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CD8 T Cell-Mediated Killing of Cryptococcus neoformans Requires Granulysin and Is Dependent on CD4 T Cells and IL-15

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Granulysin is located in the acidic granules of cytotoxic T cells. Although the purified protein has antimicrobial activity against a broad spectrum of microbial pathogens, direct evidence for granulysin-mediated cytolysis has heretofore been lacking. Studies were performed to examine the regulation and activity of granulysin expressed by CD8 T cells using Cryptococcus neoformans, which is one of the most common opportunistic pathogens of AIDS patients. IL-15-activated CD8 T cells acquired anticytotoxic activity, which correlated with the up-regulation of granulysin. When granules containing granulysin were depleted using SrCl2 or when the gene was silenced using 21-nt small interfering RNA duplexes, the antifungal effect of CD8 T cells was abrogated. Concanamycin A and EGTA did not affect the antifungal effect, suggesting that the activity of granulysin was perforin independent. Following stimulation by the C. neoformans mitogen, CD8 T cells expressed granulysin and acquired antifungal activity. This activity required CD4 T cells and was dependent upon accessory cells. Furthermore, IL-15 was both necessary and sufficient for granulysin up-regulation in CD8 T cells. These observations are most consistent with a mechanism whereby C. neoformans mitogen is presented to CD4 T cells, which in turn activate accessory cells. The resultant IL-15 activates CD8 T cells to express granulysin, which is responsible for antifungal activity. The Journal of Immunology, 2002, 169: 5787–5795.

1 Although the ability of cytotoxic lymphocytes to kill tumor cells or infected host cells is well recognized, direct lymphocyte-mediated microbial killing is currently an underinvestigated enigma of these cells. One of the important mechanisms by which lymphocytes could directly kill or inhibit the growth of microbes is via the release of granulysin, which is a member of the saposin-like protein family of molecules, including amoebapore, pulmonary surfactant protein-B, saposins, and Nk-lysin (1). Granulysin is contained within the granules of cytotoxic lymphocytes and exists in 15- and 9-kDa forms (2, 3). The 15-kDa protein is the product of granulysin mRNA following cleavage of a hydrophobic leader sequence, while the 9-kDa active form of granulysin is the product of processing of the 15-kDa form at the carboxyl and amino termini (3). Granulysin interacts with lipids in the cell membrane and also activates lipid-degrading enzymes (4), which leads to lesions in the cell membrane that result in death of the targets (5, 6). Although previous studies have shown that purified granulysin has antimicrobial activity (7, 8), it is still unknown whether granulysin expression is the mechanism by which CTL exert their antimicrobial activity.

Studies of the role of granulysin have been thwarted by the inability to specifically deplete granulysin in human lymphocytes without affecting the other contents of the granules. Furthermore, blocking Abs to granulysin are ineffective, presumably because they do not gain access to the synapse between the cytotoxic lymphocyte and the target cell. Thus, it was necessary to apply a new technique to inhibit granulysin. For this purpose, RNA interference (RNAi) might hold promise. RNAi is a newly developed method of gene silencing that was initially applied with success in Caenorhabditis elegans (9–11), plants (12, 13), Drosophila melanogaster (14, 15), and Trypanosoma brucei (16). Until recently, mammalian cells were not amenable to RNAi, because in vitro transcribed long dsRNAs (>30 bp) led to activation of a nonspecific response that blocked initiation of protein synthesis and mRNA degradation (17). However, recently, it has been reported that dsRNA is processed to shorter fragments that mediate specific gene-silencing activity (18). This activity can be mimicked by double strand 21-nt RNA with 2-nt 3’ overhang, which specially inhibit gene expression and bypass the sequence-independent response of mammalian cells to long dsRNA (19). These short RNA duplexes are referred to as small interfering RNA (siRNA) (18) and present an opportunity to use RNAi in mammalian cell systems.

Among the potential microbial pathogens, Cryptococcus neoformans is one of the most common life-threatening fungal infections in patients who have compromised cell-mediated immunity, including AIDS (20–22). This microbe has previously been shown to be susceptible to recombinant granulysin (7), and therefore, it is an excellent target for studies of expression and regulation of granulysin. Previous studies have shown that cytotoxic lymphocytes possess anticytotoxic activity, but these studies failed to identify the antimicrobial mechanism (23). We questioned whether
granulysin might be the previously unidentified factor responsible for lymphocyte-mediated anticytotoxic activity (24). Furthermore, T cells are activated by the C. neoformans mitogen (CNm), which is contained in the cell wall of C. neoformans (25). Thus, C. neoformans provides a simple system, in which it can serve as both the stimulus and the target for studies of granulysin regulation and activity.

