed with AB.1 in V-bottom microtiter plates at indicated E:T ratios in triplicate. For bystander killing assay, 10\textsuperscript{5} 53Cr-labeled non-Ag–bearing target and 10\textsuperscript{5} unlabeled cognate target were mixed with AB.1 at indicated E:T ratios. After a 4-h incubation at 37˚C, plates were centrifuged for 5 min at 500 × g, and 25 μl of supernatant was collected and counted in a MicroBeta Trilux liquid scintillation counter (PerkinElmer). Percent-specific lysis was determined as (experimental release – spontaneous release)/(maximal release – spontaneous release) × 100.

Results

Stored FasL cell surface translocation requires a lower threshold for activation than does de novo FasL expression and degranulation

When the alloreactive CTL clone AB.1 (H-2d anti-H-2b) was stimulated with the L1210 lymphoma target cell sorted for expression of different levels of H-2K\textsuperscript{b} class I MHC (L1210/K\textsuperscript{K}), we found that target cells expressing a lower level of H-2K\textsuperscript{b} induced less de novo FasL cell surface expression measured at 2 h, at which time only de novo, but not stored, FasL is expressed on the cell surface (3), and target cells expressing a higher level of H-2K\textsuperscript{b} induced more de novo FasL cell surface expression (Fig. 1A).
FIGURE 1. Stored FasL translocation is triggered at lower density of antigenic H-2Kb over de novo FasL cell surface expression and degranulation in an allospecific CTL clone. A, CTL clone AB.1 cells were stimulated with cognate target cells L1210/Kb expressing different levels of H-2Kb indicated as Kb-lo (solid line) and Kb-hi (dashed line), as measured by flow cytometry (top panel). Also shown is the isotype control (shaded) and the untransfected L1210 stained with anti-Kb (dotted/dashed line). After AB.1 cells were stimulated with the indicated H-2Kb–transfected L1210 (solid line) or L1210-negative control (dashed line), FasL cell surface expression from the stored pool (15 min) and degranulation were measured (bottom panel). B, CHX-pretreated allospecific CTL clone AB.1 was stimulated with latex beads coated with various concentrations of purified class I MHC or BSA as a control and a fixed concentration of ICAM-1. After 30 min, the bead/cell conjugates were separated with 5 mM EDTA, and the percentage of surface FasL, or CD107a positive AB.1 was determined by flow cytometry. The results were shown as the percentage of the maximum response obtained for stored FasL translocation or degranulation at plateau stimulation for 30 min. Data are representative of three independent experiments.

However, both high and low H-2Kb-expressing target cells induced a similar level of FasL translocation from the stored pool at 15 min (Fig. 1A). The expression at 15 min is entirely from a presynthesized pool of FasL, because these cells were pretreated and assayed in the presence of the protein synthesis inhibitor CHX (3). These data suggested that stored FasL translocation has a lower signaling threshold than de novo-synthesized FasL.

Given that it is difficult to control the Ag levels on the transfected target cell because the cell lines are not stable with respect to levels of class I MHC expression, we next decided to quantitatively compare the stored FasL translocation and degranulation under different stimulation conditions by titrating Ag concentration. To directly measure the effect of class I MHC density on stored FasL translocation, AB.1 was stimulated with latex beads coated with different concentrations of H-2Kb and a level of ICAM-1, which is similar to that expressed on the EL4 target cell as assessed by flow cytometry of the beads (data not shown). We found that AB.1 was capable of translocating stored FasL at low H-2Kb concentration (2 μg/ml), a condition where degranulation was barely detectable (Fig. 1B). Furthermore, stored FasL translocation approached its maximum at a lower H-2Kb concentration than that required for degranulation (Fig. 1B), indicating that stored FasL translocation has a lower signaling threshold of activation than degranulation by AB.1. This experiment was repeated four times, and the average concentration of class I MHC for a 50% response to trigger-stored FasL translocation was 2.5 ± 0.5 μg/ml whereas that required for degranulation was 5.7 ± 0.6 μg/ml. This difference was significant (p < 0.01) and shows that the signaling threshold for FasL translocation is lower than that required for degranulation for an allospecific CTL clone.

