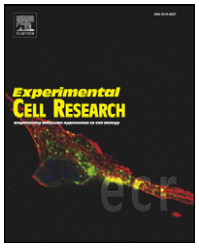
EXPERIMENTAL CELL RESEARCH 317 (2011) 531–538

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/yexcr



Research Article

The human cathelicidin, LL-37, induces granzyme-mediated apoptosis in cytotoxic T lymphocytes

Jamie S. Mader[a,*], *Marcelo Marcet-Palacios*[a], *Robert E.W. Hancock*[b], *R. Chris Bleackley*[a,*]

[a]*Department of Biochemistry, School of Molecular and Systems Medicine, Faculty of Medicine, University of Alberta, Edmonton, Alberta, Canada T6G 2H7*

[b]*Department of Microbiology & Immunology, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z4*

ARTICLE INFORMATION

Article Chronology:
Received 29 September 2010
Revised version received
23 November 2010
Accepted 29 November 2010
Available online 3 December 2010

Keywords:
Cytotoxic T lymphocytes
Apoptosis
Host defense peptide
Granzyme

ABSTRACT

LL-37 is a human cationic host defense peptide (antimicrobial peptide) belonging to the cathelicidin family of peptides. In this study, LL-37 was shown to kill stimulated $CD8^+$ T cells (Cytotoxic T lymphocytes; CTLs) via apoptosis, while having no cytotoxic effect on non-stimulated $CD8^+$ or $CD4^+$ T cells or stimulated $CD4^+$ T cells. Of interest, the $CD8^+$ cells were much more sensitive to LL-37 than many other cell types. LL-37 exposure resulted in DNA fragmentation, chromatin condensation, and the release of both granzyme A and granzyme B from intracellular granules. The importance of granzyme family members in the apoptosis of CTLs following LL-37 treatment was analyzed by using C57BL/6 lymphocytes obtained from mice that were homozygous for null mutations in the granzyme B gene, the granzyme A gene, or both granzymes A and B. Granzymes A and B were both shown to play an important role in LL-37-induced apoptosis of CTLs. Further analysis revealed that apoptosis occurred primarily through granzyme A-mediated caspase-independent apoptosis. However, caspase-dependent cell death was also observed. This suggests that LL-37 induces apoptosis in CTLs via multiple different mechanisms, initiated by the LL-37-induced leakage of granzymes from cytolytic granules. Our results imply the existence of a novel mechanism of crosstalk between the inflammatory and adaptive immune systems. Cells such as neutrophils, at the site of a tumor for example, could influence the effector, activity of CTL through the secretion of LL-37.

Introduction

Cationic host defense peptides are evolutionarily conserved throughout the animal and plant kingdoms where they play an important role in innate immunity [1]. Many of these peptides display immunomodulatory functions, influencing processes such as cytokine and chemokine release, and cell proliferation [2]. LL-37 is a cationic, amphipathic host defense peptide released by proteinase 3 proteolytic processing of the C-terminal domain of the cathelicidin, human cationic antimicrobial protein (hCAP)-18 [3]. It is endogenously expressed by a variety of cells, including keratinocytes, epithelial cells, and neutrophils [4], being released in response to multiple inflammatory stimuli [5,6]. Upon release, LL-37 acts as an effector molecule of innate immunity, displaying anti-infective and immunomodulatory activities, including the enhancement of neutrophil survival [7,8] and the induction of neutrophil, $CD4^+$ T cell, and monocyte chemotaxis [9]. However, a concentration of 100 μg/mL of LL-37 resulted in the death of 60% untransformed human airway epithelial cells [10], while other studies have shown that LL-37 can induce secondary necrosis in neutrophils [11,12]. These variations on the cytotoxic nature of LL-37 may potentially be related to peptide-mediated augmentation of innate immunity [8].

* *Corresponding authors.* Fax: +1 902 492 0886.
E-mail addresses: jmader@ualberta.ca (J.S. Mader), chris.bleackley@ualberta.ca (R.C. Bleackley).

0014-4827/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexcr.2010.11.015

The significance of lymphocyte-mediated cytotoxicity in immune regulation, defense against foreign pathogens, and the elimination of transformed cells are well-known [13,14]. CTLs utilize two distinct methods of target cell elimination, engagement of cell surface death receptors and the granule exocytosis pathway [15–17 for review]. Granule-mediated cell death involves a family of granule-associated serine proteases, known as granzymes. Granzymes A (grA) and B (grB) are stored within cytotoxic granules as active enzymes. When a CTL binds to a target cell, the granzymes are released and taken up by the target where they activate two distinct death pathways. GrA is a tryptase that triggers death in a caspase-independent manner by acting on a number of substrates, including poly ADP ribose polymerase-1 (PARP-1) [16], Ku70 [18], and NDUFS3 [19]. Following substrate cleavage, grA exposure leads to the induction of reactive oxygen species (ROS) and nuclear translocation of the SET complex [20], resulting in DNA single-strand breaks, p53 activation, and loss of cell membrane integrity [20,21]. In contrast, grB is a serine protease that cleaves pro-apoptotic substrates, including caspase-3 and Bid, after aspartate residues [22–25], resulting in caspase-mediated apoptosis.

