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Jin-Shu He and Hanne L. Ostergaard

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# CTLs Contain and Use Intracellular Stores of FasL Distinct from Cytolytic Granules<sup>1</sup>

Jin-Shu He and Hanne L. Ostergaard<sup>2</sup>

CTL lyse target cells through the release of cytolytic granule contents and cell surface expression of Fas ligand (FasL). Current models suggest that FasL is stored in cytolytic granules and that FasL cell surface expression would be subject to the same controls as degranulation. We demonstrate that murine CTLs undergo two waves of FasL cell surface expression after stimulation. The first wave is from a pre-existing pool of FasL, and the second wave requires new protein synthesis. Signaling for FasL expression appears to be finely tuned as a weak signal preferentially induced surface translocation of the stored FasL, whereas a strong signal preferentially triggered the expression of de novo synthesized FasL. The early FasL is differentially regulated from degranulation, as there were multiple circumstances whereby rapid FasL cell surface expression and FasL-dependent killing occurred in the absence of detectable degranulation. Furthermore, we found through confocal microscopy that stored FasL resides in vesicles distinct from cytolytic granules. Our data clearly show that CTL degranulation and FasL lytic mechanisms are fully independent with respect to stored component localization and regulation. *The Journal of Immunology*, 2007, 179: 2339–2348.

CTL are differentiated CD8<sup>+</sup> T cells that eliminate tumor cells and cells infected with intracellular pathogens. CTLs lyse target cells by at least two distinct well-characterized general mechanisms: extracellular Ca<sup>2+</sup>-dependent degranulation of cytolytic molecules, including perforin and granzyme B; and expression of Fas ligand (FasL),<sup>3</sup> which induces apoptosis of Fas-expressing target cells (1). In addition to their defense function, these two cytolytic mechanisms also play crucial roles in homeostatic regulation and contribute to pathogenesis in many different systems (2).

CTLs have specialized secretory lysosomes (3). Upon engagement of a target cell bearing appropriate peptide-MHC complexes, cytolytic granules move along microtubules to the contact point with the target cell (4), then a fraction of the granules fuse with the plasma membrane (5, 6), and the contents are directionally released to the target cell resulting in rapid target cell death (3). In contrast, the regulation of FasL expression on the CTL cell surface is not fully established, and there are several conflicting studies in the literature. FasL is not constitutively expressed on CTLs and must be induced (7, 8). FasL up-regulation was shown to require extracellular Ca<sup>2+</sup> (7, 8); however, the lytic phase of FasL-mediated lysis could occur in the absence of extracellular Ca<sup>2+</sup>. In contrast, our data indicate that both the induction phase and lytic

phases of degranulation-independent lysis can occur in the absence of extracellular Ca<sup>2+</sup> (9, 10). FasL up-regulation was also shown to require protein synthesis (7, 8); however, Li et al. (11) suggested that FasL expression on ex vivo CTL did not require protein synthesis and that there were pre-existing pools of FasL. Consistent with the existence of presynthesized FasL pools, FasL was shown to localize in lysosomal lytic granules and was subject to degranulation-controlled cell surface delivery (12, 13). These conflicting observations regarding FasL expression remain to be resolved.

In the current study, we investigated TCR-regulated FasL cell surface expression upon target cell engagement in alloreactive CTL clones and in vivo primed CD8<sup>+</sup> T cells. We reveal that there are two waves of FasL cell surface expression: a rapid, extracellular Ca<sup>2+</sup>-independent, preformed FasL expression and a delayed, extracellular Ca<sup>2+</sup>-dependent, de novo FasL expression. Also, our data indicate that FasL is stored in a regulated secretion compartment distinct from cytolytic granules, by confocal microscopy. Furthermore, we show that preformed functional FasL undergoes TCR-regulated cell surface expression that can be distinguished from the exocytosis of cytolytic granules. Finally, further investigation of FasL expression in different CTL clones reveals that not all CTLs are equal in their ability to mobilize preformed FasL to the cell surface upon target cell engagement, suggesting an additional level of regulation leading to cell surface transport of preformed FasL.

## Materials and Methods

### Cells

The murine alloreactive CD8<sup>+</sup> CTL clones AB.1 (H-2<sup>d</sup> anti-H-2<sup>b</sup>) and c111 (H-2<sup>k</sup> anti-H-2<sup>b</sup>) were described previously (14, 15). Clones were maintained by weekly stimulation with irradiated C57BL/6 splenocytes and a minimum amount of IL-2 and used 5–6 days later. The L1210 lymphoma cell line expressing chimeric class I MHC (L1210/K<sup>b</sup>) was a gift from Dr. Kevin Kane (University of Alberta, Edmonton, Canada) (16). Cell lines L1210, L1210/K<sup>b</sup>, EL4, NIH3T3, and COS-1 were grown in DMEM supplemented with FBS.

### Abs and reagents

The hybridoma-producing 145-2C11 (anti-CD3e) was obtained from the American Type Culture Collection. PE-conjugated anti-FasL mAb (MFL3), biotin-conjugated anti-FasL mAb (MFL3), anti-CD107a mAb (1D4B), FITC-conjugated anti-CD107a mAb (1D4B), PE-Cy5-conjugated

Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, Canada

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<sup>2</sup> Address correspondence and reprint requests to Dr. Hanne L. Ostergaard, Department of Medical Microbiology and Immunology, 670 Heritage Medical Research Centre, University of Alberta, Edmonton, Alberta, T6G 2S2, Canada. E-mail address: hanne.ostergaard@ualberta.ca

<sup>3</sup> Abbreviations used in this paper: FasL, Fas ligand; CHX, cycloheximide; LAMP1, lysosomal-associated membrane protein-1; MMP, matrix metalloprotease; PEL, peritoneal exudate lymphocytes; CS, calf serum.

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anti-CD8 $\alpha$  (Ly-2) mAb (53-6.7) and anti-active caspase-3 mAb (C92-605) were purchased from BD Pharmingen. Anti-cathepsin D polyclonal Ab (G-19), anti-granzyme B polyclonal Ab (N-19) and anti-perforin1 polyclonal Ab (H-315) were purchased from Santa Cruz Biotechnology. Rabbit anti-hamster IgG and FITC-conjugated donkey anti-rabbit IgG were purchased from Jackson ImmunoResearch Laboratories. Alexa Fluor 546- and Alexa Fluor 488-conjugated streptavidin and secondary Abs were purchased from Molecular Probes. Cycloheximide (CHX), EGTA, and colchicine were purchased from Sigma-Aldrich.

#### *In vivo* alloantigen priming and preparation of peritoneal exudate lymphocytes (PEL)-derived CD8<sup>+</sup> T cells

Female BALB/c mice 8–10 wk old were primed by i.p. injection of  $2 \times 10^7$  EL4 cells. From 10 to 12 days after priming, the mice were sacrificed, and PEL from five mice were harvested by rinsing peritoneal cavities with PBS and pooled. No tumor cells were detectable in the crude PEL. CD8<sup>+</sup> T cells were purified with MACS CD8<sup>+</sup> T cell isolation kit (Milteny Biotech) and used immediately for assay. All animal studies have been approved by the University Animal Policy and Welfare Committee at the University of Alberta.

#### COS-1 cell transient transfection

COS-1 cells were seeded on glass coverslips 24 h before transfection. Transient transfection of COS-1 cells with mouse FasL cDNA in PSR $\alpha$ SD7 (gift from Dr. John Elliott, University of Alberta) was conducted using Effectene (Qiagen). FasL expression was examined by confocal microscopy 24 h posttransfection.