We next examined peptide-specific CTL clones, which allowed a better comparison of the effects of TCR signaling strength on the induction of individual cytolytic functions by CTLs through simple peptide titrations. To this aim, CTL clone 3/4, which is specific for the H-2Dd–restricted nucleoprotein NP366-374 epitope (ASNSNEKMTM) derived from the influenza virus A/PR/8/34 (17), was used. We found that clone 3/4 translocated stored FasL at a low NP366-374 concentration (30–60 ng/ml) where degranulation was barely detectable (Fig. 2A). Furthermore, stored FasL translocation approached its maximum at a lower NP366-374 concentration than that required for degranulation (Fig. 2A), indicating stored FasL translocation has a lower signaling threshold of activation than degranulation by clone 3/4. Also, we observed that at a low concentration of NP366-374 (125 ng/ml), stored FasL translocation reached 80% of the maximum, whereas de novo FasL only reached 20% of the maximum response (Fig. 2B), indicating stored FasL translocation to the cell surface has a lower signaling threshold of activation than de novo FasL synthesis by clone 3/4. The differences between the amount of peptide required for a 50% response is significantly different between stored FasL translocation compared with degranulation and de novo FasL synthesis (Fig. 2C). These data demonstrate that stored FasL has a lower threshold for induction of translocation than for degranulation or FasL synthesis on a peptide-specific CTL clone.

Both AB.1 and clone 3/4 are in vitro-cultured untransformed CTL clones. We next wanted to examine whether the observations with these cultured CTL clones can be reproduced with freshly generated CTLs. To this aim, OT-I CTLs, which is specific for the H-2Kb–restricted SIINFEKL peptide (OVAp), were generated by coculture of purified splenic CD8+ T cells derived from OT-I (RAG1−/−) transgenic mice with LPS-matured BMDCs. By day 11 after initiation of the culture, FasL disappeared from the cell surface and was stored intracellularly (Fig. 3A), similar to in vivo-primed allospecific splenic CD8+ T cells (data not shown). We also detected a significant amount of granzyme B stored in these cells (Fig. 3A), indicating both effector mechanisms are developed in the freshly generated OT-I–derived CTLs. Similar to the results from AB.1 and clone 3/4, we found that with OT-I CTLs, stored FasL was preferentially translocated over degranulation (Fig. 3B) and de novo FasL synthesis (Fig. 3C) at low SIINFEKL concentration (0.1 nM). Taken together, these results imply that low-level TCR stimulation singularly stimulates stored FasL translocation and thus is an inherent property of CTLs because it is observed with CTLs from multiple sources with differing Ag specificities.

SIINFEKL (OVAp) is a high-affinity agonist for OT-I TCRs, whereas SIIIFKEKL (G4) is a low-affinity agonist for OT-I TCRs (22, 23), which provided an additional method to determine the effect of decreasing TCR signaling strength on the induction of individual effector mechanisms. We found that 1 μM G4 elicits
stored FasL translocation to ∼75% of the level that is induced by 1 μM OVAp (Fig. 3D). De novo FasL cell surface expression was also stimulated by G4; however, this was just >30% of the response triggered by OVAp (Fig. 3D). Cells exhibited only minimal degranulation in response to G4 (Fig. 3D). Overall, these results indicate that stored FasL is preferentially translocated over de novo FasL cell surface expression and degranulation. Overall, these results indicate that stored FasL is preferentially translocated over de novo FasL cell surface expression and degranulation.