Caspases are a family of intracellular cysteine proteases that are normally found in an inactive, zymogen form in healthy cells. During apoptosis, the proteolytic activation of initiator and subsequent effector caspase family members often results in the degradation of molecules that leads to the characteristic morphology associated with apoptosis, including inhibitor of caspase activated DNase (ICAD), PARP, and proteins involved in membrane stability (i.e. gelsolin), cellular shape (i.e. fodrin), and nuclear structures [26–28].

Our previous work on LL-37 demonstrated peptide-induced cell death in Jurkat cells. The mechanism involved the activation of a pro-apoptotic member of the Bcl-2 family, Bax, and its translocation to the mitochondria. This step appeared to be dependent on calpain and resulted in the release of AIF from mitochondria. Knockdown of AIF significantly reduced death after LL-37 treatment [29].

In a survey of different cell types, we were surprised to discover that $CD8^+$ T lymphocytes were appreciably more sensitive to LL-37 than Jurkat cells. Using lymphocytes obtained from granzyme knockout mice, we have determined that LL-37 induces apoptosis in CTLs through the release and consequent actions of grA and grB, resulting in death through caspase-independent and -dependent mechanisms. LL-37 does not kill non-stimulated T cells or stimulated $CD4^+$ T cells, which infers a selective cytotoxic effect. This points to the potential use of LL-37 as a treatment option in conditions where CTL involvement becomes detrimental, such as that found in various autoimmune diseases.

Materials and methods

Cell culture and reagents

The mouse lymphocytes were generated from control mice (strain ID: C57BL/6 12953/SV1mJ; background: C57BL/6 and 129; obtained from Dr T. J. Ley, Washington University, St Louis, MO) and mice homozygous for null mutations in the grB gene (strain ID: B6.129S *Gzmb* [Tm1 Ley]; background: B6/129; obtained from Dr T. J. Ley), the grA gene (strain ID: C57.129S*GzmA*; background: C57/129; obtained from Dr T. J. Ley), or both granzyme A and B genes (129/SUJ-GzmABKO line 64/18 CRE). Cells were maintained at 37 °C in a 5% CO_2 humidified atmosphere in RPMI 1640 medium (Sigma-Aldrich Canada, Oakville, ON, Canada) supplemented with 10% (vol./vol.) heat-inactivated fetal calf serum (FCS) (Hyclone, Logan, UT), 100 μg/mL streptomycin, 100 U/mL penicillin, 2 mmol/L L-glutamine, and 5 mmol/L HEPES buffer (pH 7.4) (Invitrogen, Burlington, ON, Canada), from this point on referred to as RHF.

Synthetic LL-37 (amino acid sequence: NH_2-LLGDFFRKSKEKIG-KEFKRIVQ-RIKDFLRNLVPRTES-COOH) was synthesized by F-moc (*N*-(9-fluorenyl)-methoxycarbonyl) chemistry at the Nucleic Acid/Protein Synthesis Unit at the University of British Columbia (Vancouver, Canada). Lyophilized peptide was resuspended in endotoxin-free water (Sigma-Aldrich) and aliquots were stored at −20 °C. Bovine serum albumin (BSA), paraformaldehyde (PFA), saponin, Ac-LLnL-CHO (calpain inhibitor 1), staurosporine, and Hoechst 33342 trihydrochloride dye were purchased from Sigma-Aldrich. Caspase inhibitors (Q-VD-OPh and Z-VAD-fmk) were purchased from Kamiya Biomedical Company (Seattle, WA). TUNEL in situ cell detection kits (fluorescein) were from Roche Diagnostics (Laval, PQ). PE-Cy7-conjugated rat anti-mouse CD4 (L3T4), FITC-conjugated rat anti-mouse CD8 (Ly-2), and PE-conjugated rat anti-mouse CD25 (BD Bioscience) were from BD Biosciences (Mississauga, ON). Purified anti-mouse CD28 and purified anti-mouse CD3e were from Cedarlane Laboratories Ltd. (Burlington, Ontario,). Goat anti-mouse granzyme A (D-15) and goat anti-mouse granzyme B (C-19) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor® 488 donkey anti-goat IgG (H + L) secondary antibody was from Invitrogen.

