Activation of primary T lymphocytes results in lysosome development and polarized granule exocytosis in CD4⁺ and CD8⁺ subsets, whereas expression of lytic molecules confers cytotoxicity to CD8⁺ T cells

Abstract: Lytic granule exocytosis is the major cytotoxic mechanism used by CD8⁺ cytotoxic lymphocytes. CD8⁺ T cells acquire this effector function in the process characterized by lysosomal biogenesis, induction of expression of cytolitic molecules, and their selective sorting into the lysosomal vesicles. However, temporal relation of these differentiation stages during T cell activation has not been defined precisely. Also, although CD4⁺ T cells typically do not express lytic molecules as a consequence of activation and therefore, do not acquire granule exocytosis-mediated lytic function, it is not clear whether CD4⁺ T cells are able to degranulate. By using in vitro TCR stimulation of primary mouse lymphocytes, we found that polyclonally activated CD4⁺ T cells degranulate upon TCR ligation and polarize enlarged lysosomal granules in response to target cell recognition, despite the lack of granule exocytosis-mediated cytotoxicity. Upon TCR stimulation, resting CD8⁺ T cells rapidly express lytic molecules and acquire potent lytic function early in activation. Maximal cytolytic potential, however, depends on enlargement of lysosomal granules during the subsequent activation stages. Thus, polyclonal TCR stimulation of resting T cells results in development of lysosomal granules and their release upon TCR engagement in CD4⁺ and CD8⁺ T cells, but only CD8⁺ T cells acquire lytic function as a result of induction of expression of lytic molecules. J. Leukoc. Biol. 80: 000–000; 2006.

Key Words: mouse · cell activation

INTRODUCTION

CD8⁺ CTL play one of the central roles in adaptive immunity. They mediate the immune response by secreting cytokines, which can be cytotoxic and/or engage other types of immune cells, and by directly killing the target cells using Fas-mediated or granule exocytosis-mediated cytotoxic mechanisms [1, 2]. Granule exocytosis is the dominant pathway used by CTL to kill tumor or virally infected cells. Degranulation releases the pore-forming protein, perforin, and several proteases or granymes, which are stored in the granules of CTL [3]. The granules of effector CTL can be characterized as secretory lysosomes, as they, in addition to the cytolytic proteins, contain lysosomal proteins such as cathepsins B and D, β-hexosaminidase, lysosome-associated membrane protein (Lamp)-1 (CD107a), Lamp-2 (CD107b), and Lamp-3 (CD63) [4].

Granules and their constituent cytolytic proteins are generated during antigen-induced maturation of CD8⁺ T cells. In this process, CTL progress through sequential stages of lysosomal biogenesis, induction of expression of cytolitic molecules, and selective sorting of lytic enzymes into the lysosomes [4]. One study has demonstrated that in a human CD8⁺ CTL clone, maximal cytolytic activity correlates with the peak of activation-dependent development of lysosomes [5]. However, detailed kinetics of lysosomal development during T cell activation in a human or mouse model has not been determined. It is more important that comparative studies concerning the kinetics of lysosome development, expression of lytic molecules, and cytolytic activity during primary T cell activation have not been performed to date. Results of such studies would help in the identification of check-points for each CTL maturation stage, which would, in turn, offer opportunities for selective regulation of these stages.

In contrast to CD8⁺ T cells, effector CD4⁺ lymphocytes typically do not possess granule exocytosis-mediated cytotoxicity. CD4⁺ T cells predominantly use Fas-mediated cytotoxicity to terminate T cell immune responses and to regulate T cell homeostasis [6, 7]. Concomitantly, long-term CD4⁺ T cell clones and short-term, nonspecifically activated CD4⁺ T cells have been shown to use primarily Fas-mediated cytotoxicity as a mechanism of killing [8–10]. However, granule exocytosis-mediated cytolytic activity of CD4⁺ T cells has been reported
in several mouse and human systems. The existence of antigen-specific CD4⁺ T cells, which use perforin-mediated cytotoxicity in response to certain pathogens, has been well-documented in humans [11–13]. Also, it has been demonstrated that a population of primary mouse alloantigen-specific CD4⁺ T cells uses perforin-mediated cytotoxicity [14]. It remains unclear, however, whether the absence of granule exocytosis-mediated cytotoxicity in effector CD4⁺ T cells is solely a result of the lack of the expression of cytolytic molecules or may also be a result of their inability to acquire degranulation potential.