#### Target cell stimulation of CTL

To discriminate between effector and target cells, target cells were stained with PKH67 green or PKH26 red fluorescent dye (Sigma-Aldrich) according to the manufacturer's instructions, with modification. Briefly,  $10^7$  cells were washed in PBS and resuspended in 400  $\mu$ l of the diluent C provided with the dye. The dye was diluted to 10  $\mu$ M in 400  $\mu$ l of diluent C. Equal volumes of cells and dye were combined and incubated for 3 min at room temperature, followed by addition of 2 ml of calf serum (CS) and further incubation for 1 min to adsorb the excess dye and stop further uptake. Cells were then extensively washed, incubated for 1 h in 4% CS in PBS at 37°C, and then washed once and resuspended at  $1 \times 10^6$  cells/ml in ice cold 4% CS in RPMI for assay.

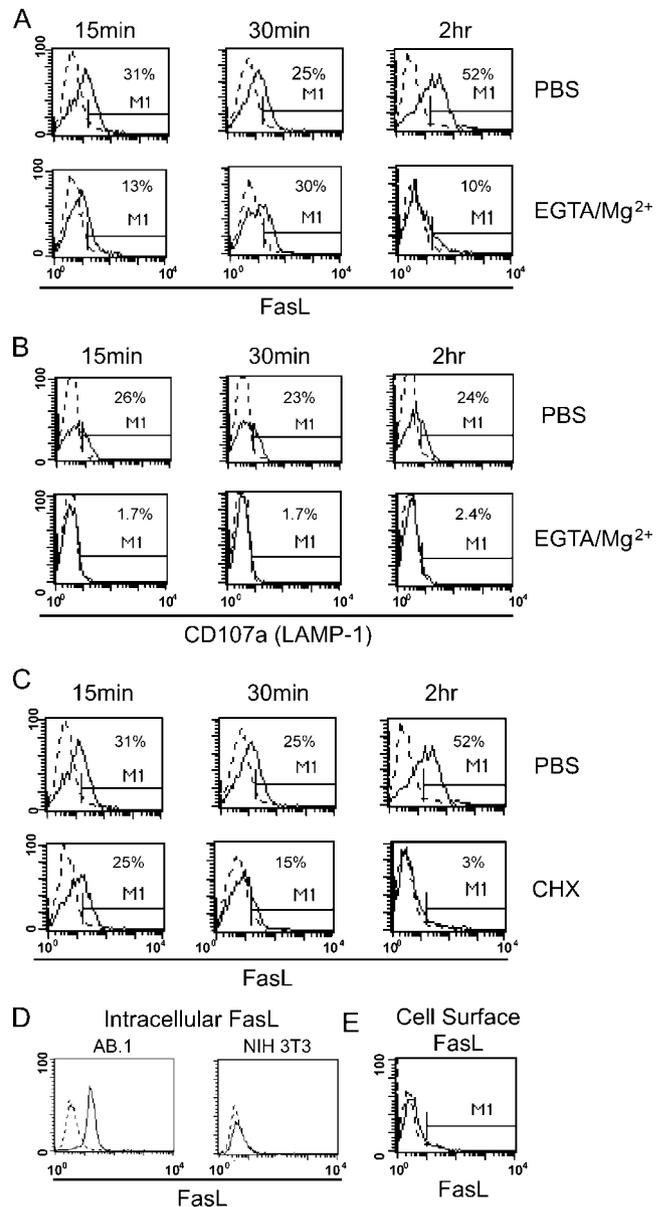
CTL clone or purified CD8<sup>+</sup> T cells were conjugated with stained target cells in ice cold 4% CS in RPMI at a ratio of 1:1 and centrifuged at  $200 \times g$  for 3 min at 4°C and then incubated at 37°C for the indicated times. Where indicated, cells were preincubated with 10  $\mu$ g/ml CHX, colchicine at the indicated concentration, 4 mM EGTA, 3 mM MgCl<sub>2</sub>, or carrier control for 20–45 min and included in the assay. After stimulation, effector and target cell conjugates were separated with 5 mM EDTA in PBS and then stained for FasL and CD107a.

#### Ab stimulation of CTLs

For immobilized anti-CD3 mAb stimulation, wells of a 24-well flat bottom Falcon 3912 microtiter plate (BD Biosciences) were coated with 10  $\mu$ g/ml 145-2C11 overnight at 4°C. Wells were washed and blocked with 2% BSA in PBS; then  $10^6$  CTLs were added to each well and incubated at 37°C for the indicated times. After stimulation, cells were detached from the plate with ice cold 5 mM EDTA in PBS and then stained for FasL and CD107a. For soluble, cross-linked anti-CD3 mAb stimulation,  $10^7$  cells/ml in PBS containing 2% CS were incubated with 20  $\mu$ g/ml 145-2C11 on ice for 15 min and then washed and resuspended at  $2 \times 10^6$  cells/ml. Rabbit anti-hamster IgG was added to 2  $\mu$ g/ml, and cells were incubated at 37°C for the indicated time, washed and then stained for FasL and CD107a.

#### Cell staining and flow cytometry

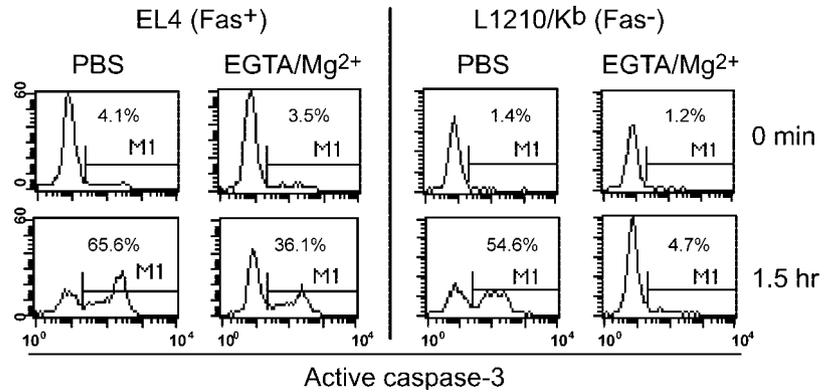
After stimulation and disruption of CTL-target cell conjugates as described above, degranulation of CTL was measured by cell surface expression of CD107a (17) by staining with FITC-conjugated anti-CD107a mAb or FITC-conjugated rat IgG isotype control (BD Pharmingen). In contrast to the previously described method (17), we did not add brefeldin A as a Golgi stop because there was no difference in CD107a expression with or without the Golgi stop (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 mM 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



**FIGURE 1.** Target recognition by CTLs stimulates both extracellular Ca<sup>2+</sup>-independent, stored pools of FasL and extracellular Ca<sup>2+</sup>-dependent, de novo synthesized FasL cell surface expression. CTL clone AB.1 was incubated in the presence or absence of 4 mM EGTA/3 mM Mg<sup>2+</sup> with cognate target cell L1210/K<sup>b</sup> (—) or untransfected L1210-negative control (---). After the indicated time, the specific conjugates were separated with 5 mM EDTA, and surface FasL (A) or CD107a (B) on gated AB.1 was determined by flow cytometry. Indicated values are the percentage of FasL (A) or CD107a (B)-positive AB.1. C, AB.1 was preincubated with 10  $\mu$ g/ml CHX for 45 min, then stimulated with cognate target cells L1210/K<sup>b</sup> (—) or negative control L1210 (---), and assessed for surface FasL expression at the indicated time. (Data presented in A and C are from the same experiment.) D, Unstimulated AB.1 or NIH3T3 cells as a FasL-negative control were subjected to intracellular FasL staining (—) or isotype Ab control staining (---) and analyzed by flow cytometry. E, Basal cell surface FasL level on unstimulated AB.1 was measured (—). Background (---) represents the isotype Ab staining. In all cases, data are representative of at least three independent experiments. The M1 gate contains positive events.

temperature and then stained in permeabilization buffer (0.2% saponin/4% CS in PBS) and analyzed. Data were acquired on a BD Biosciences FACScan flow cytometer and analyzed with CellQuest software (BD Biosciences).

