Cytotoxic T Lymphocytes from Cathepsin B-deficient Mice Survive Normally in Vitro and in Vivo after Encountering and Killing Target Cells*

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The lysosomal protease cathepsin B has been proposed to protect cytotoxic T lymphocytes from the membrane-disruptive effects of perforin secreted during the execution phase of target cell death. Accordingly, cathepsin B that translocates to the lymphocyte surface upon degranulation has been postulated to cleave and inactivate perforin molecules that diffuse back to the killer cell. We have found that recombinant perforin is cleaved inefficiently by cathepsin B and shows no significant reduction in its lytic activity following co-incubation. Furthermore, purified CD8+ cytotoxic T lymphocytes of cathepsin B-null gene-targeted mice were able to induce normal death of target cells both in vitro and in vivo and to survive the encounter with target cells as efficiently as cathepsin B-expressing killer cells. We conclude that cathepsin B is not essential for protection of cytotoxic lymphocytes from the toxic effects of their secreted perforin.

Cytotoxic T lymphocytes (CTL)3 recognize virus-infected or transformed cells and induce their apoptotic death through two independent mechanisms. The first involves death receptor triggering to bring about classic caspase-dependent apoptosis of the target cell (1). The granule exocytosis pathway constitutes the second mechanism and involves the secretion of cytotoxic proteins from lysosome-like granules toward the target cell following conjugate formation (2). The granule constituents cooperate to induce both caspase-dependent and -independent death of the target cell (3). Perforin is a key component of cytotoxic granules, and its crucial role in the clearance of intracellular pathogens and immune surveillance is evident from studies on gene-engineered perforin-deficient mice. These mice are unable to clear a variety of viruses despite having a functional death receptor pathway, and the majority develop spontaneous B cell lymphoma as they age (4, 5). Perforin’s precise mechanism of action is unclear. It is commonly believed that following target cell recognition, perforin and other components of the cytotoxic granules are released into the immunological synapse where exposure to millimolar calcium levels and neutral pH results in perforin monomers binding and inserting into the target membrane, forming transmembrane channels that can lead to loss of plasma membrane homeostasis and osmotic lysis (6). Perforin is also essential for the induction of apoptotic death in cooperation with granule proteases called granzymes, especially granzyme B, which (like caspases) can cleave its substrates adjacent to key aspartate residues (3, 7, 8). It has been proposed that granzyme uptake occurs in response to “repair” of plasma membrane perturbations brought about by perforin (9). Therefore, target cells exposed to high concentrations of purified granzyme B remain healthy unless perforin is also present in low concentrations (10).

CTL are relatively resistant to their granule toxins, allowing them to consecutively kill several target cells in vitro (11, 12). To date, it remains unclear how CTL are protected from the toxic effects of perforin, particularly following its exocytosis into the immunological synapse. Perforin biosynthesis and trafficking are closely regulated to minimize the possibility of inadvertent damage to the CTL organelles. It is thought that perforin is synthesized as an inactive precursor and that its lytic activity is achieved only when a heavily glycosylated C-terminal oligopeptide is cleaved after packaging within the secretory granules (13). Thereafter, the acidic pH of this compartment prevents perforin from binding calcium ions, an obligate requirement for lipid binding and insertion (14). But how perforin is able to selectively damage the target cell and spare the CTL after its release remains unclear. One hypothesis suggests that a CTL granule component may act as a specific inhibitor of perforin following degranulation. Recently, a role for the lysosomal protease Cathepsin B (CatB) was proposed (15). CatB is a papain-like cysteine protease that has been reported to participate in various biological processes such as apoptosis induction, enzyme activation, and tissue remodeling (16). Following granule
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exocytosis, enzymatically active CatB was found to be expressed transiently on the surface of degranulating T cells, and inhibition of CatB in vitro resulted in the death of CD8+ T cells, resulting in reduced target cell killing. The authors concluded that CatB may cleave and inactivate perforin molecules that diffuse back to the CTL surface following their release; however, the efficiency of this interaction was not studied (15).

In the current study, we further investigated the possible role for CatB in CTL protection from perforin in a more physiological setting by studying the lymphocytes of CatB-deficient mice. We found that the CTL are able to induce normal target cell death both in short- and long-term killing assays and are not compromised in inducing target cell death in vivo. We therefore conclude that CatB is dispensable for the protection of CTL from their secreted perforin.