Although granulysin protein expression has been detected in CTL and NK cell lines, gene transcription and protein expression are induced after activation of PBL (26, 27). This suggests that activation is required for the expression and activity of granulysin in CD8 T cells. However, activation of primary CD8 T cells is a complicated process in which CD4 T cells are required. CD4 T cells are in turn activated by an accessory cell and a costimulation-dependent mechanism. The activated CD4 T cells provide T cell growth factors that activate CD8 T cells (28). Alternately, CD4 T cells can signal accessory cells to a heightened state of activation; the accessory cells then acquire the ability to stimulate CD8 T cells (29).

It has been demonstrated that lymphocyte-mediated antifungal activity was critically dependent on IL-15 (30). IL-15 is a member of the four-helix bundle cytokine family with growth factor activity for T cells and NK cells (31, 32). IL-15 is produced by many cell types including monocytes/macrophages (33, 34) and dendritic cells (35), but not by T cells (36). IL-15 also promotes the survival and proliferation of memory lymphocytes (36–38), and induces NK cells to be cytolytic effector cells (39). However, little is known about the mechanisms by which IL-15 induces direct anticytotoxic activities of CD8 T cells. Furthermore, it was not known whether CD4 T cells are required or whether accessory cell signals, such as the accessory cell-derived IL-15, might be sufficient for expression of granulysin and anticytotoxic activity.

To determine whether granulysin is responsible for the antifungal activity of CD8 T cells, cells were stimulated with IL-15 and the expression of granulysin was detected by immunoblot with an Ab that recognizes both the 15- and 9-kDa forms of granulysin. The level of expression of granulysin was correlated with the anticytotoxic activity of CD8 T cells by assessing the number of viable organisms after incubation with the cytotoxic lymphocytes. To determine whether granulysin is necessary for the anticytotoxic activities of IL-15-activated CD8 T cells, granulysin was depleted with SrCl2, and gene silencing by siRNA was used to block granulysin. To determine whether perforin is required for the antifungal effect of granulysin, CD8 T cells were treated with concanamycin A and EGTA, which inhibit the perforin-mediated cytotoxicity pathway. Finally, the regulation of granulysin during activation by the CNm was examined. By comparing the activation in the presence of CD4 T cells to that in the absence of CD4 T cells, we assessed the contribution of CD4 T cells to the activation of CD8 T cells. Additionally, the requirement for IL-15 was assessed using a neutralizing Ab to IL-15.

Materials and Methods
Preparation of C. neoformans and CNm

C. neoformans CAP 67 (ATCC 52817, acapsular mutant of 3501) was obtained from the American Type Culture Collection (Manassas, VA) and was used in the present study. The organisms were maintained on Sabouraud dextrose slants (Difco, Detroit, MI) and passaged to fresh slants every month as previously described (40). CNm was prepared as previously described (25, 41). Briefly, C. neoformans was disrupted by rotating the organisms with 0.5-mm diameter glass beads in a bead mill (Bead Beater; Biospec Products, Bartlesville, OK). The cell wall of these disrupted organisms was digested overnight by using β-1,3-glucanase (ICN, Aurora, OH) and solubilized in 50 mM phosphate buffer containing 1% SDS (BDH, Toronto, Ontario, Canada), 100 μM pepstatin A (Sigma–Aldrich, St. Louis, MO), and 100 mM EDTA (BDH) for 1 h. SDS was removed by acetone precipitation by centrifugation at 4°C. The protein concentration was determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL).

Isolation and stimulation of PBMC

PBMC were isolated as described previously (25). Briefly, peripheral blood was obtained by venipuncture from healthy adults who had no history of cryptococcosis and had not worked with C. neoformans. PBMC were isolated by centrifugation (800 × g, 20 min) over a Ficoll-Hypaque density gradient (C-SIX Diagnostics, Mequon, WI). PBMC were harvested and washed three times in HBSS (Life Technologies, Burlington, Ontario, Canada) and then resuspended in medium containing RPMI 1640 (Life Technologies), 5% human AB serum (BioWhittaker, Walkersville, MD) 100 U of penicillin per ml, 100 μg of streptomycin per ml, 0.25 μg of amphotericin B per ml, 2 mM l-glutamine, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids (all from Life Technologies).