**FIGURE 2.** The early extracellular  $\text{Ca}^{2+}$ -independent FasL expression is sufficient to induce apoptosis of  $\text{Fas}^+$ , but not  $\text{Fas}^-$ , target cells. AB.1 was incubated for the indicated time with EL4 ( $\text{Fas}^+$ ) or L1210/ $\text{K}^b$  ( $\text{Fas}^-$ ) target cells in the presence or absence of 4 mM EGTA, 3 mM  $\text{MgCl}_2$ . Cells were stained with an Ab specific for activated caspase-3 as an early marker of target cell apoptosis. Data signify expression of intracellular active caspase-3 on the gated target cell population and are representative of at least three independent experiments.



#### Target cell active caspase-3 assay for CTL-mediated cytotoxicity

Target cell apoptosis was assessed by the activation of caspase-3 as described (18), with modification. Briefly, CTL was labeled with PKH26 red dye as described earlier. CTLs ( $8 \times 10^5$ ) were combined with  $2 \times 10^5$  target cells at a ratio of 4:1 and pelleted at  $200 \times g$  for 3 min at  $4^\circ\text{C}$ , followed by incubation for 1.5 h at  $37^\circ\text{C}$  in the presence or absence of 4 mM EGTA, 3 mM  $\text{MgCl}_2$ . After incubation, conjugates were disrupted with 5 mM EDTA in PBS. Cells were stained in permeabilization buffer with anti-active caspase-3 mAb followed by FITC-conjugated donkey anti-rabbit IgG and analyzed by flow cytometry.

#### Conjugate assay

CTL were labeled with PKH26 red dye and equal numbers of CTL clone AB.1 and target cells were mixed, pelleted at  $200 \times g$  for 3 min at  $4^\circ\text{C}$ , and incubated for 15 min at  $37^\circ\text{C}$ . After incubation for the indicated times, the cells were vortexed to separate nonspecific binding and fixed with 2% paraformaldehyde and analyzed by FACS. The percentage of conjugates was evaluated on red-positive cells by analyzing forward scatter vs red fluorescence (19).

#### Confocal microscopy

CTLs were adhered to BD BioCoat poly-L-lysine coverslips (BD Bioscience) for 10 min at room temperature, and fixed with methanol for 10 min at  $-20^\circ\text{C}$ . Cells were washed and blocked with 1% FBS in PBS. Cells were stained with the indicated primary Ab followed by the appropriate labeled secondary Ab. When biotinylated Abs were used, coverslips were blocked with 0.1 mg/ml NeutrAvidin (Pierce Biotechnology) and 1% BSA diluted in PBS. As secondary Ab background and cross-reactivity control, each cell image was stained with one first Ab and appropriate two secondary Abs. In all cases, secondary Ab background was negligible and showed species-specific staining. The coverslips were mounted with glycerol-PBS (9:1). Samples were examined with a Zeiss LSM510 confocal microscope. Plan-Apochromat  $63 \times / 1.4$  oil DIC objective lens was used. Z-stack images (interval,  $0.2 \mu\text{m}$ ) were acquired and subjected to three-dimensional reconstruction and deconvolution using SoftWoRx software (Applied Precisions). For each marker image, five areas under the microscope were randomly selected; three cells in each area were further randomly selected to undergo Z-stack scanning.

## Results

### Both extracellular $\text{Ca}^{2+}$ -independent and -dependent FasL cell surface expression occur on CTLs upon target cell recognition

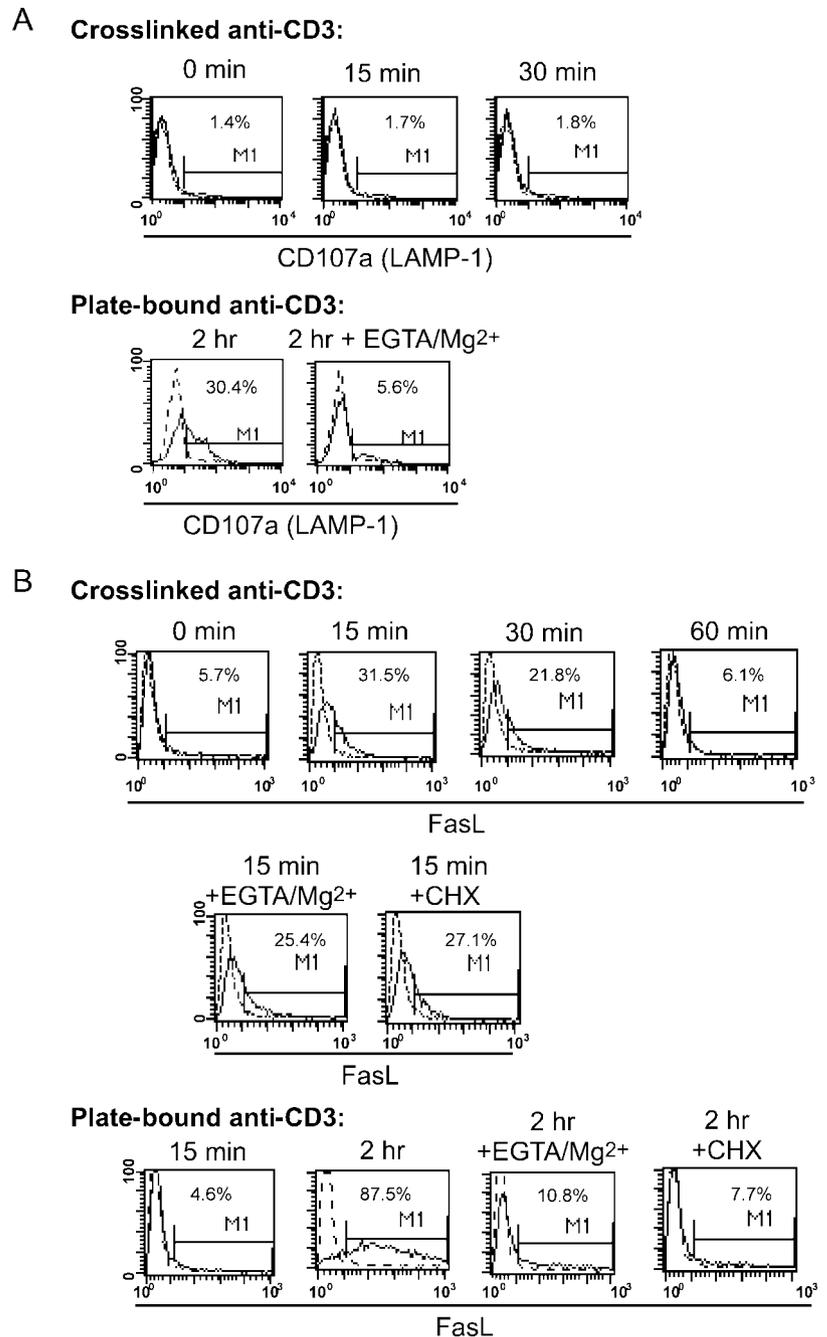
When alloreactive mouse AB.1 CTL clone cells were stimulated with its cognate target cells, L1210/ $\text{K}^b$ , there was rapid expression of FasL on the cell surface (Fig. 1A). Substantial expression was detected at 15 min and increased until 2 h. Surprisingly,  $\text{Ca}^{2+}$  chelation with EGTA revealed that there are two phases of FasL expression on the cell surface: a rapid extracellular  $\text{Ca}^{2+}$ -independent phase, followed by a later extracellular  $\text{Ca}^{2+}$ -dependent phase. In contrast, no FasL cell surface expression was observed upon negative control target cell L1210 conjugation (Fig. 1A). Although the early phase of FasL expression is extracellular  $\text{Ca}^{2+}$  independent, its expression exhibited some delay under  $\text{Ca}^{2+}$  che-