To study granule exocytosis potential in T cells, we looked at lysosome development, degranulating ability, lytic molecule expression, and cytolytic function at different stages of activation. We demonstrate that CD4⁺ and CD8⁺ T lymphocytes gain the ability to execute granule exocytosis as a consequence of in vitro TCR activation. Also, activation-dependent lysosomal biogenesis and lysosomal granule polarization induced by target cell recognition occur in both T cell subtypes. Finally, induction of expression of lytic molecules and acquisition of granule exocytosis-mediated cytotoxicity occur early in CD8⁺ T cell activation, but maximal lytic activity requires full development of lysosomal granules.

MATERIALS AND METHODS

Mice, cells, antibodies, and reagents

C57BL/6 mice were purchased from Taconic (Germantown, NY). Splenocytes were stimulated in vitro with culture supernatants containing anti-CD3 antibody produced by 2C11 hybridoma (American Type Culture Collection, Manassas, VA). CD4⁺ or CD8⁺ spleen T cells were purified using the magnetic bead-coupled antibody MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany). Purified spleen T cells were stimulated with plate-bound anti-CD3 antibody, Clone 2C11 (BD PharMingen, San Diego, CA). Hamster IgG1 (BD PharMingen) was used as an isotype control. The same antibodies were used in chromium release assay, FITC-conjugated, anti-CD107a antibody or the isotype-matched control, FITC-conjugated rat IgG2a, anti-mouse CD4allophycocyanin (APC), CD8-APC, CD25-PE, CD44-PE, CD69-PE, and annexin V-APC (all from BD PharMingen) were used for the cell surface staining, followed by flow cytometry. PE-conjugated anti-human granuzyme B (which cross-reacts with mouse granzyme B) or mouse IgG1-PE isotype control antibody (both from Caltag Laboratories, Burlingame, CA) and PE-anti-mouse perforin or PE-rat IgG2a isotype control antibody (both from eBioscience, San Diego, CA) were used for intracellular staining followed by flow cytometry. The Flowcoretrack Green (LTG) and Lysotracker Red (LTR; Molecular Probes, Eugene, OR) were used for labeling of lysosomes in T cells, and Cell Tracker Blue (Molecular Probes) was used for labeling P815 cells, Monensin, cyclohexamide, concanamycin A, brefeldin A, and n-p-nitrophenyl N-acetyl-b-D glucosaminide were from Sigma Chemical Co. (St. Louis, MO). 7-Amino-actinomycin D (7-AAD; Sigma Chemical Co., St. Louis, MO) was used for labeling of lysosomes in T cells, and Cell Tracker Blue (Molecular Probes) was used for labeling of P815 cells, monensin, cyclohexamide, concanamycin A, brefeldin A, and n-p-nitrophenyl N-acetyl-b-D glucosaminide were from Sigma Chemical Co. (St. Louis, MO). 7-Amino-actinomycin D (7-AAD; Sigma Chemical Co., St. Louis, MO) was used for labeling of lysosomes in T cells, and Cell Tracker Blue (Molecular Probes) was used for labeling of P815 cells, monensin, cyclohexamide, concanamycin A, brefeldin A, and n-p-nitrophenyl N-acetyl-b-D glucosaminide were from Sigma Chemical Co. (St. Louis, MO). 7-Amino-actinomycin D (7-AAD; Sigma Chemical Co., St. Louis, MO) was used for labeling of lysosomes in T cells, and Cell Tracker Blue (Molecular Probes) was used for labeling of P815 cells, monensin, cyclohexamide, concanamycin A, brefeldin A, and n-p-nitrophenyl N-acetyl-b-D glucosaminide were from Sigma Chemical Co. (St. Louis, MO). 7-Amino-actinomycin D (7-AAD; Sigma Chemical Co., St. Louis, MO) was used for labeling of lysosomes in T cells.
RESULTS

