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Impaired Cytolytic Activity in Calreticulin-Deficient CTLs

Simonetta Sipione,* Catherine Ewen,† Irene Shostak,* Marek Michalak,* and R. Chris Bleackley2*

Calreticulin is an endoplasmic reticulum-resident chaperone that is stored in the cytotoxic granules of CTLs and NK cells and is released with granzymes and perforin upon recognition of target cells. To investigate the role of calreticulin in CTL-mediated killing, we generated CTL lines from $crt^{+/+}$ and $crt^{-/-}$ mice expressing a constitutively active form of calcineurin in the heart. $Crt^{-/-}$ CTLs showed reduced cytotoxic activity toward allogeneic target cells despite normal production, intracellular localization, and activity of granzymes and despite perforin overexpression. Comparable or higher amounts of granzymes were degranulated by $crt^{-/-}$ cells in response to immobilized anti-CD3 Abs, indicating that calreticulin is dispensable for the signal transduction that leads to granule exocytosis. The ability to form conjugates with target cells was affected in the $crt^{-/-}$ CTLs, explaining the observed reduction in cytotoxicity. Conjugate formation and cytotoxicity were completely restored by treatments that facilitate recognition and contact with target cells, a prerequisite for degranulation and killing. Therefore, we conclude that calreticulin is dispensable for the cytolytic activity of granzymes and perforin; however, it is required for efficient CTL-target cell interaction and for the formation of the death synapse.


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Calreticulin knockout ($crt^{-/-}$) mice have been generated (24), but they die at the embryonic stage due to impaired heart development, making it impossible to study mature $crt^{-/-}$ CTLs and NK cells. The nature of the cardiac defect observed in $crt^{-/-}$ mice was found to reside in the failure to mobilize ER $Ca^{2+}$ stores and to activate the serine/threonine phosphatase calcineurin (25). By crossing a transgenic mouse expressing a cardiac-specific, constitutively active form of calcineurin (26) with $crt^{+/+}$ mice, viable

3 Abbreviations used in this paper: ER, endoplasmic reticulum; BLT, N-α-benzyloxy-carbonyl-L-lysine thiobenzyol ester; CMA, concanamycin A; LDH, lactic dehydrogenase; $crt^{+/+}$, calreticulin wild type; $crt^{-/-}$, calreticulin knockout.
transgenic mice were obtained. In these animals, normal cardiac development was restored, and embryonic lethality was suppressed (25). Because the expression of active calcineurin occurred only in heart tissue, noncardiac phenotypes specifically due to ablation of the calreticulin gene were not corrected (25). Therefore, this mouse represents an excellent model to study calreticulin functions in systems other than heart. To specifically investigate the role of calreticulin in CTL lytic function, we generated CTL lines from splenocytes derived from these mice. In this study we show that in the absence of calreticulin, CTL cytotoxicity is impaired. Because calreticulin ablation does not seem to affect components of the lytic machinery, the ability of CTLs to degranulate, or their survival during degranulation and attack of target cells, but does affect the efficiency of effector/target conjugate formation, we propose that calreticulin may be involved in the mechanisms underlying target recognition by CTLs and/or formation of the death synapse.

Materials and Methods

Cells and reagents

Mouse mastocytoma P815 cells, mouse lymphocytic leukemia L1210 cells, and mouse lymphoma EL4 cells were obtained from American Type Culture Collection and were maintained in RPMI 1640 medium supplemented with 20 mM HEPES, 50 µM penicillin, 50 µg/ml streptomycin, 1 mM sodium pyruvate (Invitrogen Life Technologies), 0.1 mM 2-ME (Sigma-Aldrich), and 10% heat-inactivated FBS (HyClone; RHFM medium). Granzyme A/granzyme B double-knockout mice were generated in our laboratory by crossing granzyme A knockout (27) with granzyme B knockout mice (28) (The Jackson Laboratory) and subsequently inbreeding F1 animals. CTL lines were generated from these mice as described below. The hybridoma 145-2C11 producing anti-CD3ε Ab was obtained from American Type Culture Collection. All reagents were purchased from Sigma-Aldrich.