lation when L1210/ $\text{K}^b$  was used as a target (Fig. 1A). This delay, however, did not occur when AB.1 was stimulated with EL4 target cells (data not shown). We can conclude that at the population level, there are two waves of FasL surface expression on the CTL clone in response to target cell engagement. It is unclear whether it is the same cells that undergo two waves of FasL expression, but of the two waves of FasL that are expressed on the CTL surface, the early wave is likely translocated from internal  $\text{Ca}^{2+}$ -independent stores and the latter wave is  $\text{Ca}^{2+}$ -dependent.

Conventional assays to examine CTL exocytosis of cytolytic granules, which generally measure granzyme A activity from CTL supernatants, evaluate degranulation at a population level, whereas the FasL experiments determine the expression on individual cells by FACS. To more directly compare FasL cell surface expression and degranulation, we used a modification of the sensitive FACS-based degranulation assay used by Wolint et al. (17) whereby degranulation is measured by the appearance of cell surface CD107a (lysosomal-associated membrane protein-1; LAMP1), which is found in CTL granules, to re-examine the extracellular  $\text{Ca}^{2+}$  requirement for degranulation. Consistent with conventional enzymatic assays, CTL degranulation, as measured by CD107a cell surface expression, stimulated by target cells, absolutely required extracellular  $\text{Ca}^{2+}$  (Fig. 1B). Taken together, these observations indicate that the early FasL cell surface expression and degranulation occur downstream of distinct signaling pathways with respect to their extracellular  $\text{Ca}^{2+}$  requirements.

### Presynthesized FasL undergoes rapid TCR-regulated mobilization to the cell surface upon target cell engagement

Given the rapid expression of extracellular  $\text{Ca}^{2+}$ -independent FasL at the cell surface, we next examined whether de novo protein synthesis is required for this to occur. Pretreatment with the protein synthesis inhibitor CHX did not have a significant impact on early FasL cell surface expression (15 min) by AB.1 CTL upon target cell engagement (Fig. 1C), indicating that new protein synthesis was not required to observe cell surface FasL at the early time point. In contrast, de novo protein synthesis was absolutely required for the delayed (2-h), extracellular  $\text{Ca}^{2+}$ -dependent FasL cell surface expression (Fig. 1C). Flow cytometry revealed a significant basal level of intracellular FasL (Fig. 1D), but there was little cell surface FasL expressed in resting CTL clone AB.1 (Fig. 1E). Constitutive cellular FasL expression was confirmed by immunoblotting (data not shown). Because of the high basal level of cellular FasL protein, but only inducible expression on the cell surface, we conclude that there is a pre-existing pool of FasL, which is stored in a TCR-regulated secretory compartment in CTL,



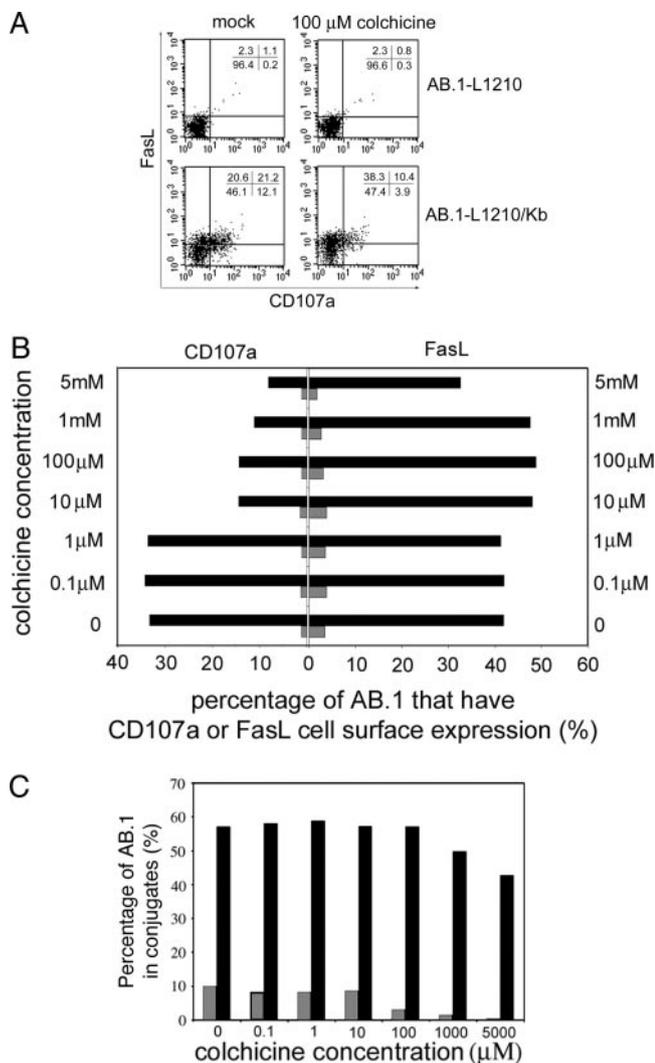
**FIGURE 3.** Soluble cross-linked and plate-bound anti-CD3 $\epsilon$  induce distinct patterns of FasL cell surface expression; however, only plate-bound anti-CD3 triggers CTL degranulation. CTL clone AB.1 was treated for the indicated time with either soluble cross-linked or plate-bound anti-CD3 (145-2C11). Cells were stimulated in the presence or absence of 4 mM EGTA, 3 mM MgCl<sub>2</sub>, or 10  $\mu$ g/ml CHX. AB.1 was detached from the anti-CD3-bound plate with 5 mM EDTA treatment at 4°C for 10 min before staining. Cell surface CD107a (A) or FasL (B) expression was measured on stimulated cells (—) or control cells (---). Data are representative of at least three independent experiments. The M1 gate contains positive events.

that undergoes immediate, extracellular Ca<sup>2+</sup>-independent cell surface transport upon target cell engagement.