EXPERIMENTAL PROCEDURES

Antibodies and Other Reagents—Rat anti-perforin monoclonal antibody P1–8 was used as described (17). A polyclonal antiserum detecting human fibronectin was purchased from Auspep. Anti-CD8 and anti-TCR Vα2 antibodies and followed mouse CD107a (lamp-1) was purchased from BD Biosciences. Propidium iodide was purchased from Sigma. Anti-mouse CD107a (lamp-1) was purchased from BD Biosciences. Recombinant mouse perforin was purified from cathepsin B transgenic mice to generate OT-I transgenic mice. CatB-null mice were crossed with OT-I (OVA-specific, H-2Kb-restricted T cell receptor transgenic) mice to generate OT-I transgenic CatB+/− mice, CatB−/− mice, and CatB+/− mice (21). Genotyping of the mice for CatB status was carried out by PCR using the following primers: 5′-GGTTCGTTCCGGTGAGG-3′ and 5′-AACAA-GAGCCGGAGGACC-3′. Expression of the OT-I transgenic T cell receptor was assessed by co-staining peripheral blood lymphocytes with anti-CD8 and anti-Vα2 antibodies and followed by flow cytometry.

Cells and Cell Culture—All cells used in this study were cultured at 37 °C in a humidified CO2 incubator in medium supplemented with 2 mM glutamine and 10% fetal bovine serum. The mouse lymphoma cell line EL4 was maintained in Dulbecco’s modified Eagle’s medium and Jurkat human lymphoma cells in RPMI. For the generation of mouse CTLs, splenocytes from naïve C57BL/6 mice were pulsed with the cognate peptide SIINFEKL (presented on Kb, 1 μg/ml) for 60 min at 37 °C, lethally irradiated, and cultured at a 1:1 ratio with responder splenocytes harvested from CatB-sufficient or -deficient mice. After 5 days, viable CD8+ cells were purified by positive bead selection using CD8 microbeads and the AutoMacs cell sorting system (Miltenyi Biotech). BALB/c CTL were generated by one-way mixed lymphocyte reaction as described (22). Activation of CTL was confirmed by staining for CD8 and the activation markers CD25, CD44, and CD69 and analysis by flow cytometry using a FACSCalibur (BD Biosciences).

Enzymatic Activity of Cathepsin B—Purified Cathepsin B, claimed by its manufacturers to be >95% active was in fact found to be 94% active as determined by an active site titration assay performed with the irreversible inhibitor E64 as described (23). In substrate cleavage assays, various dilutions of CatB were incubated with 20 μM Z-Arg-Arg-aminomethylcoumarin substrate for 15 min at 37 °C in 0.1 M sodium acetate buffer (pH 5.5 or 7.0) containing 1 mM diithiothreitol. In some assays, the specific CatB inhibitor CA074 (50 μM) was also added. Substrate turnover was estimated on a fluorometer (PolyStar Optima; BMG Lab Technologies) at 460 nm.

For cleavage of mouse perforin, substrate and protease were co-incubated at a molar ratio (CatB:perforin) of 2.5:1 under the same buffer conditions, but for 40 min. For cleavage of fibronectin, the same conditions were used as for perforin cleavage, but the 10-fold less CatB was used, on a molar basis. Cleavage products of perforin and fibronectin were separated on 10% SDS-PAGE and 4–20% gradient SDS-PAGE, respectively, transferred to nylon membranes, and probed with anti-perforin (P1–8) or anti-fibronectin antibodies. The lytic activity of perforin following cleavage was determined by incubating perforin treated with CatB with 2 × 104 51Cr-labeled Jurkat cells for 2 h at 37 °C, followed by measurement of 51Cr release into the supernatant using a γ counter (see cytotoxicity assays below).

Cytotoxicity Assays—Sorted CD8+ CTL were mixed at various effector:target ratios (in triplicate) with 51Cr-labeled EL4 target cells pulsed with SIINFEKL (1 μg/ml for 1 h at 37 °C). After 4 or 16 h, the supernatant was harvested and 51Cr release was estimated on a γ counter. For each labeling, the spontaneous release of radiolabel over the time of the assay was not greater than 10% of incorporated radioactivity. To examine CTL survival during the cytotoxicity assay, purified CTL were labeled with 51Cr in the same way and mixed with unlabeled SIINFEKL-pulsed EL4 targets or with allogeneic CTL (H2d anti-b) raised in BALB/c mice. Cytotoxicity in vivo was assessed by measuring the survival of SIINFEKL-pulsed or mock-treated CFSE-labeled syngeneic splenocyte target cells recovered from the spleen of CatB-deficient or -sufficient recipient mice 4 h following intravenous injection into the tail vein. The methodology was essentially identical to that described (24). In some experiments, the SIINFEKL-loaded cells were labeled with high CFSE and the mock-treated cells with low CFSE, but in other experiments the CFSE concentrations were reversed. The following formula was used to calculate specific lysis. Ratio was defined as %

\[
\text{Ratio} = \left( \frac{I_{	ext{experimental}} - I_{	ext{spontaneous}}}{I_{	ext{maximum}} - I_{	ext{spontaneous}}} \right) \times 100
\]

where

- \( I_{	ext{experimental}} \) is the percentage of specific lysis measured in the experiment,
- \( I_{	ext{spontaneous}} \) is the percentage of spontaneous release,
- \( I_{	ext{maximum}} \) is the percentage of maximum release.