Generation of the c−/− CTL line

c−/− mice expressing a cardiac-specific constitutively active form of calcineurin have been previously described (25). Briefly, homozygote mice bearing the activated calcineurin transgene (Fv/BN strain) (26) were crossed with c−/− mice (C57BL/6J strain) (24). Mice of the resulting progeny were interbred to generate c−/− and c+/− mice expressing activated calcineurin in the heart. CTL lines were generated from these mice by coculture of isolated splenocytes with irradiated (2500 rad) BALB/c splenocytes (1:1 ratio) in RHFM medium supplemented with 80 µM rHIL-2 (Chiron) for 3 days. Irradiated stimulators were removed by Ficoll–Paque (Amershams Biosciences) centrifugation, and CTLs were maintained in tissue culture medium supplemented with 80 µM rHIL-2 for 2 additional days before further stimulation. Subsequent stimulations with irradiated BALB/c splenocytes were performed weekly at a ratio of 1:14 (CTLs: stimulators). CTLs that had been exposed to 4–13 cycles of stimulation before further stimulation. Subsequent stimulations with irradiated BALB/c splenocytes were performed weekly at a ratio of 1:14 (CTLs: stimulators). CTL lines from splenocytes derived from these mice. In this study we show that in the absence of calreticulin, CTL cytotoxicity is impaired. Because calreticulin ablation does not seem to affect components of the lytic machinery, the ability of CTLs to degranulate, or their survival during degranulation and attack of target cells, but does affect the efficiency of effector/target conjugate formation, we propose that calreticulin may be involved in the mechanisms underlying target recognition by CTLs and/or formation of the death synapse.

Killing assays

CTL cytolytic activity was evaluated as previously described (29), measuring membrane damage (51Cr release) and DNA fragmentation (l[3H]Thymidine release) induced in target cells. Briefly, allogeneic target cells (10⁴) were labeled with 50 Ci of Na2[3H]Thymidine, washed, and incubated at 37°C with effector CTLs at the indicated E:T cell ratio in 96-well plates. Where indicated, Co A was added to the samples at the final concentration of 2 µg/ml. To block perforin activity and to assess the contribution of Fas-mediated killing, CTL were preincubated for 2 h with 500 nM concanamycin A (CMA; Sigma-Aldrich), then added to the target cells in the presence of the inhibitor. [3H]Release in the medium was determined by lysis of target cells with 2% SDS in 0.2 M NaOH. The percent membrane lysis and DNA fragmentation were calculated as: [(sample cpm – spontaneous cpm)/(maximum cpm – spontaneous cpm)] × 100. In some experiments CTL were preincubated for 4 h with soluble anti-mouse CD3ε Ab (clone 145-2C11; 1/500 dilution of hybridoma supernatant) before performing the killing assay. Cells were then centrifuged, resuspended in RHFM medium, counted, and incubated with the target cells.

Flow cytometry

CTL were washed with PBS and fixed in 2% paraformaldehyde in PBS for 15 min at room temperature. After three washes in PBS, cells were permeabilized for 5 min at room temperature with 0.3% saponin in PBS containing 1% FCS. PE-conjugated anti-granzyme B mAb (clone CLB-GB12; PeliCluster) was added to each sample at a final concentration of 5 µg/ml and incubated at room temperature for 30 min. Cells were washed three times with PBS containing 1% FCS and analyzed with a FACScan flow cytometer equipped with CellQuest software (BD Biosciences).

Granzyme B enzymatic assay

Cell lysates were prepared by resuspending CTL in 20 mM Tris-HCl (pH 7.2), 150 mM NaCl, and 1% Triton X-100 at a concentration of 10⁷ cells/ml and incubating for 30 min on ice. Lysates were freeze-thawed once, centrifuged at 10,000 rpm for 7 min to remove cell debris, and stored at −80°C until used. Granzyme B activity in supernatant and total cell lysates was assayed as previously reported (30). Briefly, aliquots of incubation medium or cell lysates were dispensed into 96-well, flat-bottom tissue culture plates (Nalge Nunc International). Enzymatic reaction was conducted at 37°C in 20 med HEPES (pH 7.5), 10% (v/v) sucrose, 0.05% (v/v) CHAPS, and 5 mM DTT containing 200 µM acetyl-Ile-Glu-Pro-Asp-paranitroanilide (Kamiya Biomedical) as substrate. Hydrolysis of acetyl-Ile-Glu-Pro-Asp-paranitroanilide was measured at 405 nm using a Multiskan Ascent spectrophotometer (Thermo Lab-system). The absorbance of a blank containing no proteins was subtracted from each sample.