CD8 T cells were isolated by the MACS negative selection system using the CD8 T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) for 3 days or with 10 μM 2′,2′-diamino-6′-tericin B per ml, 2 mM l-glutamine, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids (all from Life Technologies).

CD8 T cells were stimulated with IL-15 (50 ng/ml) (R&D Systems, Toronto, Ontario, Canada) (48 h). In preliminary experiments, granulysin was detected when CD8 T cells were stimulated with 30 ng/ml IL-15. The amount of granulysin increased up to the maximum amount of IL-15 tested (100 ng/ml). However, 30 ng/ml IL-15 did not induce granulysin expression in CD8 T cells in all of the donors; thus, 50 ng/ml was used for all of the experiments. For some experiments, purified stimulated CD8 T cells were treated with 25 mM strontium chloride (Sigma–Aldrich) for 18 h (42, 43), or 10 nM concanamycin A (Sigma–Aldrich) for 2 h, or 4 nM EGTA (Sigma–Aldrich) for 12 h. The cells were washed three times in medium and placed in culture for the experiment. The viability of cells was not altered by these treatments as assessed by trypan blue exclusion.

For some experiments, PBMC were stimulated with PHA (1 μg/ml; Sigma–Aldrich) for 3 days or with Cm (1-1000 ng/ml) for 7 days before flow cytometry using FITC-labeled Abs to CD8, PerCp-labeled Abs to CD3, and FITC-labeled Abs to CD56 (BD Biosciences, Mountain View, CA). For CD8 T cell depletion experiments, CD8 T cells were depleted from PBMC by using CD4 microbeads (Miltenyi Biotec) (the remaining population was 25% CD8 T cells), followed by stimulation with CNm and IL-15 or with CD8 T cells and CD4 T cells were purified from unstimulated or CNm-stimulated PBMC by CD8 T cell and CD4 T cell isolation kits, respectively (Miltenyi Biotec).

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Immunoblot analysis

Cells were placed in a lysis buffer (50 mM Tris (pH 6.8), 1% SDS, 0.025% bromophenol blue, 10% glycerol, 20 mM DTT) and were sonicated. The nuclei were removed by centrifugation at 10,000 × g for 30 min. Protein from the lysates of 5 × 10⁶ cells were loaded in each lane of an 16% Tris-glycine gel (Invitrogen, San Diego, CA), separated by electrophoresis, transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA) and blotted with polyclonal anti-granulysin Ab 519/GST rabbit serum (1/1000 dilution) (7). The reactive bands were visualized using HRP-conjugated anti-rabbit IgG (1/5000 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA) with the ECL plus Western blotting detection system (Amersham Pharmacia Biotech, Piscataway, NJ). The membranes were then labeled with a mouse monoclonal anti-β actin Ab (Chemicon International, Temecula, CA) and bands were detected as described above. For the detection of perforin, the membranes were blotted with a polyclonal anti-perforin Ab (Research Diagnostic, Flanders, NJ) (1/1000 dilution), and the bands were visualized as described above.

siRNA preparation and gene silencing assays

To design target-specific siRNA duplexes, we selected a sequence of the type AA(N₃₀)₄dTdT (N, any nucleotide) from the open reading frame of the granulysin mRNA, to obtain a 21-nt sense and 21-nt antisense strand with symmetric 2-nt 3′ overhangs of identical sequence. The selected siRNA sequence was also submitted to a search by basic local alignment search tool against the human genome sequence to ensure that only one gene was targeted. The siRNA sequence targeting granulysin mRNA (GenBank accession number NM.012483) was from position 476–498. dsRNA was synthesized by Dharmacon (Dharmacon Research, Lafayette, CO). CD8 T cells were transfected by electroporation (250 V, 800 μF) (Bio-Rad) with granulysin siRNA (1 μg per well) or with CD20 siRNA (target sequence: C. neoformans).
5′-AACACTCTTCAGGAGGTGT-3′ (Dharmacon Research, Lafayette, CO) or a nonsilencing siRNA (target sequence: 5′-AATCTCC GAACGTGTCACGT-3′) (Xeragon, Huntsville, AL), which served as negative controls. It has been shown that CD20 siRNA could decrease the constitutive expression of CD20 on Ramos B cells (a B cell line that constitutively expresses CD20) by ∼50% (H. D. Li and Dr. J. P. Deans, personal communication). The treated cells were stimulated with IL-15 as described above.