*The rapidly expressed pool of FasL is sufficient to lead to CTL-mediated killing of Fas-expressing target cells*

We next sought to determine whether the arguably low levels of early Ca<sup>2+</sup>-independent FasL expression on CTL was sufficient to induce target cell death. In contrast to a previous study examining FasL-dependent killing by CTL clones (11), we used an assay that could measure induction of cell death more rapidly than a standard 4-h killing assay to minimize contributions of possible low levels of de novo FasL expression in a longer cytolytic assay. The triggering of apoptosis via Fas involves a caspase activation cascade, within which caspase-3 is activated downstream of caspase-8 activation (1). Additionally, caspase-3 can be a direct substrate for granzyme B in intact cells (20). Therefore, we selected caspase-3

activation as an early indicator of target cell apoptosis via both the FasL and degranulation pathways. CTL clone AB.1 induced caspase-3 activation of target cell apoptosis was investigated by flow cytometry and the level of cleaved, active caspase-3 in Fas<sup>+</sup> EL4 (H-2<sup>b</sup>) cells and Fas<sup>-</sup> L1210/K<sup>b</sup> cells was compared in the presence or absence of EGTA-Mg<sup>2+</sup>. We reasoned that, in the absence of extracellular Ca<sup>2+</sup>, the Ca<sup>2+</sup>-dependent FasL and degranulation mechanisms would be excluded and the cytolytic effect of the extracellular Ca<sup>2+</sup>-independent, preformed FasL pool alone could be selectively examined. We first confirmed that AB.1 shows both rapid Ca<sup>2+</sup>-independent and delayed Ca<sup>2+</sup>-dependent FasL cell surface expression upon EL4 engagement (data not shown). We observed that, after a 1.5-h incubation with CTL clone AB.1 in the presence of 4 mM EGTA, EL4 still undergoes a significant level of caspase-3 activation, which, in contrast, is completely blocked in the Fas<sup>-</sup> target cells L1210/K<sup>b</sup> (Fig. 2). This



**FIGURE 4.** Cell surface expression of preformed pool of FasL is microtubule independent. AB.1 cells were pretreated with ethanol carrier control or the indicated concentration (0.1  $\mu\text{M}$ –5 mM) of colchicine, then stimulated with cognate target cell L1210/K<sup>b</sup> (■) or negative control L1210 (□) for 15 min. Cells were stained with CD8 $\alpha$  (PE.Cy5), FasL (PE), and CD107a (FITC). After gating on the CD8<sup>+</sup> cells to examine only the CTLs, the expression of FasL and CD107a was assessed. *A*, Typical FACS plot. *B*, CD107a and FasL surface expression was determined at different colchicine concentrations. Indicated values are percentage of AB.1 positive for FasL or CD107a expression. Data are representative of three independent experiments. *C*, PKH26 red dye-stained AB.1 cells were treated with colchicine and stimulated with unstained L1210 or L1210/K<sup>b</sup> as in *A*. After 15 min of stimulation, cells were vortexed to eliminate nonspecific binding and fixed. The percentage of AB.1 in conjugates was assessed by focusing on red fluorescence-positive cells and analyzing forward scatter.

observation indicates that the rapidly expressed pool of FasL is indeed sufficient to induce target cell apoptosis. These data are consistent with our previous study showing that AB.1 is a potent mediator of extracellular Ca<sup>2+</sup>-independent target cell killing assessed by <sup>51</sup>Cr release assay (10).

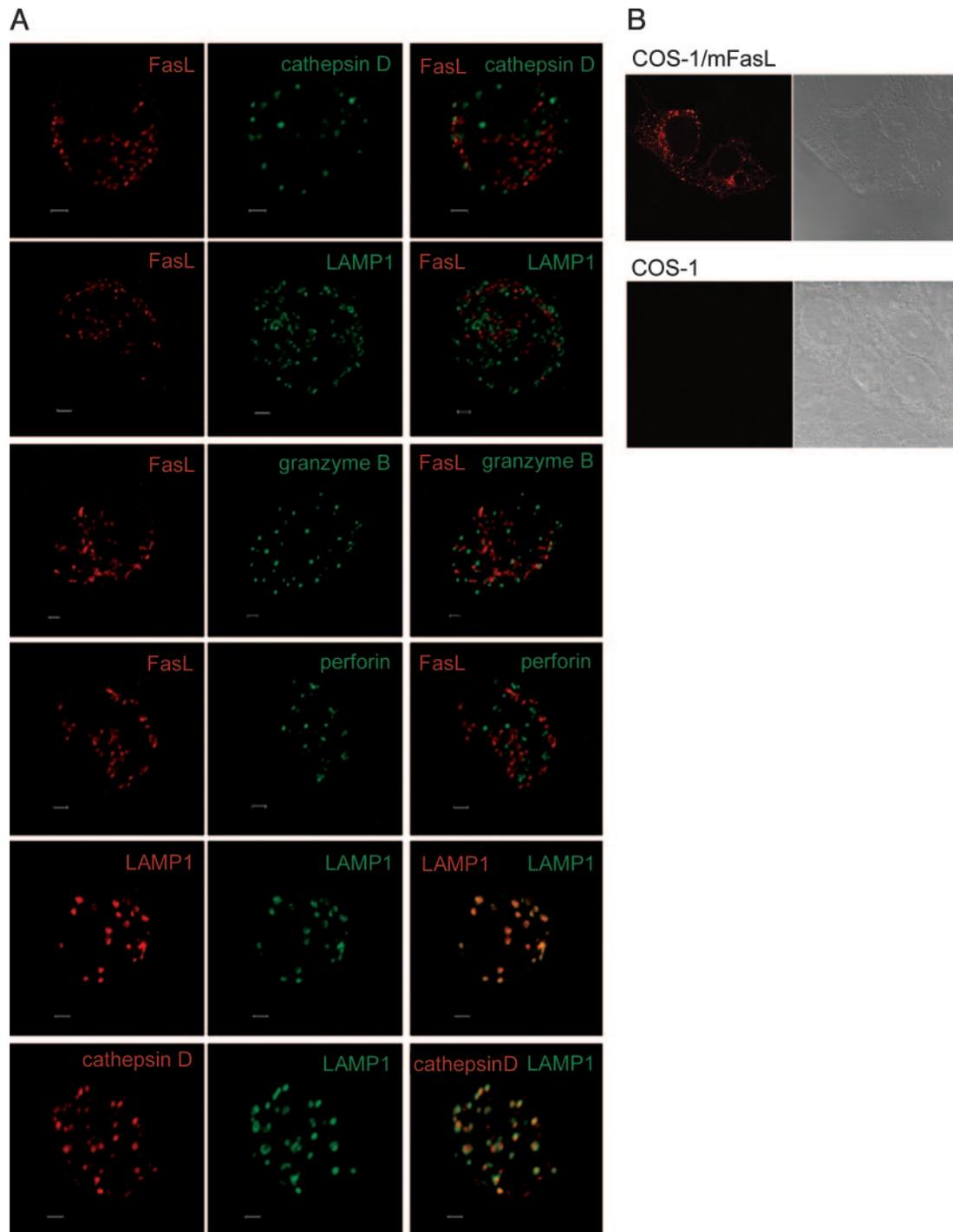
#### *Anti-TCR stimulates two distinct patterns of FasL surface expression*

We next examined whether TCR stimulation alone can induce the same pattern of FasL cell surface expression induced by target cells. Our laboratory has previously shown that solid-phase or plate-bound anti-TCR/CD3 is required to trigger degranulation;

cross-linked anti-TCR/CD3 is unable to stimulate any detectable degranulation by granzyme A enzymatic assay (21). However, both stimulation methods can induce tyrosine phosphorylation and activation of other downstream signaling pathways (21). Similarly, we determined which TCR stimulation method can trigger FasL cell surface expression, as it had previously been shown that plate-bound anti-TCR triggers Ca<sup>2+</sup>-dependent cell surface expression of de novo synthesized FasL (7, 8). Consistent with our earlier study (21), only plate-bound, but not cross-linked anti-CD3 induces Ca<sup>2+</sup>-dependent degranulation as measured by CD107a cell surface expression (Fig. 3*A*). In contrast, we observed that both cross-linked and immobilized anti-CD3 can induce FasL cell surface expression on the CTL (Fig. 3*B*). Moreover, FasL cell surface expression patterns induced by these two different methods are characteristically distinct. TCR stimulation by anti-TCR Ab cross-linking induces extracellular Ca<sup>2+</sup>-independent, rapid and transient, preformed FasL cell surface expression, which mimics the rapidly expressed pool of FasL upon target cell stimulation. In contrast, immobilized anti-CD3 stimulation results in an extracellular Ca<sup>2+</sup>-dependent, delayed, de novo FasL cell surface expression pattern as was previously published (7, 8), which is similar to the late FasL cell surface expression upon target cell conjugation. An intriguing observation is that, although immobilized anti-CD3 provides a more sustained stimulation than cross-linked anti-CD3 (21), it did not elicit the early (15-min) FasL cell surface expression (Fig. 3*B*), indicating that perhaps there are distinct signaling requirements for the two different FasL expression patterns. We also examined surface FasL expression 5 and 10 min post-immobilized anti-CD3 stimulation and detected no increased expression (data not shown), excluding the possibility that there is rapid FasL cell surface expression followed by rapid endocytosis. It should be pointed out that CTLs undergo relatively rapid attachment and TCR signaling within 10 min of addition to the immobilized anti-CD3 (21), suggesting that CTL are adhered and signaling at the early time point. Taken together, these data indicate there are two TCR-dependent signaling pathways that lead to different patterns of FasL CTL cell surface expression upon TCR engagement.