N-a-benzoyloxycarbonyl-l-lysine thioenzymester (BLT) esterase activity assay

Serine esterase activity in total cell lysates and medium was measured spectrophotometrically in 96-well, flat-bottom tissue culture plates. Enzymatic reaction was conducted at 37°C for 1 h at 30 mM Blt and 0.2 mM dithiobis-2-nitrobenzoic acid. Absorbance at 405 nm was read with a Multiskan Ascent spectrophotometer. The absorbance of total cell lysate from granzyme A/granzyme B double-knockout CTLs was subtracted from sample readings. Serine esterase activity was calculated based on the extinction coefficient of dithiobis-2-nitrobenzoic acid (ε₄₃₀ = 13,600).

Immunoblotting

CTL were washed in PBS, counted, and resuspended in SDS sample buffer at a concentration of 10⁷ cells/ml. Forty microcenters of sample was resolved by electrophoresis on a 12% SDS-polyacrylamide gel under reducing conditions. Proteins were transferred to a polyvinylidene difluoride membrane. The membrane was blocked for 1 h with 5% (w/v) milk/0.1% Tween 20 in TBS, then incubated overnight at 4°C with rabbit anti-perforin Ab (1/1000; Torrey Pines Biologabs). The secondary Ab was HRP-conjugated goat anti-rabbit IgG (Bio-Rad). Immunoblotting was performed by ECL Plus (Amershams Biosciences).

Immunocytochemistry

Cells were washed and resuspended at a concentration of 10⁷ cells/ml in PBS. Fifty microliters of the cell suspension was spotted onto silane-treated coverslips (Marianet Laboratory Glassware) and incubated at 37°C for 10 min. PBS was gently withdrawn, and adherent cells were fixed with 2% paraformaldehyde in PBS for 15 min at room temperature. After three washes with PBS, cells were permeabilized with 0.1% Triton X-100 for 3 min and blocked for 1 h with PBS containing 5% donkey serum. Incubation with the primary Abs was performed for 1 h at room temperature. The following Abs were used: mouse monoclonal anti-granzyme B (clone CLB-GB12; 1/50), rabbit anti-perforin (1/50), mouse monoclonal anti-perforin (provided by Dr. G. Griffiths, University College London, London, U.K.), and goat anti-ly-sosomal-associated membrane protein-1), and goat anti-LAMP-1 (1/20; Santa Cruz Biotechnology). Secondary Abs were: donkey anti-rabbit Alexa 594 anti-mouse Alexa 488 and Alexa 594, and donkey anti-goat Alexa 488, all used at 1/400 dilution, except for Alexa 594-conjugated Abs, which were used in 1/200 dilution (Molecular Probes). Slides were mounted with ProLong (Molecular Probes) and analyzed with an LSM510 laser scanning confocal microscope mounted on a Zeiss Axiovert 100M microscope.
Induction of granule exocytosis by immobilized anti-CD3ε Ab

Ninety-six-well, U-bottom plates were coated overnight at 4°C with anti-CD3ε mAb (clone 145-2C11; BD Biosciences) at the indicated dilutions in 0.1 M bicarbonate buffer; pH 9.6. The following day, wells were washed four times with cold PBS and blocked for 30 min at room temperature with 2% FCS in PBS. Blocking solution was then replaced with 100 μl/well CTL suspension in phenol-free RPMI 1640 medium containing 2% FCS (5 × 10^6 cells/ml). CTL were allowed to degranulate at 37°C for 5 h. After centrifugation at 1200 rpm for 5 min in an Allegra 6R centrifuge (Beckman Coulter), 50 μl of supernatant was transferred to a 96-well, flat-bottom plate for detection of granzyme B, BLT esterase, or lactic dehydrogenase (LDH) enzymatic activity.

LDH

LDH released by cells into the medium (50 μl) was measured at room temperature in a reaction volume of 200 μl and in the presence of 0.4 mM sodium pyruvate and 0.2 mM NADH. The kinetics of the enzymatic reaction were monitored spectrophotometrically, and the rate of absorbance decrement at 344 nm over 2 min was used to calculate LDH activity in the samples. Total LDH activity was measured in fresh cell lysates and used to derive the percentage of LDH released into the medium.