T cell isolation

$CD8^+$ and $CD4^+$ T cells were isolated from mouse spleen cell suspension using the MACS™ $CD8^+$ or $CD4^+$ T cell isolation kits (Miltenyi Biotec, Auburn, CA) and a MidiMACS™ separator. Briefly, spleens were passed through 30 μM nylon mesh, followed by RBC depletion using RBC lysis buffer (10 mM $KHCO_3$, 150 mM NH_4Cl, and 0.1 mM EDTA (pH 8.0) in distilled H_2O) (5 min, 4 °C). Cells were washed 2 times with ice-cold isolation buffer (0.5% BSA and 2 mM EDTA in PBS, pH 7.2), counted, and resuspended in ice-cold isolation buffer (40 μL/10^7 total cells) containing biotin-antibody cocktail (10 μL/10^7 total cells, 10 min, 4 °C), followed by the addition of isolation buffer (30 μL/10^7 total cells) and anti-biotin microbeads (20 μL/10^7 total cell, 15 min, 4 °C). Cells were then washed with isolation buffer and centrifuged at 300*g* (10 min, 4 °C). Supernatant was removed; cells were resuspended in isolation buffer (500 μL/10^8 cells) and loaded onto a pre-rinsed LS MACS separator column. The enriched $CD8^+$ or $CD4^+$ T cell fraction was collected following column passage and washes. $CD4^+$ 25^+ T cells were then removed by further enrichment. The purity of the enriched T cell fractions was analyzed by flow cytometry using anti-CD8–FITC or anti-CD4–PE and was determined to be greater than 90% and 95 %, respectively (data not shown). Cell debris, dead cells, and non-$CD8^+$ cells were excluded from analysis based on scatter signals and PI fluorescence.

T cell activation

Six-well plates (Becton Dickinson Labware, Franklin Lakes, NJ) were coated with 5 μg/mL hamster anti-mouse α-CD3e/αCD28 in 1 mL PBS (overnight, 4 °C). Cells were stimulated (1×10^6 cells/well) in a 5% CO_2 humidified atmosphere with plate-bound hamster anti-mouse α-CD3e/anti-CD28 monoclonal antibodies in RHF supplemented with 60 U/mL IL-2 for 48 h (37 °C).

DNA fragmentation assay

TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay was used to measure DNA fragmentation in cells undergoing cell death [30]. Briefly, cells (2×10^5 cells/well) were exposed to media or LL-37 (10, 20, and 40 μg/mL) in triplicate wells of a 96-well round-bottom tissue culture plate (Becton Dickinson Labware, Franklin Lakes, NJ) for 4 h at 37 °C. At the end of the experimental time point, cells were resuspended in 2% PFA in phosphate-buffered NaCl solution (NaCl, KCl, $Na_2HPO_4\times2H_2O$ and KH_2PO_4) (PBS) (o/n, 4 °C). Following fixation, cells were permeabilized by exposure to 0.1% saponin for 15 min at room temperature. Cells were then resuspended in 20 μL of TUNEL–FITC pre-mix and incubated for 1 h at 37 °C. Cells were then washed (2 times), resuspended in IF buffer (1% BSA in PBS), and analyzed by flow cytometry.

Variations of this experiment were performed. Initial analysis of apoptosis in $CD4^+$ and $CD8^+$ T cells was performed using TUNEL–FITC and antibodies against CD4, CD8, and CD25. The addition of broad-spectrum caspase inhibitors (Q-VD-OPh, 20 μM or Z-VAD-fmk, 50 μM) 1 h prior to LL-37 addition was used to determine the requirement of caspase involvement in LL-37-mediated cell death. The addition of a specific calpain inhibitor (1 μM) was performed 2 h prior to LL-37 exposure to determine the requirement for Ca^{2+}-dependent calpain in LL-37-mediated apoptosis. Analysis of the effect of high serum concentration on LL-37-induced apoptosis was performed using FCS at "high" (10%) and "low" (0.5%) serum concentrations. Total cell counts were performed prior to and following LL-37 treatment; cells were not lost due to washes or lysis in control or LL-37 treated samples (data not shown). Media control values were negligible and were subtracted from treatment values prior to graphing.