#### *Stored FasL cell surface delivery is microtubule independent*

CTL cytolytic granules have been shown to move along microtubules to accumulate at the target cell contact point (4) and MHC class I-stimulated degranulation is disrupted with colchicine (22). We wanted to determine whether microtubules are required for stored FasL cell surface translocation to further distinguish stored FasL translocation from degranulation. To disrupt microtubule-based granule movement, AB.1 was pretreated with colchicine (0.1  $\mu\text{M}$ –5 mM), which binds tubulin and blocks microtubule polymerization; then FasL and CD107a cell surface expression was assessed at 15 min after target cell stimulation. For these experiments, we performed simultaneous staining of FasL and CD107a and the FACS profiles of cells treated with and without 100  $\mu\text{M}$  colchicine is shown (Fig. 4*A*), along with a colchicine titration (Fig. 4*B*). At all concentrations examined, colchicine did not inhibit rapid FasL cell surface delivery (Fig. 4*B*). In contrast, degranulation as measured by CD107a cell surface expression was significantly impaired by colchicine in a concentration-dependent manner (Fig. 4*B*). The same concentrations that blocked CD107a cell surface expression also inhibited degranulation as measured by release of granzyme A enzymatic activity from cells and reorientation of the microtubule organizing center toward the contact point (data not shown). We further confirmed that colchicine treatment did not impair effector-target conjugate formation at 15 min after target cell incubation ensuring that lack of degranulation is not due to insufficient conjugate formation (Fig. 4*C*). These data



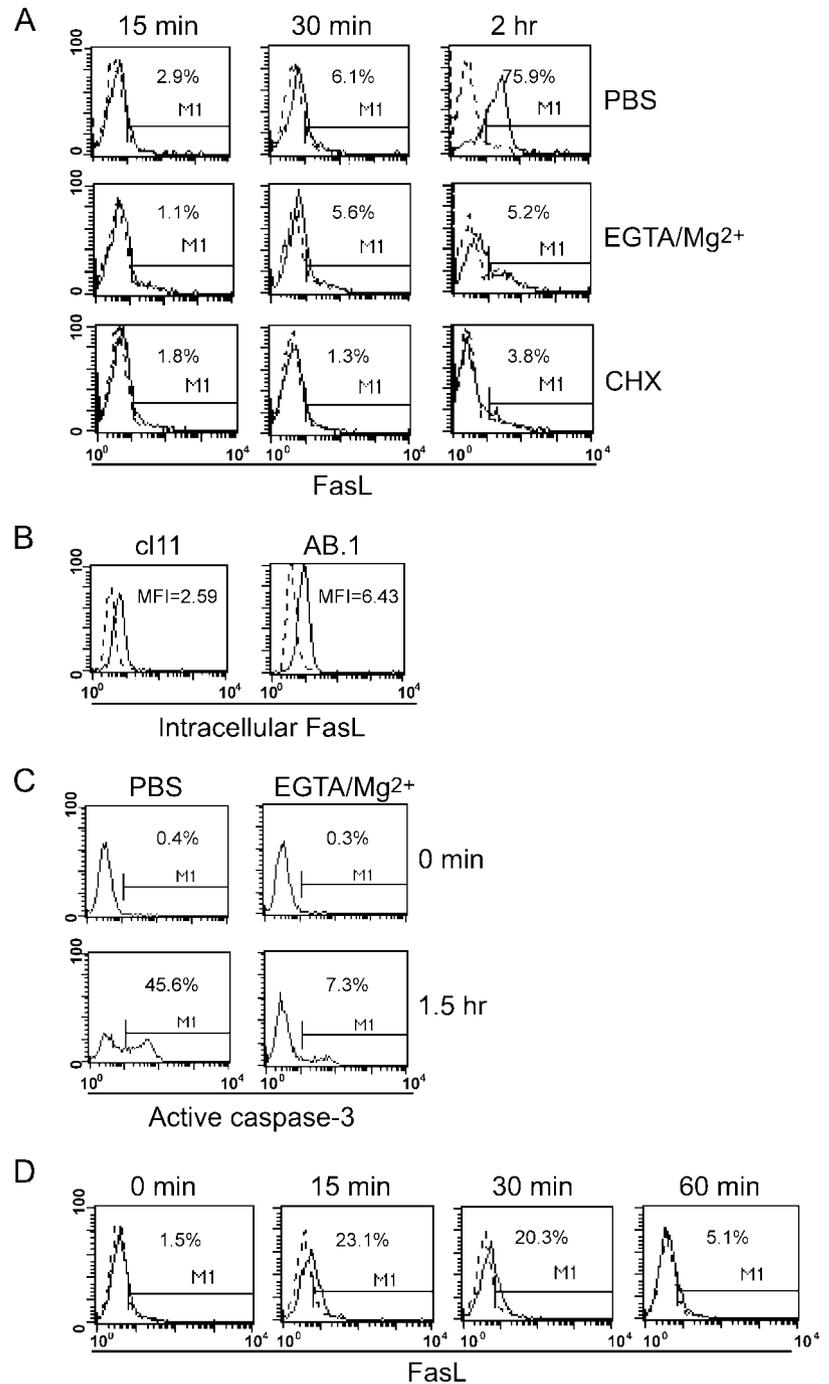
**FIGURE 5.** Stored FasL segregates from cytolytic granules by confocal microscopy. *A*, AB.1 cells were stained with Abs specific for FasL (red) and the cytolytic granule markers cathepsin D, LAMP1, granzyme B, or perforin (green) and the appropriate secondary Abs and analyzed by confocal microscopy. As a positive control, AB.1 cells were stained with rat anti-LAMP1 primary Ab followed by both Alexa Fluor 488 donkey anti-rat IgG and Alexa Fluor 594 donkey anti-rat IgG. Another positive control was cathepsin D (red) and LAMP1 (green) costaining. Z-stack images were acquired (interval, 0.2  $\mu\text{m}$ ) and subjected to three-dimensional reconstruction and deconvolution. Representative projections of the reconstructed three-dimensional images are shown. Scale bars represent 1  $\mu\text{m}$ . *B*, COS-1 cells were either transiently transfected with mouse FasL cDNA (COS-1/mFasL), or mock transfected (COS-1); then cells were stained with biotinylated anti-FasL Ab (MFL3) followed by Alexa Fluor 546 streptavidin and detected by confocal microscopy.

indicate that preformed FasL expression is not dependent on microtubules, unlike CTL degranulation, and further illustrate the differential control of exocytosis of cytolytic granules and rapid FasL transport to the cell surface.