Analysis of conjugate formation

CTL were labeled with 2 μM Orange Cell Tracker (Molecular Probes), and P815 cells were labeled with 1 μM Green CellTracker (Molecular Probes) according to the manufacturer’s instructions. Labeled CTLs were left resting for 3 h at 37°C in regular medium or in the presence of soluble anti-CD3ε Abs (1/500), then washed in cold phenol-free HBSS. After resuspension in cold phenol-free HBSS containing 5% FCS, CTL and target cells were mixed at the ratio of 1:3 and centrifuged at 1000 rpm in an Allegra 6R centrifuge (Beckman Coulter) for 3 min at 4°C to facilitate cell-cell contact. Cells were then incubated at 37°C for the indicated time to allow conjugate formation. At the end of the incubation, samples were gently vortexed for 2 s and fixed by addition of an equal volume of 4% paraformaldehyde solution. Analysis of double-labeled cell conjugates was performed with a FACScan flow cytometer.

Statistical analysis

Data were analyzed by two-tailed Student’s t test for paired samples. A value of p < 0.05 was considered significant.

Results

Crt⁻/⁻ CTLs exert reduced cytotoxic activity on target cells

Crt⁺⁺⁺ and crt⁻⁻⁻ CTL lines were generated from transgenic mice expressing a constitutively active form of calcineurin in the heart. Because the expression of the transgene was under control of the cardiac specific α-myosin H chain promoter, we expected the transgene to be silent in the CTL lines. Western blotting analysis confirmed that the transgene was not expressed at any time in the CTLs (data not shown).

The cytolytic potential of the crt⁺⁺⁺ and crt⁻⁻⁻ CTLs was assessed in in vitro killing assays in which the two CTL lines were challenged with allogeneic target cells, the mouse mastocytoma cell line P815 and the lymphocytic leukemia L1210 line (Fig. 1). Compared with wild-type cells, crt⁻⁻⁻ CTLs showed reduced cytotoxicity toward target cells at all E:T cell ratios tested (shown in Fig. 1A for L1210 cells; killing of P815 targets occurred with a similar trend). Both membrane lysis (Fig. 1B) and DNA fragmentation (Fig. 1C) in target cells exposed to the CTL attack were measured. In all cases the cytotoxic activity of crt⁻⁻⁻ CTLs was significantly reduced with respect to that of wild-type cells. The difference was limited to the perforin-dependent killing mechanism, rather then Fas-mediated killing, because in the presence of CMA (31) cytotoxicity was greatly reduced, with no significant differences between the residual killing activities of the crt⁺⁺⁺ and crt⁻⁻⁻ CTLs.

It has been previously demonstrated that the level of granzyme B activity released by CTLs into the medium during incubation with target cells strictly correlates to the levels of degranulation and killing (30). As expected, the amount of granzyme B released by crt⁻⁻⁻ CTL during incubation with target cells was 50 ± 23% (p = 0.0032) lower than the amount secreted by wild-type cells. In light of these data, three possible explanations could be considered for the reduced cytotoxicity of crt⁻⁻⁻ CTLs: 1) CTL death during the killing assay, 2) lower expression of granzymes and perforin, or 3) inefficient target-induced degranulation.

Crt⁻⁻⁻ CTLs are viable during the attack of target cells

Because it has been proposed that calreticulin may have a protective role on CTLs against their own perforin (21), we investigated whether the lower level of killing observed with crt⁻⁻⁻ CTLs could be attributed to CTL death during incubation with target cells. Labeling of CTLs with Na₂⁵¹CrO₄ allowed us to monitor CTL death during the killing assay. No difference in CTL survival
was observed (Fig. 1D). CTL death was as low as 6–7% for both crt+/+ and crt−/− cells and was comparable to the level of death observed in the absence of target cells (spontaneous death), indicating that CTLs were viable during the assay.

Crt−/− CTLs have normal granzyme content and express high levels of perforin

To test the second hypothesis, we measured granzyme and perforin expression in crt−/− CTLs. As shown in Fig. 2, granzyme B expression, as measured by FACS staining, was not significantly different between crt+/+ and crt−/− cells (Fig. 2A). Moreover, the enzymatic activity of granzyme B was comparable, suggesting that in the absence of calreticulin, granzyme B can still fold properly without loss of activity (Fig. 2B). Crtp+/+ and crt−/− CTLs also contained the same amount of serine esterase (granzyme A) activity (Fig. 2C). In contrast, levels of perforin expression were higher in crt−/− CTLs than in wild-type cells (Fig. 2D). No differences in granzyme B and perforin intracellular localization were evident by confocal microscopy, as assessed by colocalization with lysosomal-associated membrane protein-1, a lysosomal marker also present in lytic granules (32) (Fig. 3).