Intracellular calcium is required for stored FasL translocation

Degranulation depends on both intracellular and extracellular Ca2+ (24–26). We previously showed that de novo FasL cell surface expression requires extracellular Ca2+, whereas stored FasL cell surface expression requires intracellular calcium. Intracellular calcium is required for stored FasL translocation.
surface translocation is extracellular Ca\textsuperscript{2+} independent (3). In the current study, we sought to further address whether Ca\textsuperscript{2+} flux from intracellular stores is necessary for FasL translocation from internal stores. The membrane-permeant intracellular Ca\textsuperscript{2+} chelator BAPTA-AM was used to pretreat AB.1 CTLs, then its effect on stored FasL cell surface translocation and de novo FasL cell surface expression was examined upon target cell engagement. We found that, as with de novo FasL cell surface expression and degranulation, BAPTA-AM does inhibit stored FasL cell surface expression (Fig. 4), suggesting that Ca\textsuperscript{2+} flux from intracellular stores is necessary and sufficient for cell surface translocation of the stored pool of FasL, whereas both intracellular and extracellular sources are required for both degranulation and de novo FasL synthesis.

Cell surface FasL derived from intracellular stores has a fast turnover rate

Because only a transient calcium signal is required for translocation of the stored FasL, we hypothesized that the expression of the translocated FasL on the cell surface would be very transient. We found that kinetics of the stored FasL cell surface translocation depends on TCR signaling strength. Target cells expressing higher levels of class I MHC induced faster stored FasL translocation in CTL AB.1, which peaked at 15 min then underwent a rapid turnover, whereas target cells expressing lower levels of class I MHC elicited slower FasL kinetics of appearance at and disappearance from the cell surface (Fig. 5). Nonetheless, FasL derived from the intracellular vesicles disappeared from the cell surface by one hour after the initiation of target cell conjugation (Fig. 5). It is clear that FasL surface expression occurs quickly after target cell conjugation and appears to remain on the surface as long as conjugation is occurring. It is very difficult to measure the duration of conjugation using standard conjugation assays, however we found that an intact cytoskeleton, which is required to maintain conjugation, is required for \textasciitilde30–40 min for AB.1 to degranulate (27). Taken together, we found FasL cell surface expression from the stored pool has a fast turnover rate, and the natural kinetics of translocated FasL on the cell surface mirrors conjugation and might ensure the directional mobilized FasL is being presented exclusively to the specific target cells that initiate its translocation.

De novo synthesized FasL, but not stored translocated FasL, mediates bystander killing

Previous studies have shown that once FasL is expressed on the cell surface, it can mediate killing of any target that expresses cell surface Fas (28–30); however, the rapid turnover of the FasL from the internal stores makes it unlikely that this would mediate efficient bystander lysis. However, the two different sources of FasL expression could contribute to the death of Fas-expressing target cells in a short-term (4 h) killing assay, so the relative contribution of the stored or de novo FasL to various aspects of CTL-mediated lysis is unknown. We have also shown that the transient FasL translocation from internal stores to the cell surface, although low, is sufficient to induce target cell killing (3). Because stored FasL remains only transiently on the cell surface during conjugation, we

![FIGURE 4. Intracellular calcium is required for FasL translocation. AB.1 cells were pretreated with carrier control or the indicated concentrations of BAPTA-AM, then stimulated with cognate target cell L1210/K\textsuperscript{b} or negative control L1210 for either 15 min for stored FasL (A) and CD107a expression (C) or 2 h for de novo FasL, expression (B). Cells were stained with CD8a (PE.Cy5), FasL (PE), and CD107a (FITC). After gating on the CD8\textsuperscript{+} cells to examine only the CTLs, the expression of FasL and CD107a was assessed. The percentage of AB.1 positive for stored FasL (A), de novo FasL (B), or CD107a (C) expression upon L1210/K\textsuperscript{b} stimulation was determined at different drug concentrations by subtracting the percentage of positive cells by that of L1210 engagement alone, which was never \textasciitilde10\% for FasL and 0\% for CD107a. Representative data are shown from two independent experiments where excess BAPTA-AM was washed out after the preincubation. Three independent experiments with BAPTA-AM present during stimulation were also conducted, with similar results. Cell viability, as measured by trypan blue exclusion, was not affected by BAPTA-AM treatment during the period of the assay.](http://www.jimmunol.org/)