*FasL is found in vesicles that are distinct from cytolytic granules*

The distinct  $\text{Ca}^{2+}$  (Fig. 1) and TCR (Fig. 3) stimulation requirements and microtubule dependence (Fig. 4) for rapid FasL cell

surface expression and degranulation suggest that these two processes are independently controlled. It has been suggested that FasL is stored in cytolytic granules (12, 13), implying that signaling requirements for degranulation and FasL expression would be identical. However, our results above, indicating distinct signaling requirements for these two mechanisms, strongly suggest that colocalization would be unlikely. We therefore compared the intracellular localization of preformed FasL and cytolytic granules in



**FIGURE 6.** CTL clone c111 contains stored FasL but expresses only newly synthesized FasL on the cell surface in response to cognate target cells and exhibits limited extracellular  $\text{Ca}^{2+}$ -independent killing. *A*, CTL clone c111 was stimulated with L1210/K<sup>b</sup> (—) or L1210 (---) target cells for the indicated time in the presence or absence of 4 mM EGTA, 3 mM  $\text{Mg}^{2+}$  or 10  $\mu\text{g}/\text{ml}$  cycloheximide and then stained for surface FasL. Indicated values are the percentage of cell surface FasL-positive AB.1 upon L1210/K<sup>b</sup> engagement subtracted by that of L1210 engagement. *B*, Unstimulated c111 and AB.1 were subjected to intracellular FasL staining (—) or isotype control Ab staining (---). Indicated values are mean fluorescent intensity (MFI) of FasL-PE staining subtracted by background staining. *C*, c111 was incubated for the indicated time with EL4 (Fas<sup>+</sup>) target cells in the presence or absence of 4 mM EGTA, 3 mM  $\text{MgCl}_2$ . Cells were stained with an Ab specific for activated caspase-3. Indicated values represent expression of intracellular active caspase-3 on the gated target cell population. *D*, c111 was treated for the indicated time with soluble cross-linked anti-CD3 (145-2C11). Cell surface FasL expression was measured. Indicated values are percentage of cells with FasL-PE staining (—) subtracted by isotype-PE staining (---). Data are representative of at least three independent experiments. The M1 gate contains positive events.

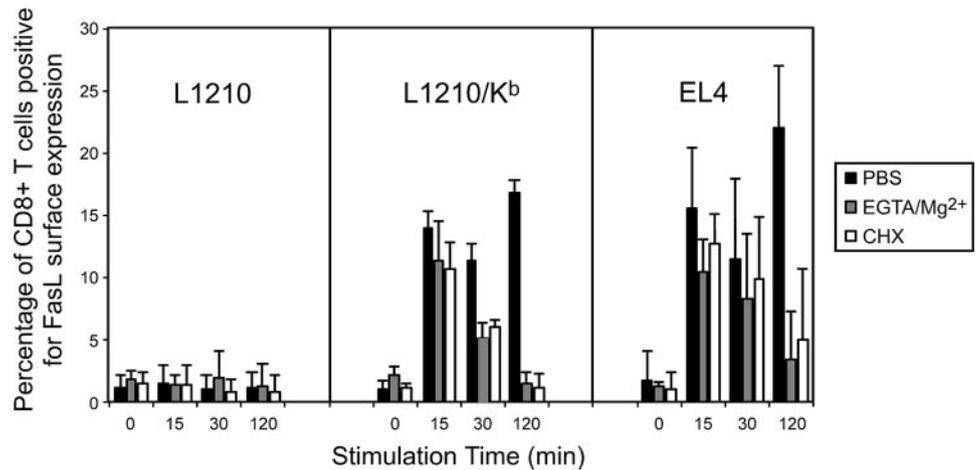
AB.1 by confocal microscopy. Using cathepsin D, LAMP1, perforin, and granzyme B as markers for cytolytic granules, we demonstrated that the FasL intracellular storage compartment completely segregates from cytolytic granules (Fig. 5A). Cytolytic granules stained with granule cargo marker cathepsin D and granule membrane marker LAMP1 as a positive control (Fig. 5A) demonstrated that the cytolytic granule structure was conserved and that the majority of this staining colocalized to the same granules. To confirm the specificity of the FasL Ab used for this study, we transfected COS-1 cells with mouse FasL and stained with the Ab. The anti-FasL (MFL3) stained the transfected COS-1 but not the untransfected cells (Fig. 5B). Furthermore, negligible staining of cells was detected with biotinylated isotype control Ab (data not shown). These confocal data demonstrate that stored FasL does not localize with the cytolytic granules. We also stained cells 2 h after

stimulation and detected no colocalization of FasL with the cytolytic granules (data not shown), suggesting that the newly synthesized FasL also does not colocalize with the granules; however, in these experiments we were unable to distinguish stored from newly synthesized FasL.

*Stored FasL that is rapidly transported to the cell surface is found in other clones and ex vivo CTL*

We have observed that not all CTL clones are competent to lyse Fas<sup>+</sup> EL4 in the absence of extracellular  $\text{Ca}^{2+}$  ((23) and data not shown). C111 is one such clone, and we hypothesized that this CTL clone would be deficient in the rapid mobilization of stored FasL to the cell surface. Upon conjugation with L1210/K<sup>b</sup> target cells, there is no immediate,  $\text{Ca}^{2+}$ -independent FasL cell surface expression on c111 (Fig. 6A). Nevertheless, the  $\text{Ca}^{2+}$ -dependent, de novo

**FIGURE 7.** Purified CD8<sup>+</sup> CTL from in vivo-primed PELs exhibit rapid FasL expression independent of extracellular Ca<sup>2+</sup> or new protein synthesis. In vivo-primed alloreactive CD8<sup>+</sup> T cells from BALB/c mice were isolated from the peritoneum and stimulated for the indicated time with specific target cells L1210/K<sup>b</sup> or EL4 or the negative control target cell L1210 in the presence or absence of EGTA or CHX. Cell surface FasL expression on the gated CD8<sup>+</sup> CTL population was analyzed by flow cytometry. The percentage of FasL-positive CD8<sup>+</sup> T cell population was shown. Data are the average of three independent experiments; bars, SE.



FasL cell surface expression is as robust as that of AB.1 (Fig. 6A). The possibility that there was no preformed FasL in c111 was excluded by the detection of intracellular FasL by FACS analysis (Fig. 6B) and by immunoblotting (data not shown). However, the expression level of intracellular FasL in c111 is only about one-half that of AB.1 (Fig. 6B), which could be below a threshold for sufficient expression for Ca<sup>2+</sup>-independent killing. Target cell apoptosis, measured as caspase-3 cleavage, in the presence of EGTA revealed that the preformed pool of FasL in c111 was not able to efficiently induce lysis of the EL4 Fas-expressing target cell (Fig. 6C). However, we are able to detect rapid and transient expression of FasL in response to cross-linked anti-CD3 (Fig. 6D), which was not blocked or delayed by CHX or EGTA-Mg<sup>2+</sup> treatment (data not shown), demonstrating that this pool can be mobilized and detectable on the cell surface. Confocal microscopy also showed no colocalization of stored FasL with cytolytic granule markers in c111 (data not shown). These results reveal that not all CTL are equivalent in their ability to rapidly mobilize stored FasL to the cell surface in response to target cells in the absence of extracellular Ca<sup>2+</sup>.