Crt−/− CTLs degranulate more efficiently than crt+/+ cells when stimulated with immobilized anti-CD3e Ab

To investigate whether impaired transduction of the signal from the TCR-CD3 complex could account for the reduced cytotoxicity observed in the absence of calreticulin, we triggered activation of the TCR-CD3 complex and exocytosis of lytic granules by exposing CTL to immobilized anti-CD3e Ab (33, 34). In these experi-

mental conditions, the crt−/− CTLs degranulated and released granzyme B and BLT esterase activity efficiently (Fig. 4). Although at a saturating concentration (10 μg/ml) of immobilized Ab, the release of granzyme B and BLT esterase was comparable in crt+/+ and crt−/− CTLs, at lower Ab concentrations crt−− cells secreted even more granzyme B than wild-type cells (Fig. 4A). CTL death during the 5 h of incubation was <7% of the total cell number, as assessed by measuring LDH release into the medium, with no significant difference between cell lines or between control (no Ab) and stimulated samples (data not shown). Therefore, the higher secretion of granzymes by crt−/− cells was due to specific triggering of TCR-CD3 signaling by immobilized anti-CD3 Ab.

Effector/target conjugate formation is impaired in crt−/− CTLs, and cytotoxicity is restored in conditions that favor cell adhesion

Impaired cytotoxicity may result from defective recognition and/or adhesion to target cells. Indeed, we found that crt−/− CTLs were
less efficient than wild-type cells in forming conjugates with target cells (Fig. 5A). If reduced conjugate formation (or stability) was the cause of the low levels of killing observed with \( \text{crt}^{-/-} \) CTLs, we expected to see increased cytotoxicity after treatments and in conditions that enhance cell adhesion. Therefore, we prestimulated CTLs with soluble anti-CD3 Abs before washing and exposing them to target cells. Soluble anti-CD3 Abs do not induce CTL degranulation (34), but are known to activate adhesion of CTLs to substrates and target cells (35). As expected, anti-CD3-stimulated \( \text{crt}^{-/-} \) CTLs became indistinguishable from wild-type cells (Fig. 5B). The restored cytotoxicity to kill target cells (Fig. 6C), further demonstrating that restoration of the \( \text{crt}^{-/-} \) CTL activity was due to the increased ability to degranulate upon recognition of target cells.

Similar results were obtained when the killing assay was performed in the presence of Con A (Fig. 7A). Con A is known to induce lectin-dependent, cell-mediated cytotoxicity, a process in which target cells are killed by CTLs in an Ag-independent fashion. This is achieved through the bridging action of Con A that binds to glycoproteins on the surface of both effectors and target cells and at the same time cross-links the TCR on the CTL membrane (36–40). In the presence of Con A, \( \text{crt}^{-/-} \) CTL cytotoxicity increased significantly, allowing for the killing of allogeneic targets (P815) at levels close to those observed with wild-type CTLs. Moreover, Ag-independent cytotoxicity toward syngeneic target cells (EL-4) was observed for both \( \text{crt}^{+/+} \) and \( \text{crt}^{-/-} \) CTLs (Fig. 7B).

**Discussion**

Previous studies have identified calreticulin, an ER-resident chaperone and a \( \text{Ca}^{2+} \)-binding protein, as a component of CTL and NK cell lytic granules (15). Upon activation of CTLs, calreticulin gene expression is up-regulated (23), and the protein is targeted to lytic granules (20) and the plasma membrane (41). Although several studies have reported on functions played by the protein outside the ER (reviewed in Refs. 16 and 17), there is no information on its role in the lytic granules and CTL physiology. In this regard, our study provides the first direct evidence of calreticulin involvement in the cytotoxic activity of CTLs. Because the calreticulin knockout is embryonically lethal (24), we generated a CTL line from \( \text{crt}^{-/-} \) mice that were able to survive due to the ectopic expression of active calcineurin in the heart (25). The transgenic constitutively active form of calcineurin was not expressed in tissues other than heart, allowing for the specific analysis of calreticulin deficiency in CTLs. The \( \text{crt}^{-/-} \) CTLs were healthy, providing evidence that calreticulin function is dispensable during development and maturation of CTLs. Our study also indicates that