![FIGURE 5. Translocated FasL expression peaks at 15 min and is rapidly cleared from the cell surface. The top panel shows the flow cytometry histogram for the different H-2K\textsuperscript{b} levels on the transfected L1210 cells used for these experiments, as was done in Fig. 1. The lower panel shows the kinetics of stored FasL cell surface expression upon target cell engagement. CHX-pretreated AB.1 cells were stimulated with the indicated cognate L1210/K\textsuperscript{b} target cell or negative control L1210 for indicated time. The percentage of cells positive for surface FasL-positive AB.1 cells upon L1210/K\textsuperscript{b} engagement was assessed, with the value subtracted by that of L1210 engagement. Data are representative of three independent experiments.](http://www.jimmunol.org/)
wanted to determine whether translocated FasL from intracellular stores mediates cognate target cell killing or bystander killing of non-Ag–bearing cells.

AB.1 (anti-H-2b) CTLs were mixed with 51Cr-labeled target cells L1210/Kb, EL4 (H-2b), L1210 (H-2d), or L.Fas (H-2d) target cells to confirm the specificity of target cell killing (Fig. 6, top left panel). The killing assay was also performed in the presence of EGTA to inhibit degranulation-dependent killing and de novo FasL-dependent killing but would allow for killing by the rapidly translocated stored FasL. EL4, which expresses significant levels of Fas, was killed in the absence of Ca2+ as has been reported previously (25); however, the L1210/Kb target cell, which expresses very low levels of Fas, was only marginally killed, confirming the Fas dependence of this killing (Fig. 6, top right panel).

To examine bystander killing of noncognate target cells, AB.1 was mixed with 51Cr-labeled L.Fas (Fas-transfected L1210) bystanders together with unlabeled L1210/Kb or EL4. 51Cr-labeled L1210 bystander cells (with very low Fas) were used as a negative control. We found that cognate target cells induced significant bystander killing of Fas+ L.Fas but not Fas− L1210 by AB.1 (Fig. 6), indicating the bystander killing was mediated by FasL in this system and not degranulation. To further distinguish the source of FasL that contributed to the bystander killing, we examined this killing in the presence of EGTA, which completely inhibited all bystander killing (Fig. 6). These data suggest that de novo-synthesized FasL, which is extracellular Ca2+ dependent, is mediating this bystander killing.

To confirm the contribution of the different sources of FasL to bystander killing, we performed the same assay as described above but used CHX to inhibit de novo synthesis of FasL. CHX had a minimal effect on direct lysis of both EL4 and L1210/Kb (Fig. 7), suggesting that newly synthesized FasL contributes minimally to direct lysis by this CTL clone. However, as was observed above, CHX completely abolished all bystander killing of the L.Fas cell (Fig. 7), confirming that the bystander killing is mediated primarily by the newly synthesized FasL. These data indicated that stored FasL translocation induces Ag-specific but not bystander killing, whereas de novo-synthesized FasL induces significant bystander killing. These results also suggested that, in this system, degranulation does not contribute to bystander killing, for which conflicting data exist (31, 32). Taken together, these observations clearly demonstrate that translocated FasL from intracellular stores and degranulation mediate specific target cell killing, and in contrast, significant bystander killing of innocent target cells is attributed to de novo-synthesized FasL.