To ascertain whether the observations obtained with AB.1 actually reflect FasL expression in in vivo-derived CTL, isolated CD8<sup>+</sup> T cells from PEL from BALB/c mice on days 10–12 after i.p. injection of EL4 lymphoma were assessed for target cell-induced FasL expression. We elected to examine PEL-derived CTL, because these can be easily examined ex vivo without any potential influence of culture in the presence of cytokines. Similar results to those found above for CTL clone AB.1 were obtained with in vivo-derived CTL (Fig. 7). Namely, the ex vivo CD8<sup>+</sup> T cell population showed no basal FasL cell surface expression but rapidly expressed pre-existing FasL at 15 min that was Ca<sup>2+</sup>-independent for its induction. Furthermore, as with the CTL clones, there is later expression of Ca<sup>2+</sup>-dependent, newly synthesized FasL at 2 h after cognate target cell L1210/K<sup>b</sup> or EL4 conjugation (Fig. 7). These results demonstrated that in vivo-derived CTL are triggered to rapidly express internal stores of FasL on the cell surface via an extracellular Ca<sup>2+</sup>-independent pathway and confirm that in vivo-derived CTL express two distinct waves of FasL at the cell surface after target cell engagement.

## Discussion

The primary finding of this study is that CTL contain intracellular pools of FasL that are rapidly mobilized to the cell surface to trigger degranulation-independent, FasL-mediated lysis. This intracellular pool of FasL does not localize to the cytolytic

granules, and mobilization of this stored FasL to the cell surface occurs independently of degranulation. We provide substantial experimental evidence to support our conclusion that FasL is stored and regulated independently of the cytolytic granules. First, we could not detect degranulation by cells stimulated with cross-linked Ab even though these cells mobilized pre-existing FasL to the cell surface (Fig. 3). Second, target cell-stimulated degranulation was inhibited by extracellular Ca<sup>2+</sup> depletion, whereas the rapid FasL surface expression occurred under these same conditions (Fig. 1). Third, we detected Fas-dependent target cell apoptosis in the absence of granule-dependent lysis (Fig. 2). Fourth, microtubule disruption blocked degranulation but not FasL cell surface expression upon target cell engagement (Fig. 4). Finally, we did not detect costaining of FasL with the cytolytic granules (Fig. 5).

Our data clearly indicate that FasL expression and degranulation are under distinct controls and likely represent two independent pathways rather than FasL being a component of granule-mediated lysis. This is consistent with and provides further insight into a number of previous studies. Degranulation, but not all CTL-mediated killing, is inhibited with extracellular Ca<sup>2+</sup> depletion (10, 24). Additionally, perforin-dependent cytotoxicity is classical protein kinase C-dependent, whereas TCR-CD3-induced FasL expression is not (25). In another study, a CTL clone that failed to mobilize intracellular Ca<sup>2+</sup> after TCR engagement was unable to kill via the degranulation mechanism of killing, yet was still able to kill via the FasL/Fas mechanism in an Ag-specific manner (26). Furthermore, CTL from *Ashen* mice are defective in degranulation, but are still able to kill via the FasL pathway (27). These data support our conclusion that FasL is stored in vesicles distinct from the perforin/granzyme-containing granules.

The nature of the compartment containing the stored FasL remains unknown. We have not detected costaining with markers for endoplasmic reticulum, Golgi, or mitochondria. We have also found by density centrifugation that these FasL-containing vesicles are of low density and cofractionate with Golgi membranes but are distinct from the cytolytic granules (data not shown). The translocation of the stored FasL is clearly signal regulated. There is precedent for such a vesicle type, given that the chemokine RANTES is found in distinct storage granules in human CTLs (28). These vesicles rapidly released the stored RANTES upon T cell activation, similar to what we detect with FasL; however, the nature of the storage vesicles was not further characterized. Our CTL clones do not store RANTES (data not shown), so we could not determine whether RANTES and FasL are stored in

the same compartment in CTLs. Further studies will be required to identify the TCR-regulated compartment in which the FasL is stored.

We further demonstrated in the current study that there are two waves of FasL cell surface expression, at the population level, in response to cognate target cell binding; a rapid mobilization of internal stores of FasL and a delayed de novo synthesis of FasL. There are conflicting studies in the literature describing the control of FasL expression on the CTL cell surface. Anti-TCR-induced FasL up-regulation was shown to require protein synthesis and  $Ca^{2+}$  (7, 8). In contrast, up-regulation of surface FasL expression on ex vivo PEL CTLs did not require protein synthesis but was derived from pre-existing pools of FasL (11). Our studies reconcile these apparently disparate data because we showed that cell surface FasL is both expressed from existing pools and newly synthesized after TCR engagement (Fig. 1), similar to what has been reported after phorbol ester and calcium ionophore treatment of human CTL (29). Cell surface expression of stored FasL or de novo synthesized FasL depends on the presence of extracellular  $Ca^{2+}$ , time of analysis after TCR stimulation, and potentially the strength of the TCR generated signal.

Our observation that TCR cross-linking can only induce preformed FasL cell surface expression, whereas plate-bound anti-TCR can only induce de novo FasL expression revealed that FasL cell surface expression might be finely tuned in CTL, perhaps by differential TCR signaling strength or kinetics. Cross-linked anti-TCR stimulates a shorter duration of signaling and a generally less robust signal compared with plate-bound anti-TCR (21). We have examined cells stimulated with plate-bound anti-CD3 at early time points, as early as 5 min after stimulation, and at no time do we detect FasL translocation from internal stores (data not shown). We therefore think it is likely that signaling thresholds are key in determining if FasL is translocated or synthesized. Consistent with this, we have sorted L1210- $K^b$  target cells for high and low  $K^b$  expression and found that the low  $K^b$ -expressing cells stimulate stronger FasL translocation and the high  $K^b$ -expressing cells stimulate stronger de novo synthesis of FasL (data not shown). This low-level signaling requirement for translocation of stored FasL may also explain why some CTLs are unable to mediate  $Ca^{2+}$ -independent killing of Fas-expressing cells (e.g., C111 in Fig. 6). Even though all CTL clones and in vivo-derived CTL that we have examined store FasL, not all are able to mobilize the FasL to the cell surface in response to cognate Ag on target cells. It is possible that some of these CTL, such as C111, signal very strongly in response to target cells such that only degranulation and de novo FasL expression is triggered. Consistent with this C111 are triggered to degranulate with very low concentrations of purified class I MHC alloantigen compared with a number of other CTL clones (15).

Interestingly, previous reports also indicated that activation of FasL-dependent cytotoxicity required weaker TCR signaling than the induction of degranulation or cytokine production. For example, epitope modification or blocking of CD8 resulted in a  $\geq 8$ -fold decrease in TCR ligand binding, greatly impaired CTL degranulation and IFN- $\gamma$  release but had no effect on Fas-dependent cytotoxicity (30). The authors of this study proposed that the FasL-dependent cytotoxicity in the CTL examined was possibly by translocation of preformed FasL to the cell surface, although this was not further investigated (30). Cao et al. (31) reported that CD8<sup>+</sup> T cells partially activated by a self-derived peptide killed only by FasL/Fas pathway but not degranulation. These results are consistent with our hypothesis that a low level of signal preferentially induces surface translocation of stored FasL compared with degranulation and de novo FasL expression.

In summary, we demonstrate that there are two waves of FasL expression that are differentially controlled in CTLs. We further show that CTL degranulation and FasL lytic mechanisms are fully independent with respect to stored component localization and regulation and suggest that CTL contain distinct storage vesicles that are independently regulated downstream of the TCR. Further studies will need to be done to uncover the nature of the FasL storage compartment and to determine why CTLs express two distinct waves of FasL expression after TCR stimulation.

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## Disclosures

The authors have no financial conflict of interest.

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