![Image](http://www.jimmunol.org/DownloadedFrom.png)
calreticulin is not necessary to protect CTLs from lytic activity of stored perforin, a hypothesis that was proposed when the ER chaperone calreticulin, with consequent accumulation of misfolded proteins that would otherwise be degraded. Alternatively, higher gene expression or increased protein half-life (or decreased protein turnover) could account for the increased perforin content in the cmt−/− CTLs. Whether all the perforin molecules produced by cmt−/− CTLs were correctly folded is not known. However, we believe that at least an amount of active perforin comparable with the wild-type content must have been present in the cmt−/− CTLs, otherwise we would not have been able to restore cytotoxicity by treatment with aCD3 or Con A. The data on perforin are intriguing, and they suggest that the lack of calreticulin may generate a multifaceted phenotype in CTLs. However, the higher levels of the pore-forming protein in the cmt−/− CTLs did not seem to have an impact on CTL viability or their cytolytic function.

It has been proposed that calreticulin may be involved in sorting granule components, perforin in particular, to the lytic granules (20). However, our data argue against such a hypothesis, because intracellular localization of perforin and granzyme B in cmt−/− CTLs appeared to be similar to that in wild-type cells.

Calreticulin has been shown to block the lysis of erythrocytes induced by perforin (21, 22). Consequently, it was proposed that calreticulin might bind to the CTL surface upon degranulation, preventing polymerization of perforin within the CTL plasma membrane and consequent lysis of the effector cells. If that hypothesis were correct, one would have expected to observe cmt−/− CTL lysis during the killing of target cells and overall reduced

FIGURE 6. Killing assays after prestimulation with anti-CD3e Ab. CTLs were exposed to soluble anti-CD3e Ab (aCD3 +) for 4 h, washed, and then incubated with target cells (E:T cell ratio, 2:1 for P815 and 5:1 for L1210 cells). □, cmt+/+ CTLs; ■, cmt−/− cells. A, DNA fragmentation in target cells. Data are expressed as the percentage of cytotoxicity with respect to the killing induced by cmt+/+ CTLs. Values are the mean ± SD of six experiments, each performed in triplicate. *, p = 0.01; **, p = 0.0008. B, Membrane lysis in target cells. Data are expressed as the percentage of cytotoxicity with respect to the killing induced by cmt+/+ CTLs. Values are the mean ± SD of five experiments, each performed in triplicate. *, p < 0.02. C, Levels of degranulation (measured by granzyme B activity released in the medium) during the killing of P815 cells. CTLs were preincubated with anti-CD3e Ab (aCD3 +) or with regular medium (aCD3 −) before exposing them to target cells. Data are expressed as the percentage of granzyme B released over the amount released from cmt+/+ CTLs. Values are the mean ± SD of four experiments, each performed in triplicate. *, p = 0.00006.

FIGURE 7. Killing assay in the presence of Con A. □, cmt+/+ CTLs; ■, cmt−/− cells. A, CTL cytotoxicity in the presence of Con A is expressed as the percentage of killing (DNA fragmentation) of target cells (P815) with respect to the level of killing induced by cmt+/+ CTLs. CTRL, assay performed under control conditions; Con A, assay performed in the presence of Con A. B, Killing of syngeneic target cells (EL4). Where indicated, Con A was added to the incubation medium. Fas-mediated killing was assessed by preincubation of CTLs with 500 nM CMA (CMA +). Data represent the percentage of target cells killed (DNA fragmentation). Values are the mean ± SD of two independent experiments, each performed in triplicate. *, p = 0.0002.
target killing. However, when we measured \textit{crt} \textsuperscript{−/−} CTL lysis during incubation with target cells, we did not find any specific increase in CTL death relative to wild-type cells or with respect to the levels of cell death spontaneously occurring in CTL populations in the absence of targets. Similar results were obtained when CTL lysis was measured during immobilized anti-CD3 Abs-induced degranulation. These data argue against a protective role of calreticulin on CTLs. Other molecules must be involved in CTL protection, such as the recently identified cathepsin B (42).