**Discussion**

In this study, we quantitatively examined signal thresholds leading to stored FasL and de novo-synthesized FasL expression and degranulation, and we have shown that at a low level of signaling strength, stored FasL is preferentially translocated to the cell.
surface over de novo FasL synthesis and degranulation. We further demonstrated that the stored, translocated FasL mediates highly specific CTL-mediated killing, whereas the significant bystander killing that has been documented for FasL (10, 12, 33, 34) we have attributed to the de novo-synthesized FasL. These results suggest that stored FasL and newly synthesized FasL are used in distinct biological contexts. Interference with Ag presentation is used by some viruses and tumors as an immune evasion strategy (35–38). FasL from stored pools could contribute significantly to the antiviral, antitumor immunity when class I MHC expression or the presented viral/tumor Ag level is reduced. Additionally, FasL from the intracellular stores might be used under circumstances of low-level infection and may be required to clear the final pathogens after infection to play a preferential role during low-level chronic infections. In contrast, when there is a massive virus infection that has infected large numbers of cells, it might be advantageous for the CTL to kill all surrounding cells, sacrificing some noninfected cells to quickly clear the virus. Indeed, recent studies have suggested that FasL may be important for clearing chronic infections (7, 8). It would be of interest to investigate the contribution from particular FasL pools (stored versus de novo synthesized) for the antiviral immunity during these infections.

Recently, Jenkins et al. (39) compared D^bNP^366- and D^bPA^224-specific CTLs recovered directly from the lungs of mice with influenza pneumonia and found that despite the higher avidity and longer duration of contact between D^bPA^224-specific CTLs and target cell, it took significantly more time to induce target cell apoptosis than lower-avidity, D^bNP^366-specific CTLs, perhaps as a result of the longer duration of contact between the two cell types. This observation suggests that lower TCR/epitope avidity may be more beneficial than higher epitope avidity for cell-mediated immunity. This lower avidity might result in a shorter contact time that may allow individual CTLs to lyse more targets. Our data presented here raise the possibility that different TCR signaling strengths may induce qualitatively and quantitatively different killing mechanisms in different CTLs.

TCR signal strength plays a critical role in regulating various aspects of T cell biology, including lineage fate decisions (40), differentiation (41, 42), cytokine production (43), cytotoxicity (44, 45), and the induction of distinct tissue-homing receptors (46). At a very low Ag level, subtle changes in Ag dose can be translated into significant biological effect. Purbho et al. (47) showed that CTLs are able to detect even a single peptide/MHC complex at the T cell–target interface but required as few as three specific peptide/MHC complexes to induce cytotoxicity, whereas ~10 complexes of peptide/MHC were required to achieve maximal calcium increase and to form a mature synapse. It was shown that OT-I CTLs induced target cell death accompanied by intense membrane blebbing, an early marker of apoptosis, 5–15 min after initiation of conjugation (47). Interestingly, they found that blocking of Fas-mediated killing reduced the occurrence of membrane blebbing in target cells containing 3–30 peptide/MHC complexes at the CTL–target interface from 100 to 15%, implying that FasL-induced killing may predominate at the lowest Ag density (47). On the basis of our studies, we predict that this killing was mediated by FasL from intracellular stores and not newly synthesized FasL.

Directional delivery of cytolytic granule contents toward Ag-bearing cells is considered to be an important property of CTL-mediated target cell killing. However, it appears that T cells use two distinct pathways for directional cytokine and chemokine secretion; some target to the immunological synapse to ensure a local Ag-specific effect, and others deliver biologically active molecules in a multidirectional way, which might facilitate the establishment of an inflammatory milieu to affect cells at a distance (48). In the current study, we provide evidence to suggest that FasL from a stored pool undergoes transient, Ag-specific killing, whereas de novo-synthesized FasL leads to significant bystander killing. There are precedents for a single cytokine/chemokine to undergo both directional polarization and less confined delivery, depending on the pool from which it is derived (stored versus de novo synthesized). Human CD8^+ T cells polarized prestored RANTES (CCL5) toward the target cell contact area (49), whereas de novo-synthesized RANTES underwent multidirectional secretion by murine Th1 cells (48), although different cell sources might account for the disparate results observed. Also, it was shown in the same Th1 cells that TNF was detected at the immunological synapse 30 min after stimulation but distributed throughout the cell 2–3 h after initial stimulation (48).