Semiquantitative RT-PCR

Total RNA was extracted from individual cell samples using the RNA extraction kit (Qiagen, Chatsworth, CA). The extracted total RNA was quantified fluorometrically using the SYBR Green II fluorescent RNA dye (Molecular Probes, Eugene, OR) on a LS-5 fluorescence spectrophotometer (PerkinElmer, Wellesley, MA) with excitation at 468 nm and emission at 525 nm using a standard curve of rRNA (Sigma-Aldrich).

One microgram of total RNA from each sample was reverse transcribed using a Stratagene RT-PCR kit (Stratagene, La Jolla, CA), following the manufacturer’s instructions. The following granulysin-specific primers were used: sense, 5′-CATATGCATATGGGCGCGCTGACTACAGGAC-3′; and antisense, 5′-GGATCCGAGTTACCTGAGGTCTCCTCA CAGATCTG-3′ (44). The following human GAPDH-specific primers were used: sense, 5′-TCACCTTCCAGGAGCCGA-3′; and antisense, 5′-AGTGATGGCATGGACTGTG-3′. The PCR profile for granulysin and GAPDH was as follows: denaturation at 94 °C for 1 min, followed by 25 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min. A final extension at 72 °C for 10 min was used. The number of cycles was adjusted so that amplification occurred over the linear range. The PCR products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. The gels were then photographed using Polaroid Type 55 Land film and analyzed by densitometry (MasterScan. Interpretive Densitometer and RFLPscan; Scanalytics/CSPI, Fairfax, VA). Integrated density values were normalized to GAPDH values to yield a semiquantitative assessment of individual transcript levels.

Anticryptococcal activity of CD8 T cells

A CFU assay was performed as previously described (45–47). C. neoformans (2 × 10^5/well) were incubated with or without 10^5 lymphocytes. The number of CFU of C. neoformans per well was determined at 0, 24, or 48 h by lysing the effector cells with 0.1% Triton X-100 followed by diluting and spreading onto Sabouraud dextrose agar plates. Preliminary experiments established that this concentration of Triton X-100 lysed effector cells without affecting fungal viability.

Results are expressed as the percentage of growth according to the following formula: ((CFU experimental/CFU inoculum) − 1) × 100. Thus, a value of zero indicates that the number of CFU at the start and conclusion of the incubation were the same, and fungistasis was obtained. A value of +100 indicates that the number of C. neoformans doubled, while values less than zero indicate killing.

Statistics

Values are expressed as mean ± SE. Each experiment was performed with different donors on different days. Statistical analysis was performed by using the Fisher least-squares difference, when allowed by the F value (Statview; Brain Power, Calabasas, CA). For these tests, a p of <0.05 was considered significant.

Results

IL-15-enhanced anticryptococcal activity of CD8 T cells and induced granulysin expression

To determine whether IL-15 could enhance the anticryptococcal activities of CD8 T lymphocytes, purified peripheral blood CD8 T cells were stimulated with IL-15. The ability of stimulated CD8 T cells to inhibit the growth of C. neoformans was compared with that of unstimulated CD8 T cells. When C. neoformans was placed in culture without CD8 T cells, there was a >400% increase in the number of organisms after 48 h (Fig. 1A). When C. neoformans was cultured with CD8 T cells stimulated with IL-15, there was a significant anticryptococcal effect. In these cultures, the number of organisms at 24 and 48 h was significantly lower than the starting inoculum (Fig. 1A), indicating that the stimulated CD8 T cells had acquired fungicidal activity and killed C. neoformans. By contrast, the growth of C. neoformans in the presence of unstimulated CD8 T cells was not significantly different from wells containing C. neoformans alone (Fig. 1A), indicating that unstimulated CD8 T cells had not acquired anticryptococcal activity. These results indicate that CD8 T cells acquired anticryptococcal activity after IL-15 stimulation.