The ratio is a measure of the extent to which the experimental sample has lysed the target cells compared to the maximum possible lysis. Ratios greater than 100% indicate lysis of cells beyond the maximum possible release, which is not possible in practice. The ratio is thus a relative measure of lysis efficiency.
CFSE low/% CFSE high; percentage-specific lysis was calculated as: 1 - (the ratio of non-peptide pulsed/ratio peptide pulsed) × 100 (24).

Susceptibility of CTL to Purified Perforin—CTL were induced to degranulate by preincubation with plate-bound anti-CD3 antibody as described (15). Purified CD8+ 51Cr-labeled CTL were incubated with various dilutions of recombinant mouse perforin (in the presence or absence of the general cathepsin inhibitor E64, 50 μM) for 2 h at 37 °C. Degranulation was confirmed by increased expression of the lysosomal marker CD107a on the cell surface as determined by flow cytometry.

RESULTS
Recombinant Perforin Is a Poor Substrate for CatB in Vitro—It has recently been proposed that CatB redirected from the lysosomal compartment to the surface of degranulating CTL can cleave and inactivate perforin molecules that diffuse back to the CTL following exocytosis (15). This mechanism would explain the relative resistance of CTL to perforin; however, the ability of CatB to inactivate purified perforin has not been examined. To further test this hypothesis, we initially examined the ability of CatB to cleave purified, recombinant mouse perforin in vitro. CatB (with specific activity of 94% as determined by active site titration) was potently able to cleave the synthetic oligopeptide substrate Z-Arg-Arg-aminomethylcoumarin at either granule (~5.5) or neutral pH and cleaved the known polypeptide substrate fibronectin to completion under similar conditions (Fig. 1A and inset). The specificity of the proteolysis was demonstrated by the complete inhibition of cleavage observed when the specific CatB inhibitor CA074 was included in either reaction (Fig. 1A). CA074 has been modeled on the structure of the general thiol protease inhibitor E64 and is designed to bind specifically to the unique occluding loop only seen in CatB (25). When recombinant mouse perforin and purified CatB were incubated together for 40 min at 37 °C under a variety of buffer conditions and a molar protease:substrate ratio ten times greater than was used with fibronectin, most of the perforin remained intact. However, a principal cleavage product of ~45 kDa was also detected by Western blot analysis at both acidic and neutral pH, the generation of which was inhibited by CA074 (Fig. 1B). To determine whether perforin cleavage under these conditions reduced its lytic capacity, we mixed the CatB-treated perforin with 51Cr-labeled Jurkat cells, which are sensitive to perforin at pH 7.4 (14). We found that perforin activity was not substantially reduced following exposure to CatB at either acidic or neutral
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To exclude an intrinsic resistance of these CTL to the perforin pathway, we exposed the same $^{51}$Cr-labeled CTL to allogenic CTL raised in BALB/c mice and found that both CatB-sufficient and -deficient C57BL/6 target cells were killed efficiently and to a similar extent (Fig. 2A). As expected, no target cell death was observed in the absence of the cognate peptide. To more directly address the question of CTL survival during the co-incubation, a similar experiment was performed in which the purified CD8$^+$ T cells were labeled with $^{51}$Cr rather than the EL4 targets. No significant CTL death was observed at any of the E:T ratios examined (Fig. 2B).