CD4⁺ and CD8⁺ T cells acquire the ability to degranulate as a consequence of activation

We used in vitro TCR stimulation of primary mouse lymphocytes to compare the ability of naïve and activated αβ T cells to degranulate. In this system, in vitro culture of total spleen cells in the presence of anti-CD3 antibody resulted in >99% activation of CD4⁺ and CD8⁺ T cells, as evidenced by their forward- and side-scatter and their cell surface phenotype (Fig. 1A). Also, CD8⁺ T cells activated in this manner acquired cytolytic function, as determined by redirected cytolysis (Fig. 1B), indicative of mature CTL. The observed cytotoxicity was predominantly granule exocytosis-mediated, as it was almost completely blocked by concanamycin A (Fig. 1B), a selective inhibitor of perforin-mediated cytolysis [17]. Similarly, if FAS-deficient L1210 cells [18] were used as targets in the cytotoxic assays (Fig. 1C), the lytic activity was slightly lower compared with the lytic activity against the FAS-expressing P815 cells (compare Fig. 1, B and C) and was inhibited completely by concanamycin A.

Naïve or 2-day-activated CD4⁺ or CD8⁺ spleen T cells were purified and tested for the ability to release lysosome-specific degranulation marker β-hexosaminidase. As shown in Figure 2A, activated but not naïve T cells degranulated in response to secondary stimulation by plate-bound anti-CD3 antibody. Despite the lack of granule exocytosis-mediated cytotoxicity (Fig. 1, B and C), activated CD4⁺ T cells secreted β-hexosaminidase in response to TCR stimuli, albeit at slightly reduced levels compared with CD8⁺ T cells. As β-hexosaminidase release is a population-based degranulation assay, these results could not reveal whether the observed extent of degranulation is a consequence of TCR-induced granule exocytosis of most or only a fraction of the stimulated T cells. To make this determination, we decided to use a single cell-based degranulation assay. TCR-induced increase in cell surface translocation of transmembrane lysosomal proteins, such as Lamp-1 [19], CD63 [20], Fas ligand [21], and CTLA-4 [22], has been

![Fig. 1. Cell surface marker expression and cytolytic phenotype of CD3-activated CD4⁺ and CD8⁺ mouse splenocytes. Total splenocytes were cultured in the presence of anti-CD3 antibody. (A) After indicated periods of time, cell surface activation marker expression on CD4⁺ or CD8⁺ T cells was assessed by flow cytometry. After 2 days of activation, CD4⁺ or CD8⁺ T cells were purified by magnetic immunobead purification and tested for the ability to kill P815 (B) or L1210 cells (C) in the redirected cytosis assay, in the presence or absence of 1 µg/ml anti-CD3 antibody and in the presence or absence of 100 nM concanamycin A. In all experiments, specific lysis in the absence of anti-CD3 antibody was 5% or less (at E:T ratio of 30:1). Each E:T ratio was done in quadruplicate samples. Error bars represent SD.](image-url)
successfully used for this purpose. In addition, Lamp-1 has been used recently as a degranulation marker in human melanoma-specific CD8⁺ CTL, which had encountered and responded to cognate tumor [23] as well as in vitro-activated CD8⁺ T cells from lymphocytic choriomeningitis virus (LCMV)-specific or OT-1 TCR transgenic mice [24, 25]. Therefore, we chose to use a TCR-induced increase in Lamp-1 cell surface translocation as a measure of degranulation. Naïve or 2 day-activated CD4⁺ or CD8⁺ spleen T cells were purified and tested for the ability to mobilize Lamp-1 to the cell surface in response to secondary stimulation by plate-bound, anti-CD3 antibody. As shown in Figure 2, B and C, these experiments showed that the majority of activated but not naïve CD4⁺ or CD8⁺ T cells translocates Lamp-1 to the cell surface after TCR ligation.