It has been reported that recombinant granulysin can inhibit C. neoformans growth in vitro (7). Therefore, experiments were performed to examine the expression of granulysin in CD8 T cells before and after stimulation with IL-15. The 15- and 9-kDa forms of granulysin were expressed in IL-15-stimulated CD8 T cells but could not be detected in unstimulated CD8 T cells (Fig. 1B). Hence, increased anticryptococcal activity correlated with up-regulation of granulysin in CD8 T cells.

Granulysin was necessary for CD8 T cell-mediated anticryptococcal activity

After demonstrating a correlation with increased activity, experiments were performed to determine whether depletion of granulysin correlates with a reduction in antifungal activity in activated CD8 T cells. The currently available technique uses SrCl2, which depletes the granule components of the cells without causing cellular toxicity (48, 49). Unstimulated and IL-15-stimulated CD8 T cells were treated with SrCl2. The CD8 T cells expressed granulysin after stimulation by IL-15, and this expression was abrogated after treatment with SrCl2 (Fig. 2A). In parallel, the IL-15-stimulated CD8 T cells that had been SrCl2-treated were challenged with C. neoformans. Treatment of IL-15-stimulated CD8 cells with
SrCl₂ abrogated the anticryptococcal activity (Fig. 2B). Thus, depletion of granules containing granulysin correlated with a marked reduction in anticryptococcal activity.

However, strontium treatment is nonspecific, because it depletes all of the granule contents including granulysin. To specifically interfere with granulysin, RNAi was used. CD8 T cells were transfected with siRNA to granulysin. As a control, CD8 T cells were also treated with siRNA to CD20 (CD20 is a B cell marker that is not expressed in T cells) or nonsilencing siRNA. CD8 T cells were stimulated with IL-15, and granulysin mRNA was determined by semiquantitative RT-PCR. The response of treated cells was compared with that of cells that had not been electroporated (EP –) or electroporated but not treated with siRNA (EP +). Granulysin was detected by immunoblot from above groups. The antifungal effect of IL-15-stimulated CD8 T cells (IL-15+), IL-15 stimulated CD8 T cells transfected with granulysin siRNA (IL-15 + Gran), or CD20 siRNA (IL-15 + CD20), or nonsilencing siRNA (IL-15 + NSI), or sham-transfected (IL-15 + none) was compared with the growth of C. neoformans alone (Crypto alone) or untreated CD8 T cells (US). Results are expressed as mean ± SE. NS, Not significant. * p < 0.05 compared with the growth of C. neoformans alone. †, p < 0.05 compared with the IL-15-stimulated CD8 T cells. The experiments were repeated three times with similar results.
and challenged with live C. neoformans. The growth of C. neoformans was inhibited in the presence of IL-15-stimulated CD8 T cells. By contrast, anticytotoxic activity was abrogated by transfection of granulysin siRNA in IL-15-stimulated CD8 T cells (Fig. 3C). This suggests that granulysin is necessary for CD8 T cell-mediated anticytotoxic activity.

Granulysin did not require perforin to function

It has been demonstrated that perforin is required for the anticytobacterial activity of granulysin (7). For this reason, experiments were performed to determine whether perforin was required for the anticytotoxic activity of granulysin. CD8 T cells were stimulated with IL-15 in the presence or absence of concanamycin A, an inhibitor of vacuolar ATPase that is required to maintain perforin in lytic granules (50). Immunoblot analysis revealed that perforin was decreased by concanamycin A treatment but the granulysin level was not affected in IL-15-stimulated CD8 T cells (Fig. 4A). We could not detect perforin expression in peripheral blood unstimulated CD8 T cells by Western blot. Our results are consistent with previous studies in which perforin is not detected in unstimulated peripheral blood CD8 T cells (51, 52). Correspondingly, experiments were performed to determine the effect of concanamycin A treatment on the anticytotoxic activities of CD8 T cells. IL-15-stimulated CD8 T cells treated with concanamycin A showed similar levels of antifungal activity to those of concanamycin A-untreated IL-15-stimulated CD8 T cells (Fig. 4B). Similar results were obtained with EGTA, which chelates calcium and blocks perforin polymerization (Fig. 4C).