Because \textit{crt} \textsuperscript{−/−} CTLs secreted less granzyme B than wild-type CTLs in response to stimulation by target cells, we investigated the hypothesis that aberrant transduction of the signal downstream of the TCR-CD3 complex could result in impaired degranulation. When we triggered granule exocytosis by incubation with immobilized anti-CD3 Abs, thus bypassing the need for recognition and contact with target cells, \textit{crt} \textsuperscript{−/−} CTLs responded better than wild-type cells, showing a lower threshold for maximal induction of degranulation. Granule exocytosis is triggered in CTLs by initial release of Ca\textsuperscript{2+} from intracellular stores, followed by sustained influx of extracellular Ca\textsuperscript{2+} through a store-operated Ca\textsuperscript{2+} channel (reviewed in Ref. 43). In our study calreticulin was dispensable for induction of degranulation when this was triggered by CD3 cross-linking. In line with our observations are data showing that overexpression of calreticulin decreases store-operated Ca\textsuperscript{2+} influx through a mechanism that seems to be independent from its Ca\textsuperscript{2+}-buffering activity (44).

The remaining possible explanation for the reduced cytotoxic activity of \textit{crt} \textsuperscript{−/−} CTLs is impaired recognition and/or adhesion to target cells. As a matter of fact, we found that when \textit{crt} \textsuperscript{−/−} CTLs were mixed with target cells, they formed less (or less stable) effector-target conjugates than wild-type CTLs. To test whether this could be taken into account for the reduction of cytotoxicity in \textit{crt} \textsuperscript{−/−} CTLs, we used prestimulation with soluble anti-CD3 Abs (35) or addition of Con A (36–40) as experimental tools to increase the formation of effector-target conjugates during killing assays. Under these conditions, \textit{crt} \textsuperscript{−/−} CTL granule-mediated cytotoxicity was restored. These results suggest that the lytic machinery of \textit{crt} \textsuperscript{−/−} CTLs (granzymes and perforin) is functional and has the potential to kill target cells, provided that the contact between effectors and targets is efficiently established.

The effects of calreticulin on recognition and/or binding to target cells are currently under investigation. Target recognition could be affected due to reduced expression of TCR. However, this did not seem to be the case in \textit{crt} \textsuperscript{−/−} CTLs, because they expressed similar or slightly higher levels of CD3 than wild-type cells. We cannot exclude the possibility that protein misfolding or aberrant post-translational modification in calreticulin-deficient cells may alter the Ag recognition ability of the TCR. Alternatively, other mechanisms involved in the adhesion to target cells may be affected by calreticulin deficiency.

One of these mechanisms involves the family of integrin proteins, specifically LFA-1 (45, 46) and \(\beta_1\) and \(\beta_3\) integrins (47), which have been shown to promote CTL adhesion and TCR-mediated cytotoxicity (for review, see Ref. 48). The idea that integrins may be a target of calreticulin deficiency is particularly intriguing because it is supported by studies showing calreticulin involvement in cell adhesion and integrin function in different cell types (49–53). In addition, anti-CD3 Abs, which restored \textit{crt} \textsuperscript{−/−} CTL cytotoxicity in our study, are known to enhance LFA-1-dependent adhesion of CTLs to target cells (54). Calreticulin also binds thrombospondin (55–57), an extracellular matrix protein with general antiadhesive effects (58) that is involved in the regulation of processes such as T cell activation, adhesion, and homeostasis (reviewed in Ref. 59). Future studies will address the hypothesis that calreticulin deficiency may alter the integrins and/or thrombospondin-mediated adhesive properties of CTLs.

In conclusion, we have shown that despite functional lytic machinery, cytotoxicity is impaired in calreticulin-deficient CTLs, probably due to inefficient effector-target conjugate formation. Such a deficit could have important implications in the regulation of the immune response in vivo. In light of our data, it is tempting to speculate that calreticulin deficiency could result in reduced or delayed clearance of infected and transformed cells. At the same time, it could severely affect lymphocyte homeostasis and result, as do several genetic defects impeding degranulation of CTLs or inactivating perforin (60, 61), in hemophagocytic-like syndromes or autoimmune diseases. Interestingly, autoantibodies against calreticulin have been found in several autoimmune conditions (62–67).

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Disclosures

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