**Primary T lymphocyte activation results in lysosome development and targets cell-induced lysosomal granule polarization in CD4⁺ and CD8⁺ subsets**

The use of β-hexosaminidase release and Lamp-1 cell surface translocation assays indicated that activated CD4⁺ and CD8⁺ T cells have similar capacities to degranulate in response to TCR ligation. This further suggested that the extent of TCR-induced lysosomal biogenesis, which is an important component of the process of CD8⁺ CTL maturation [5], might be similar in the two T cell subtypes. To test this, we assessed the enlargement of a lysosomal compartment in CD4⁺ and CD8⁺ T cells as a function of TCR stimulation. For this purpose, lysosomes were stained with the acidotropic fluorescent dye LTG, which accumulates specifically in lysosomes and enables the assessment of relative size of the lysosomal compartment by flow cytometry. As shown in Figure 3A, kinetics and the extent of activation-dependent enlargement of the lysosomal compartment were similar in the two T cell subtypes. Confocal images of T cells loaded with Lysotracker confirmed that the reagent specifically stained lysosomes (Fig. 3B). It is interesting that after reaching a peak at 48 h, the relative size of the lysosomal compartment in both T cell subsets decreased at the later activation stages (Fig. 3A). T cell blasts at later activation stages, however, did not become smaller (Fig. 3A, lower panel), which further confirmed that the LTG loading assay used is a measure of relative size of lysosomal granules rather than the size of cells. Intracellular staining of Lamp-1 followed by confocal microscopy also showed that relative size and morphology of the lysosomal granules are similar in activated CD4⁺ and CD8⁺ T cells (Fig. 3C). Finally, double-staining of activated CD4⁺ or CD8⁺ T cells with LTR and Lamp-1 showed that the two reagents specifically label the same intracellular (lysosomal) compartment (Fig. 3D; for the simplicity of data presentation, only the results with CD8⁺ T cells are shown). To test whether enlarged lysosomal granules in activated CD4⁺ T cells polarize toward the contact site upon target cell recognition, we assessed the polarization of Lamp-1 in activated T cells using confocal microscopy. As shown in Figure 3E, Lamp-1 is highly polarized in activated T cells, with the majority of Lamp-1 localized to the plasma membrane near the contact site. This further suggested that the polarization of Lamp-1 is a key event in the activation of T cells and that it is not restricted to CD8⁺ T cells. Overall, these results indicate that Lamp-1 is a useful marker for the assessment of T cell activation and degranulation.
Fig. 3. Activation of resting CD4⁺ T cells results in lysosomal biogenesis and targets cell-induced lysosomal granule polarization. Total splenocytes were cultured in the presence of anti-CD3 antibody. After indicated periods of time, CD4⁺ or CD8⁺ T cells were purified by magnetic immunobeads, loaded with LTG, and analyzed by flow cytometry (A) or by confocal microscopy (B) or were stained for intracellular Lamp-1 followed by confocal microscopy (C). (A, lower panel) A forward-scatter (FSC) versus side-scatter (SSC) plot of total splenocytes activated with anti-CD3 antibodies for indicated periods of time. (B and C) The images of T cells activated for 2 days are shown. (D) CD8⁺ T cells activated for 2 days were loaded with LTR and stained intracellularly with Lamp-1-FITC antibody, followed by confocal microscopy. (E) After 2 days of activation, CD4⁺ or CD8⁺ T cells were purified by magnetic immunobeads and loaded with LTG. Loaded cells were then allowed to form conjugates with the Cell Tracker Blue-labeled P815 cells for 10 min in the presence of 1 μg/ml anti-CD3 antibody, and lysosomal granule polarization was monitored by confocal microscopy. (A) The results for each indicated time-point are obtained from a single experiment. Four independent experiments yielding similar results were performed.
tion, activated CD4+ or CD8+ T cells were loaded with LTR, the cells were allowed to form conjugates with P815 targets in the presence of anti-CD3 antibody, and granule movement was monitored by confocal microscopy. As shown in Figure 3E, activated CD4+ T cells polarized the labeled lysosomal granules toward the target cells in a similar manner as CD8+ T cells.