CD8 T cells isolated from CnM-stimulated PBMC acquired antifungal activity

Our previous work showed that CnM causes activation and proliferation of peripheral blood T lymphocytes (25). This provided an opportunity to study the events involved in the activation of CD8 T cells that was more similar to the activation during an infection than the activation provided by recombinant IL-15. To determine whether CD8 T cells could be activated by CnM to express granulysin and anticytotoxic activity, PBMC were stimulated and the CD8 T cells were isolated. Immunoblot analysis showed that a small amount of the inactive 15-kDa form of granulysin was detected in CD8 T cells isolated from unstimulated PBMC but failed to detect the 9-kDa active form (Fig. 5A). CD8 T cells isolated from PHA- or CnM-stimulated PBMC expressed a greater amount of the 15-kDa form, and the 9-kDa form of granulysin was easily detected (Fig. 5A). Correspondingly, CD8 T cells isolated from CnM-stimulated PBMC acquired anticytotoxic activity, while CD8 T cells isolated from unstimulated PBMC did not (Fig. 5B). A similar effect was observed when other encapsulated strains of C. neoformans were used as targets (data not shown).

CD4 T cells were required for the granulysin expression and anticytotoxic activity of CD8 T cells stimulated with CnM

Although CD4 cells are critically important for immune responses and host defense to C. neoformans (53), both CD4 and CD8 T lymphocytes are involved in the generation of protective immune responses to C. neoformans (54–57). Furthermore, CD8 cells can be activated independently of CD4 T cells in response to C. neoformans (55–58). Therefore, experiments were performed to determine whether CD4 T cells were required for the activation of CD8 cells that resulted in granulysin expression and anticytotoxic activity. CD8 T cells were isolated from CnM-stimulated PBMC that had been depleted of CD4 T cells. These cells were compared with CD8 T cells isolated from CnM-stimulated PBMC that were unstimulated (IL-15) or stimulated with IL-15 (IL-15 + ) in the absence of concanamycin A (CMA – ) or in the presence of CMA (CMA + ). B. The growth of C. neoformans in the presence of unstimulated CD8 T cells (US), unstimulated CD8 T cells treated with CMA (CMA-US), IL-15-stimulated CD8 T cells (IL-15), IL-15-stimulated CD8 T cells treated with CMA (IL-15 + CMA) was compared with the growth of C. neoformans alone (Crypto alone) or C. neoformans treated with CMA (Crypto+CMA). C. The growth of C. neoformans in the presence of unstimulated CD8 T cells (US), unstimulated CD8 T cells treated with EGTA (EGTA-US), IL-15-stimulated CD8 T cells (IL-15), IL-15-stimulated CD8 T cells treated with EGTA (IL-15+EGTA) was compared with the growth of C. neoformans alone (Crypto alone) and C. neoformans in the presence of EGTA (Crypto+EGTA). Results are expressed as mean ± SE. NS, Not significant. *, p < 0.05 compared with the growth of C. neoformans alone. The experiments were repeated three times with similar results.
Regulation of granulysin and anticyptococcal activity

Results are expressed as mean ± SE. NS, Not significant. *, p < 0.05 compared with the growth of C. neoformans alone. The experiments were repeated three times with similar results.

that had not been depleted of CD4 T cells. Immunoblot analysis confirmed the previous observation that CD8 T cells stimulated in the presence of CD4 cells expressed both the 15- and 9-kDa forms of granulysin (Fig. 6A). However, CD8 T cells that had been isolated from CD4 T cell-depleted, CnM-stimulated PBMC expressed only a trace amount of the 15-kDa form of granulysin and undetectable levels of the 9-kDa granulysin (Fig. 6A), which was similar to the expression of unstimulated CD8 T cells. Thus, CD4 T cells were required for the up-regulation of granulysin in CD8 T cells.

Similarly, depletion of CD4 T cells or accessory cells also prevented CD8 cells from acquiring anticyptococcal activity. When C. neoformans was cultured with CD8 T cells isolated from CD4 T cell-undepleted PBMC, the growth of C. neoformans was significantly lower than that in wells containing C. neoformans alone (Fig. 6B). By contrast, when C. neoformans was cultured with CD8 T cells isolated from CD4 T cell-depleted PBMC, the CD8 T cells had not acquired antifungal activity (Fig. 6B), indicating that CD4 T cells were necessary.