We showed that FasL from a pre-existing intracellular pool is rapidly (15–30 min) expressed on the cell surface in response to TCR engagement (Fig 5). This rapidly expressed FasL also appears to quickly disappear from the cell surface before target cell release. At least two, nonmutually exclusive, possibilities might account for this rapid disappearance of early FasL. One is that FasL might be cleaved by matrix metalloproteinase (MMP), because FasL has been shown to be cleared from the cell surface (50). However, we found that an MMP inhibitor did not substantially increase the amount of rapid FasL surface expression from stored pools and only slightly delayed the disappearance of FasL from the cell surface (data not shown). This is in contrast to the cell surface expression of the later de novo-synthesized FasL, which was increased by 30–40% (data not shown) in the presence of MMP inhibitors. This suggests that cleavage is not the primary means by which the rapidly expressed FasL is cleared from the cell surface. This is consistent with a previous study showing that 12-O-tetradecanoylphorbol-13-acetate/ionomycin–induced early FasL expression in human T cells was not MMP sensitive (51). We speculate that FasL molecules from different pools might undergo different modification, which endows their distinct MMP sensitivity, a possibility that needs to be investigated in the future. A second potential mechanism for the loss of FasL, from the cell surface is by endocytosis because endocytosis inhibitors prevented the loss of surface FasL (52). Interestingly, we also found significantly increased and prolonged early FasL cell surface expression by cross-linked anti-CD3 stimulation in the presence of cytochalasin D (data not shown), which might suggest that endocytosis could contribute to this rapid FasL disappearance. Further studies will need to be done to determine how the FasL transported to the cell surface from internal stores is so rapidly cleared from the cell surface.

FasL on CTLs may be important for the generation of autoimmune disease. Fas/FasL is required for the induction of diabetes in NOD mice (53, 54). Intriguingly, as NOD mice age, their islet-associated autoreactive CTLs for a β-cell epitope (55). Furthermore, it was demonstrated that despite the preferential use of the Fas/FasL pathway for cytology of β-cell targets, transgenic β-cell–specific CTLs derived from NOD mice were able to kill targets via the perforin pathway when triggered by a higher-affinity stimulus (55). These results indicate that TCR signaling strength might play an important role in determining which killing mechanisms are elicited and are consistent with our findings presented here.

Differences in CTL responses relative to differences in signaling thresholds have been observed in an infection model. Zelinsky et al. (57) showed that during acute Friend murine leukemia virus (F-MuLV) infection, the replication level of F-MuLV was the critical factor that determined the differential expression of cytotoxic molecules by CTLs and the pathway of CTL cytotoxicity;
The low-replication infection induced CTLs expressing solely FasL, but not the cytotoxic molecules granzymes A and B, whereas the high-replication infection resulted in induction of CTL-secreting molecules of the granule exocytosis pathway. It is possible that CTL signaling strength determines differential activation of CTL expressing the different lytic mechanisms in F-MuLV–specific CTLs. This suggests that differences in Ag threshold could also control the differentiation, as well as the switch of the different lytic mechanisms in CTL.

In summary, in the current study, we showed that stored FasL has a lower signaling threshold of activation than de novo FasL synthesis and degradation. We provided further evidence suggesting that stored FasL translocation mediates highly specific killing and is rapidly cleared from the surface as conjugates dissolve, whereas de novo-synthesized FasL mediates significant bystander killing. It is not clear why CTLs express FasL on the cell surface from two differentially regulated sources, but our data showing that they have different response thresholds and differentially mediate bystander killing suggest that the two sources of FasL are used in distinct biological contexts. Further studies are required to examine the biological importance of these two distinct sources of FasL in CTLs.

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Disclosures
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References