**Resting CD8+ T cells rapidly acquire granule exocytosis-mediated cytotoxicity upon TCR stimulation**

Collectively, these results showed that in CD4+ and CD8+ subsets of polyclonal resting T cells, TCR stimulation induces enlargement of lysosomal granules, which are released upon subsequent TCR engagement. It was of interest, therefore, to determine the stage of T cell activation at which the ability to degranulate is acquired. Splenocytes were stimulated for different periods of time, and CD4+ or CD8+ T cells were purified and assayed for degranulation in response to TCR ligation. We found that CD4+ and CD8+ T cells, which are activated for 24 h or longer, are able to degranulate when triggered via TCR, as determined by an increase in Lamp-1 cell surface translocation, and at the earlier activation stages, the increase in Lamp-1 cell surface translocation could not be detected (Fig. 4A). It is interesting that a TCR-induced increase in Lamp-1 cell surface translocation could not be detected at the late activation stages either, which parallels the observed changes in the size of the lysosomal compartment as a consequence of activation (Fig. 3A).

To determine whether acquisition of degranulation potential temporally correlates with the acquisition of granule exocyto-

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**Fig. 4.** Kinetics of acquisition of granule exocytosis during primary T cell activation. Total splenocytes were cultured in the presence of anti-CD3 antibody for indicated periods of time, followed by purification of CD4+ or CD8+ T cells. (A) The cells were then stimulated for 4 h with the plate-bound anti-CD3 antibody, followed by Lamp-1 cell surface translocation assessment as described in Figure 2. “ctrl IgG” refers to the isotype control for the Lamp-1-FITC staining. For the simplicity of data presentation, the isotype control staining was shown only for the 48-h time-point. MFI is mean fluorescence intensity. The experiment was repeated three times, giving the same results for CD4+ and CD8+ T cells. (B) Cumulative results of activation-dependent cytolytic activity of purified CD8+ T cells against P815 cells tested in a 4-h, redirected chromium release assay. The value for lytic activity for each indicated time-point is a mean of three or more independent experiments. (C and D) Representatives of four independent experiments testing activation-dependent lytic activity of purified CD8+ T cells against P815 or L1210 cells, respectively, by redirected chromium release assay are shown. The values for lytic activity for each indicated time-point are obtained from a single experiment. (CD8+ T cells, which originated from the same splenic culture, were tested for lytic activity after indicated periods of activation.) In all chromium release experiments, each E:T ratio was done in quadruplicate samples. Error bars represent SD.
sis-mediated cytotoxicity, we assessed cytolytic potential of CD8\(^{+}\) T cells activated for different periods of time in the system described above. Figure 4B represents cumulative data of at least three independent experiments for each indicated time-point. In Figure 4C, a representative of four independent experiments giving similar results is shown. It is surprising that the kinetics experiments showed that the onset of granule-exocytosis-mediated cytotoxicity could be detected as early as 6–8 h poststimulation and that moderate levels of lytic activity could be detected by 12 h of activation. Cytolytic activity reached a peak between 36 and 42 h and started declining after \(~48\) h of activation. Similarly to the experiments presented in Figure 1B, concanamycin A almost completely blocked the observed lytic activity at all indicated time-points (the results were not shown for the simplicity of data presentation). In addition, essentially the same results were obtained when L1210 cells were used as targets in the chromium release assays (Fig. 4D), confirming that the observed cytolytic activity was granule exocytosis-mediated.

Amplitude of granule exocytosis-mediated cytotoxicity depends on levels of expression of lytic molecule and size of the lysosomal compartment