**IL-15 was required for granulysin-mediated anticyptococcal activity**

Having observed that IL-15 was sufficient and CD4 T cells were required to induce granulysin expression and antifungal activity (Figs. 1 and 6), it was necessary to determine whether IL-15 was required for the CD4-dependent expression of granulysin and anticyptococcal activity. CD8 T cells isolated from PBMC stimulated with CnM in the presence of a neutralizing Ab to IL-15 were compared with CD8 T cells isolated from CnM-stimulated PBMC in the presence of an isotype-matched Ab. The results of the immunoblot showed that CD8 T cells isolated from CnM-stimulated PBMC expressed both forms of granulysin while anti-IL-15 Ab treatment abrogated the expression of granulysin (Fig. 6B). The anticyptococcal activity of CD8 T cells acquired after CnM stimulation was also abrogated when an anti-IL-15 Ab was added to the culture (Fig. 7B). Thus, IL-15 was necessary for the expression of granulysin and antifungal effect of CD8 T cells.

Because IL-15 is produced by accessory cells and not by T cells, the requirement for accessory cells was examined. When purified CD8 and CD4 T cells that had been depleted of accessory cells were stimulated with CnM, neither form of granulysin was detected (Fig. 7A). A similar effect was observed when CD40-CD40 ligand interactions were blocked with an anti-CD40 Ab during stimulation (data not shown). Correspondingly, purified CD4 plus CD8 T cells that had been depleted of accessory cells failed to display anticyptococcal activity. These data suggest that accessory cells are required for the expression of granulysin by CD8 T cells and anticyptococcal activity.

**Discussion**

We have made four observations: 1) IL-15 was both necessary and sufficient to up-regulate granulysin expression in human primary CD8 T cells; 2) the up-regulation of granulysin correlated with the acquisition of anticyptococcal activity, and specific inhibition of granulysin blocked the effect; 3) granulysin-mediated anticyptococcal activity was perforin independent; and 4) the expression of granulysin required CD4 T cells, accessory cells, and IL-15.

Previous studies have attempted to define the mechanism of the lymphocyte-mediated anticyptococcal activity without success. It had been observed that CD8 T cells and NK cells form conjugates with C. neoformans, and predicted that the organisms were killed by granule exocytosis (23). Hydroxyl radical scavengers (diethyl thiourea, propyl gallate), cyclooxygenase inhibitors (piroxicam, indomethacin), PGE2, and mAbs or ligands reactive with receptors on
human lymphocytes failed to demonstrate a correlation with the anticytotoxic activity of CD8 T cells (24). Although catechin (a hydroxyl radical scavenger) and salicylic acid (a cyclooxygenase inhibitor) inhibited the antifungal activity, the authors (Levitz et al. (24)) did not make a general conclusion because other related agents did not have similar effects.

It has been shown that recombinant granulysin showed a dose-dependent anticytotoxicity against a variety of organisms including Escherichia coli, Staphylococcus aureus, Mycobacterium tuberculosis, C. neoformans, and Leishmania major in vitro (6, 7). Increased expression of granulysin by cytotoxic lymphocytes correlated with cytotoxicity to M. tuberculosis and Mycobacterium leprae (8, 59, 60), and strontium ions abrogated the activity (59, 61). To confirm this correlation using C. neoformans, the expression of granulysin was established and SrCl2 was used in the current studies. SrCl2 depletes all of the granule components including granulysin without causing cellular toxicity (48, 49). However, because of the inability to specifically inhibit granulysin, definitive evidence of granulysin-mediated cytotoxicity has heretofore been lacking. When granulysin expression was blocked by granulysin-specific siRNA at the level of mRNA and protein expression, the anticytotoxic activities of CD8 T cells were abrogated. The current study shows that granulysin is necessary for the anticytotoxic activity of CD8 T cells. Further studies will be required to establish whether granulysin is required for antimycobacterial and other antimicrobial activities.

The current studies provide strong support for the utility of siRNA. The discovery that dsRNA could induce gene silencing in organisms as diverse as plants and parasitic protozoans raises the possibility that RNAi might be a nearly universal mechanism of gene silencing. This notion has been supported by the identification of homologs of proteins that participate in the silencing process in virtually all genomes examined to date, with the exception of Saccharomyces cerevisiae (62). The first indications that this response might also extend to mammals came from the observation that injection of dsRNA into early mouse embryos induced sequence-specific silencing (63, 64). Recently, RNAi mediated by 21- and 22-nt RNA has been observed in numerous mammalian cell lines, such as 293, NIH/3T3, Hela S3, Hela SS6, COS-7 cells (19), and rat fibroblast F5 and FR (wt648) (65). As an extension of previous studies, we have demonstrated that siRNA can induce potent and specific gene silencing in human primary cells. In the present study, 21-nt siRNA duplexes were used to block granulysin expression in human primary CD8 T cells. Granulysin mRNA expression was suppressed in the presence of siRNA. Granulysin protein expression was also dramatically reduced by siRNA. By contrast, granulysin mRNA and protein expression were not affected by two control siRNA, CD20 siRNA, and nonsilencing siRNA, suggesting that the effect was not due to a nonspecific effect of siRNA. Thus, siRNA may hold promise in human primary cells and provides optimism for studies of dsRNA-mediated silencing in mammalian systems.