Equivalent numbers of OT-1-specific CD8$^+$ T cells with a similar profile of activation markers were generated from CatB-deficient and -sufficient mice, as seen by co-staining with CD8/αβ2 antibodies and the activation markers CD25, CD44, and CD69 (data not shown). Killing by the purified CD8$^+$ CTL was mediated through perforin-dependent pathways, as it was completely inhibited when Ca$^{2+}$ was complexed with EGTA, even at a very high effector:target ratio (Fig. 2A). EL4 mouse thymoma cells used as targets express only very low levels of functional Fas and so are not susceptible to Fas ligand-mediated cell death. We found no difference in the killing of SIINFEKL-pulsed EL4 cells over 4 h or upon prolonged co-incubation for 16 h, irrespective of whether the CD8$^+$ CTL were derived from mice null for CatB expression or possessing either one or two wild-type alleles (Fig. 2A). As expected, no target cell death was observed in the absence of the cognate peptide. To more directly address the question of CTL survival during the co-incubation, a similar experiment was performed in which the purified CD8$^+$ T cells were labeled with $^{51}$Cr rather than the EL4 targets. No significant CTL death was observed at any of the E:T ratios examined (Fig. 2B).

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pH compared with control (Fig. 1, C and D). Perforin that had been incubated with CatB at pH 5.5 for 40 min induced potent lysis (~65% Cr release). Under acidic proteolysis conditions, perforin is unable to bind calcium ions (14) and so remains incapable of polymerization until the pH is raised to neutral. In contrast, because perforin subjected to proteolysis at pH 7.0 in the presence of calcium ions can self-associate and was incapable of polymerization until the pH is raised to neutral (6), it was necessary to assay for the effect of CatB cleavage kinetically (Fig. 1D). Accordingly, mock-treated perforin progressively lost activity over the 40-min incubation; however, exposure to active (or CA0174-inhibited) CatB did not further reduce perforin-induced lysis over the 40-min incubation (Fig. 1D).

CTL from CatB-deficient Mice Demonstrate Normal Target Cell Killing in Vitro—If CatB protects CTL from perforin-induced toxicity after degranulation, a possible adverse consequence of CatB deficiency would be the progressive depletion of CTL following sequential encounters with multiple target cells. To examine this possibility in an antigen-specific context, we crossed CatB-deficient mice with syngeneic mice expressing the transgenic T cell receptor OT-1, which can recognize the peptide antigen SIINFEKL presented on H-2K$^b$ target cells (21).
tease other than CatB may be involved in protecting the CTL following degranulation, a general protease inhibitor (E64) was added to degranulated CatB-null and CatB-expressing CTL exposed to recombinant perforin. Again, we found no difference in susceptibility of the CTL to recombinant perforin, irrespective of CatB expression (Fig. 3B).

**Normal in Vivo CTL Cytotoxicity in CatB-deficient Mice—** Finally, to determine the in vivo efficiency of target cell killing in CatB-deficient and -sufficient OT-I transgenic mice, in vivo cytotoxicity assays were performed as previously described (24). Syngeneic target splenocytes (C57BL/6) labeled with a high concentration of CFSE were mixed with a similar number of lymphocytes labeled with SIINFEKL and a low concentration of CFSE and infused into the tail veins of recipient mice. Control recipients received CFSE-labeled but SIINFEKL-unpulsed target cells. 4 h later, the mice were sacrificed, and the death of target cells assessed within the spleen. In the example shown (Fig. 4A), the death of SIINFEKL-pulsed target cells was deducted in that the ratio of high to low CFSE lymphocytes remaining in the spleen of recipient mice was markedly increased when SIINFEKL was presented on H-2Kb compared with a syngeneic mouse receiving lym-
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**DISCUSSION**

Immunologists and cell biologists have long been perplexed by the question of how lymphocytes that express perforin remain selectively resistant to its potent membrane-disruptive properties. Recent studies have provided evidence of several independent mechanisms through which the CTL or natural killer (NK) cell remains protected from perforin during its biosynthesis, trafficking, and storage. Perforin becomes capable of lytic activity only at the time of its packaging into the cytotoxic granules when a glyco-
sylated C-terminal oligopeptide is removed (13). Thereafter, storage within the acidic milieu of the lysosome-like compartment (at which critical aspartate residues are protonated) prevents perforin from binding Ca2+, thereby preventing premature activation. Compared with untransformed cells and some tumor target cells, both cloned CTL and primary killer lymphocytes are resistant to purified perforin applied extrinsically, whereas other pore-forming reagents such as streptolysin O, complement, and mellitin induce equivalent lysis of CTL and tumor targets (26–28). This implies that the resistance to lysis by perforin seen in CTL is perforin specific.

Two broad hypotheses have been put forward to explain the resistance of CTL to perforin during target cell apoptosis. First, the composition of the CTL membrane may be different from that of a target cell and thus more resistant to perforin-mediated lysis. Cytoplasts from CTL were shown to be resistant to perforin-induced lysis, and patch clamp experiments validated this observation by showing a difference in conductance between perforin-susceptible and -resistant cells (29, 30). A difference in lipid or protein composition of the plasma membrane of CTL compared with target cells has been shown and is believed to alter the ability of perforin to bind, insert, or poly-
merize to the target membrane (28, 31, 32). Second, a CTL granule component may act as a specific inhibitor of perforin following degranulation.