Comparison of the kinetics of lysosomal biogenesis (Fig. 3A) with the kinetics of acquisition of granule exocytosis-mediated cytotoxicity (Fig. 4B) in CD8\(^{+}\) T cells showed that maximal cytotoxicity is observed at the activation stage, when the enlargement of the lysosomal compartment reaches the peak. Conversely, high levels of lytic activity were detected during the initial stages of lysosomal granule development, implying that CD8\(^{+}\) T cells are able to release lytic granules efficiently before lysosomal biogenesis, as determined by the enlargement of lysosomal granules, is completed. This further suggested that it is the induction of expression of lytic molecules that determines the activation stage at which CD8\(^{+}\) T cells gain the ability to kill using granule exocytosis mechanism. To test this, expression levels of granzyme B and perforin in CD8\(^{+}\) T cells activated for different periods of time were determined by intracellular staining, followed by flow cytometry. As shown in Figure 5A, induction of granzyme B and perforin expression in CD8\(^{+}\) T cells occurred early in activation and correlated closely with acquisition of lytic function (compare Figs. 5A and 4B). In CD4\(^{+}\) T cells, the expression of granzyme B and perforin was not up-regulated upon activation (data not shown). Similar to the acquisition of lytic function, TCR-induced loss of granzyme B antigenicity in CD8\(^{+}\) T cells could be detected early in activation, as determined by intracellular staining followed by flow cytometry (Fig. 5B). It is important that the observed loss of granzyme B antigenicity could not be blocked by brefeldin A, an inhibitor of constitutive secretion, demonstrating that granzyme B was released from lytic granules by the mechanism of TCR-induced, regulated exocytosis. Double-staining of CD8\(^{+}\) T cells, activated for different periods of time, with Lysotracker and perforin confirmed that at early activation stages, the majority of CD8\(^{+}\) T cells expresses significant levels of lytic molecules when no increase in the relative size of lysosomes could be detected (Fig. 5C). Finally, levels of expression of the two lytic molecules were reduced dramatically at the later stages of activation (Fig. 5A), which temporally correlates with the observed decrease in cytolytic activity (Fig. 4, B–D) and the relative size of the lysosomal compartment (Figs. 3A and 5C).

To determine whether the observed decline in the lytic and related functions in CD8\(^{+}\) T cells is a consequence of induction of apoptosis and/or decrease in cell viability, we assessed these parameters in CD8\(^{+}\) T cells activated for 48 or 72 h. As Figure 6A shows, viability of CD8\(^{+}\) T cells activated for 72 h was decreased slightly compared with the viability of cells activated for 48 h, as determined by 7-AAD uptake. Conversely, the percentage of apoptotic CD8\(^{+}\) T cells (as determined by annexin V staining, which allows detection of early apoptotic cells) was increased significantly in the population of cells activated for 72 h compared with the cells activated for 48 h (Fig. 6A). Therefore, decline in lytic and related functions in CD8\(^{+}\) T cells can be at least partially attributed to the induction of apoptosis at the late stages of T cell activation.

Thus, upon TCR stimulation, resting CD8\(^{+}\) T cells rapidly express lytic molecules and acquire granule exocytosis-mediated cytotoxicity, the amplitude of which depends on levels of expression of lytic molecules and enlargement of the lysosomal compartment. In Figure 6B, we summarized the results of our comparative analyses of kinetics of lysosomal granule development, synthesis of lytic molecules, degranulating ability, and granule exocytosis-mediated cytotoxicity during primary T cell activation.

**DISCUSSION**

Lytic granule exocytosis is the major cytotoxic mechanism used by CD8\(^{+}\) T cells. Effector CD4\(^{+}\) T cells, conversely, are typically unable to lyse target cells efficiently via granule exocytosis, although human and mouse CD4\(^{+}\) clones with this ability have been described [11–14]. Factors, which determine whether CD4\(^{+}\) T cells will gain granule exocytosis-mediated cytotoxicity, are not well defined. There are several lines of evidence suggesting a possible role of CD8\(^{+}\) T cells in down-modulating the lytic activity of CD4\(^{+}\) T cells. For example, absence of CD8\(^{+}\) T cells in β2-microglobulin-deficient mice leads to increased cytotoxic activity of CD4\(^{+}\) T cells [26]. Furthermore, development of cytotoxicity in polyclonal human CD4\(^{+}\) T cells is more efficient if CD8\(^{+}\) T cells are removed prior to activation [14]. However, mediators of the observed inhibition have not been identified to date. It is also unclear whether the entire population or only a subset of CD4\(^{+}\) T cells has the potential to acquire cytolytic function. Also, the question remains whether a mere induction of expression of lytic molecules confers lytic function to CD4\(^{+}\) T cells, or effector CD4\(^{+}\) T cells also need to gain the ability to degranulate. In other words, is the ability to degranulate a common feature of all (or the majority) of activated T cells, or does only a subset of cells, which are programmed to become killers, gain this function?