Previous studies have shown that granulysin-mediated killing of intracellular M. tuberculosis was dependent on perforin (7). After establishing that granulysin was required for CD8 T cell-mediated anticytotoxic activity, experiments were performed to determine whether the granulysin-mediated antifungal effect is perforin dependent. CD8 T cells were treated with EGTA, a chelator of calcium, or concanamycin A, which has been shown to selectively block the perforin and granzyme cytotoxicity pathway (50, 66). The anticytotoxic activity of CD8 T cells was not affected. This observation suggests that the granulysin-mediated antifungal activity of CD8 T cells is perforin independent. A critical difference between the perforin-independent killing of Cryptococcus and the perforin-dependent killing of Mycobacteria (7) is that the cryptococi were extracellular while mycobacteria were intracellular. This is supported by the observation that perforin acts as a gateway for granzymes through the plasma membrane. Both of these events might require intimate contact of the T lymphocyte and the target, and previous studies have shown that intimate contact between the T lymphocytes and C. neoformans is required for the anticytotoxic activities (23, 46, 67). Thus, it may be that perforin is required for granulysin to access the compartment containing mycobacteria, but granulysin is directly active on extracellular C. neoformans at the conjugation region.

It has been established that CD4 T cells are required for CD8 T cell proliferation in response to C. neoformans (58). Likewise,
CD4 T cells are required for the CD8 T cells to produce granulysin and to become cytotoxic cells with anticytotoxic activity. CD4 cells could participate in a number of different ways. CD4 T cells secrete T cell growth factors such as IL-2, which activate CD8 T cells (28, 32, 68). Additionally, CD4 T cells signal accessory cells, such as dendritic cells and monocytes/macrophages, to express important cytokines such as IL-1, IL-6, IL-15, or TNF-α (69–71), or surface-expressed ligands, such as CD40, LFA-1, or ICAM-1 (72, 73), so that CD8 T cells can be activated (29). The studies of IL-15 shed some light on these two possibilities.

We considered two possibilities that might explain how both CD4 T cells and IL-15 might be required. IL-15 is required for lymphocyte-mediated anticytotoxic activity, and IL-15 is produced by cells of the monocyte and macrophage lineage (30). The first possibility was that IL-15 would be necessary for the response of CD4 T cells. This would be consistent with the requirement for IL-15 in the response to CnM. The CD4 cells would then provide IL-15 in the response to CnM. The CD4 cells would then provide T cell help for the CD8 cytotoxic T cells. In this circumstance, we would anticipate that IL-15 would be required to stimulate CD8 T cell activity in response to CnM. The CD4 cells would then provide IL-15 in the response to CnM. The CD4 cells would then provide T cell help for the CD8 cytotoxic T cells. In this circumstance, we would anticipate that IL-15 would be required to stimulate CD8 T cells, and CD4 T cells would then be required to stimulate CD8 T cells. However, we observed that CD4 T cells did not need to be present when IL-15 was used to stimulate CD8 cells. Indeed, IL-15 was sufficient to stimulate CD8 T cells. Thus, the data is most consistent with a second model in which CD4 T cells are activated, but because CD4 T cells do not release IL-15 (36), they stimulate accessory cells of the myeloid lineage to produce IL-15. The IL-15 in turn stimulates CD8 T cells to express granulysin and become cytotoxic.

In summary, we have examined granulysin function and regulation in primary CD8 T cells. Using siRNA-mediated RNAi, we found that granulysin was necessary for CD8 T cell-mediated antimicrobial activity. The granulysin-mediated antifungal effect was independent of perforin. In response to CnM, CD4 T cells were required to activate accessory cells that secrete IL-15, which up-regulates granulysin expression and enhanced the antifungal effect in CD8 T cells.

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References