Once perforin is released into the immunological synapse and becomes active, additional means of preventing CTL/NK cell damage are required. On this basis, it has been proposed that lysosomal CatB expressed on the surface of killer lymphocytes upon fusion of the granule membrane with the plasma membrane may inactivate perforin molecules that diffuse back to the CTL/NK cell surface (15). Our study has examined the capacity of purified CatB to cleave and inactivate perforin directly and also in the context of an intact CTL in vitro and in vivo. Although our experiments confirm that
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CatB has some capacity to cleave perforin, we found that CatB is not necessary for protecting CTL during and following perforin-mediated killing of target cells. We have shown using in vitro cleavage assays that recombinant perforin is a relatively poor substrate for CatB under conditions in which fibronectin is cleaved to completion. Furthermore, CatB-cleaved perforin fragments remained biologically active and maintained their lytic function. Compatible with this finding is the observation that lytic activity has been demonstrated for several short peptides derived from the perforin primary sequence, especially at the N terminus (33, 34). The previous reported experiments showing extensive cleavage of perforin by purified CatB were not performed with purified perforin but rather used perforin within a CTL cell lysate as the substrate. It is possible that the CTL lysate contained contaminating proteases capable of cleaving perforin to completion or that CatB might activate a second protease responsible for perforin digestion and inactivation. Using purified major histocompatibility complex class I-restricted CD8+ CTL derived from CatB-sufficient or -null mice, we also showed that the absence of CatB did not adversely affect the extent of target cell death or the survival of CTL for up to 16 h following encounter with target cells. The use of CatB-deficient mice allowed us to study the function of the protease in a peptide- and major histocompatibility complex-restricted cellular context without the requirement for CatB inhibitors, which the previous study heavily relied upon to induce a state of CatB inhibition (15).

Our studies with CatB-deficient lymphocytes indicate that CatB is dispensable for perforin cleavage and if CatB does play some role in perforin inactivation, alternative proteases must also be capable of achieving this function. Of the 11 identified human cathepsins, cathepsins B, H, L, W, and C are expressed by cytotoxic lymphocytes, but only CatW is expressed solely by activated CTL (35). Recently, studies using CatW-deficient mice have shown that CatW deficiency does not alter the susceptibility of cytotoxic lymphocytes to suicide or fratricide following degranulation (36). Therefore, like CatB, CatW does not have a unique role in cytotoxic cell survival. If cathepsins do play a role in CTL protection, it is likely that a high level of redundancy may exist between the proteases responsible. However, our experiments examining perforin-mediated lysis of CTL from CatB-null or -expressing mice showed no difference in susceptibility to death with or without the general cysteine protease inhibitor E64. These results imply that cysteine proteases are not involved in protecting the CTL from death following degranulation. The mechanism(s) used by CTL to achieve self-protection from perforin following degranulation therefore still remains elusive. Other studies have suggested the alternative possibility that lipid composition of CTL/NK membranes may bestow CTL survival by reducing the susceptibility to perforin lysis, and one suggestion is that certain phospholipids may be more tightly packed in the CTL membrane, reducing the capacity for perforin to bind or intercalate the lipid bilayer (31). The availability of purified recombinant perforin should facilitate future studies that aim to test these hypotheses.

FIGURE 4. Death of syngeneic, SIINFEKL-pulsed and unpulsed target lymphocytes in vivo is equivalent in CatB-deficient and -sufficient mice. A, representative data from OT-I transgenic CatB+/+ (top) and OT-I transgenic CatB−/− mice showing a marked reduction in the number of SIINFEKL-pulsed (dotted, low CFSE fluorescence) lymphocytes compared with unpulsed lymphocytes (dotted, high CFSE fluorescence) remaining in the spleen 4 h after their simultaneous intravenous infusion. The solid trace shows the corresponding populations recovered from a mouse of identical genotype that received equal numbers of high and low CFSE-labeled lymphocytes, neither of which was peptide pulsed. B, the methodology exemplified in panel A was used to calculate the percentage-specific cell death achieved in vivo, according to Ref. 24. The data represent the mean ± S.D. of assays performed on 10 CatB−/− mice (7 received SIINFEKL, 3 did not), 14 CatB+/+ mice (7 received SIINFEKL, 7 did not), and 8 CatB+/+ mice (5 received SIINFEKL, 3 did not).
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