To answer these questions, we studied granule exocytosis potential during primary T cell activation. Our results demonstrate that polyclonal population of activated mouse CD4\(^{+}\) T cells is capable of TCR-mediated degranulation. Furthermore,
we showed that activation-dependent development of lysosomes occurs in all CD4+ T cells with the kinetics and to the extent comparable with that observed in CD8+ T cells. Finally, we demonstrated that same as in CD8+ T cells, CD4+ T cells release enlarged lysosomal granules in a vectorial manner (Fig. 3E). However, in contrast to CD8+ T cells (Fig. 5A), expression of perforin and granzyme B in CD4+ T cells is not induced upon activation (data not shown). As a consequence, activated CD4+ T cells do not exert lytic granule-mediated cytotoxicity (Fig. 2, B and C). Collectively, these findings suggest that a mere induction of expression of lytic molecules confers granule exocytosis-mediated cytotoxicity to CD4+ T cells.

Contrary to our findings, Wolint et al. [24] have recently reported that activated LCMV-specific CD4+ T cells do not degranulate in response to the specific peptide recognition, as determined by Lamp-1 cell surface translocation. Yet, in another study, the ability of mouse CD4+ T cell clones to degranulate has been documented [22]. The existing discrepancies could be attributed to the differences in T cell stimulation systems (i.e., antigen-specific vs. polyclonal TCR stimulation of primary T cells vs. stimulation of T cell clones) used in the studies. These finding, however, do not necessarily have to be contradictory, as our data suggest that activated CD4+ T cell can be induced to degranulate by a strong TCR signal.
Thus, the main conclusion of the study presented here is that activated CD4\(^+\) and CD8\(^+\) T cells are able to release lysosomal granules in response to antigenic stimulation but that only CD8\(^+\) T cells possess granule exocytosis cytotoxicity as a result of expression of lytic molecules.

In this study, we also sought to precisely determine the activation stage at which T cells gain the ability to degranulate. Recently, it has been demonstrated that the majority of OT-1 TCR transgenic CD8\(^+\) T cells acquires degranulation ability after 48 h of antigenic stimulation, as determined by Lamp-1 assay [25]. The use of Lamp-1 translocation assay in our system of T cell activation indicated that this ability is gained after 24 h. However, the results of our functional granule exocytosis-mediated cytotoxicity assays demonstrate that activated CD8\(^+\) T cells are able to release lytic granules after only 6–8 h of activation. Similarly, TCR-induced loss of granzyme B antigenicity in CD8\(^+\) T cells could be detected as early as 8 h after activation (Fig. 5B), long before Lamp-1 translocation could be detected. Based on this, we conclude that CD8\(^+\) T cells are able to perform regulated exocytosis in response to TCR ligation as early as 6–8 h upon activation.

The fact that CD8\(^+\) T cells are able to execute granule exocytosis-mediated cytotoxicity efficiently at the activation stages, which precede the stage at which TCR-induced Lamp-1 cell surface translocation could be detected, implies that the degranulation assay used is not sensitive enough to detect all lysosomal granule release (or at least lytic granule release) events in T cells. Our studies of activation-induced enlargement of a lysosomal compartment indicate that detectability of TCR-induced Lamp-1 cell surface translocation positively correlates with the size of the lysosomal compartment (compare Figs. 3A and 4A). During early and late activation stages, when lysosomal granules are relatively small, a TCR-induced increase in Lamp-1 cell surface expression could not be detected. Thus, it is possible that granules need to reach a certain size for a sufficient number of lysosomal Lamp-1 molecules to be delivered to the plasma membrane, such that an increase in Lamp-1 cell surface expression could be detected. In that regard, we consistently observed (our unpublished data) a markedly high increase in Lamp-1 cell surface expression upon brief TCR ligation in human CTL line Tall-104, characterized by notably large lytic granules [27]. Conversely in human NK-like cytotoxic YT cells, which contain small lysosomal granules, Lamp-1 translocation can be barely detected (our unpublished observations).

In addition to the lysosomal granule size, expression levels of Lamp-1 on the lysosomal membranes could affect the extent...
mediated cytotoxicity is gained before significant lysosomal development in cytotoxic T lymphocytes (CTL) clone during the early stages of activation and that distribution of these molecules begins to overlap only at the later activation stages.

Finally, it is possible that during initial T cell activation stages, lytic molecules are stored in Lamp-1-negative structures, which may be small, immature granules. A population of small CD3−, CD56+ human lymphocytes, which were lytic and contained high perforin levels but no detectable dense azurophilic granules, has been described [29]. Similar to our findings in mouse T cells, this study provides evidence that dense granule morphology/development and cytotoxic capacity can be distinguished from one another.

Lysosomal transmembrane proteins other than Lamp-1 have also been used as degranulation markers in T cells. In human PBMC, Lamp-2 and CD63 have been used for this purpose, although each of these molecules was shown to be a less-sensitive degranulation marker than Lamp-1 [30]. In CD8+ or CD4+ human T cell clones, CD63 was demonstrated to translocate rapidly to the cell surface upon stimulation [21]. Our unpublished studies of human CTL line Tall-104 and human NK cell line NK-92 demonstrate that Lamp-1, Lamp-2, and CD63 translocate rapidly to the cell surface upon antigen receptor ligation, where an increase in cell surface expression was slightly higher for Lamp-1 compared with the other two markers. We also tested Lamp-2 as a degranulation marker in our system of activation of primary mouse T cells but were not able to detect a TCR-induced increase in Lamp-2 cell surface expression at any tested activation stage (data not shown). Assessment of CD63 cell surface translocation in mouse models remains difficult, as to our knowledge, antibodies specific for mouse CD63 are currently not commercially available.

Taken together, Lamp-1 appears to be the most sensitive marker of granule exocytosis in CTL. However, results of the work presented here indicate that TCR-induced Lamp-1 translocation assay does not fully reflect granule exocytosis capacity of T cells at different activation stages. As a consequence, the sole use of Lamp-1 assay for the assessment of CTL frequency in a given cell population may result in a failure to detect a subpopulation of cytolytically competent T cells.

The results of our comprehensive studies of kinetics of lysosomal development and expression of lytic molecules and lytic activity during primary T cell activation allowed us to closely define parameters that determine cytolytic capacity of CD8+ T cells. We showed that potent granule exocytosis-mediated cytotoxicity is gained before significant lysosomal biogenesis occurs. During these early activation stages, levels of expression of lytic molecules closely correlate with the height of lytic activity. However, the expression levels reach a peak relatively early in activation, before lytic activity reaches the maximum, suggesting that another factor is required for the attainment of maximal cytotoxicity. The development of lysosomal granules is likely to be such a factor, as the peak of lytic activity in activated CD8+ T cells temporally correlates with the activation stage at which relative size of lysosomal granules is maximal (Fig. 6B). This finding is in agreement with the studies of a human CTL clone, in which maximal lytic activity was shown to correlate with the peak of activation-induced lysosomal biogenesis [5]. It is interesting that although kinetics of activation was slower compared with our activation system, in this study, too, cytolytic activity and size of lysosomal granule have been shown to decline at the later activation stages.

Therefore, we conclude that CD8+ T cells exert potent granule exocytosis-mediated cytotoxicity early in activation, during the initial stages of lysosomal biogenesis, but gain maximal lytic activity only upon full development of lysosomal granules later in activation.

In summary, in this work, we dissected granule exocytosis capacity of CD4+ and CD8+ T cells at different activation stages. We showed that upon activation, lysosomal biogenesis and polarized lysosomal granule secretion occur in both T cell subsets, but only CD8+ T cells gain lytic function as a result of expression of lytic molecules early in activation. We believe that our studies will help to better understand granule exocytosis and cytolytic function in T cells, which will, in turn, offer opportunities for selective regulation of the distinct stages of these processes.

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