Hoechst staining

Murine $CD4^+$ and $CD8^+$ T cells (5×10^4 cells/treatment) were treated with media or LL-37 (20 and 40 μg/mL, 37 °C, 5% CO_2) for 2 h. Cells were washed with PBS following LL-37 treatment, resuspended in 50 μL of 4% PFA in PBS, and placed on microscope slides to air dry overnight (Corning, Corning, NY). Staining was then performed for 10 min at room temperature with Hoechst 33342 trihydrochloride dye (10 μg/mL in PBS). Slides were washed 4 times with distilled water and allowed to air dry in the dark. Confocal microscopy was used to assess chromatin condensation and nuclear fragmentation by counting 500 cells from non-overlapping fields. Total cell counts were performed prior to and following LL-37 treatment; cells were not lost due to washes or lysis in control or LL-37 treated samples (data not shown).

Confocal microscopy

Coverslips were treated with poly-L-lysine (1:5 in sterile distilled water, 30 min, room temperature). CTLs (5×10^4 cells/coverslip) were pipetted onto coverslips in combination with media or LL-37 (20 μg/mL, 30 min, 37 °C). Following incubation, cells were washed 3 times with PBS and fixed overnight (2% PFA, 4 °C). Cells were then permeabilized by treatment with 0.1% saponin (15 min, room temperature), then exposed to media alone, or stained with goat anti-mouse granzyme A (D-15, 1:50) or goat anti-mouse granzyme B (1:500) for 1 h at 37 °C. Cells were washed 3 times with PBS and exposed to Alexa Fluor 488 donkey anti-goat secondary antibody (1:300, 1 h, 37 °C) in the dark. Analysis of granzyme A and B release was performed at room temperature on a LSM510 laser scanning confocal microscope mounted on a Zeiss Axiovert 100M microscope fitted with a Plan Neofluar objective 60/1.4 and analyzed with LSM5 software (Zeiss).

Statistical analysis

One-way analysis of variance (ANOVA) and the Tukey–Kramer multiple comparisons test were performed for dose response analysis using the Instat statistics program (Graphpad Software, CA). The Student's *t*-test was used to compare differences between test and control groups. Results are considered statistically significant if the *p*-value is less than 0.05.

Results

LL-37 induces apoptosis in stimulated $CD8^+$ T cells

Apoptosis is a form of cellular death that is characterized by specific morphological features, including DNA fragmentation and chromatin condensation. Fig. 1A shows that stimulated murine $CD8^+$ T cells (CTLs) exposed to increasing concentrations of LL-37 (10, 20, and 40 μg/mL, equivalent to 2.2, 4.4, and 8.8 μM, respectively, 4 h) displayed significant DNA fragmentation as assessed by TUNEL–FITC DNA staining. The CTLs are activated as they kill target cells in both chromium release and H^3Tdr assays (data not shown). Since DNA fragmentation is an indicator of apoptotic cell death, this data supported the premise that the cells were undergoing apoptosis following LL-37 exposure. Interestingly, we saw significant fragmentation at 10 μg/mL with activated $CD8^+$ cells. In other cell types, this level of fragmentation required 100–200 μg/mL [29]. Both stimulated and non-stimulated $CD4^+$ T cells were not affected by LL-37, which suggests that the apoptosis-inducing activity of LL-37 is specific to $CD8^+$ T cells. LL-37 treatment also led to apoptosis in non-stimulated $CD8^+$ T cells, but only at the higher LL-37 treatment concentrations, and not to the same extent as in activated CTLs. This points to the importance of the stimulated state in LL-37-induced apoptosis of $CD8^+$ T cells, which will be discussed later in the text. Hoechst 33342 staining (Fig. 1B and C) revealed chromatin condensation and apoptotic body formation in CTLs treated with LL-37 ($53\%\pm3$ and $73\%\pm1$, respectively, 20 and 40 μg/mL, 2 h) but not in stimulated $CD4^+$ T cells (0 ± 0.6, 40 μg/mL, 2 h), further confirming that LL-37 is specifically targeting CTLs versus $CD4^+$ T cells and inducing an apoptotic form of cell death in CTLs, indicated by chromatin condensation and apoptotic body formation.

CTLs from granzyme A and AB knockout mice are resistant to LL-37-induced apoptosis

Apoptosis in wild type (wt), $grA^{-/-}$, $grB^{-/-}$, and $grAB^{-/-}$ CTLs was analyzed using TUNEL–FITC staining in the absence or presence of LL-37 (10, 20, and 40 μg/mL, 4 h) (Fig. 2). CTLs that were knockout in the grA and grAB genes were significantly less sensitive to apoptosis induced by LL-37, while $grB^{-/-}$ CTLs displayed modest resistance to LL-37-induced cell death. These data suggest that grA, and thus caspase-independent apoptosis, which is activated by grA [31,32], is primarily involved in LL-37-induced killing of CTLs. At the same time,

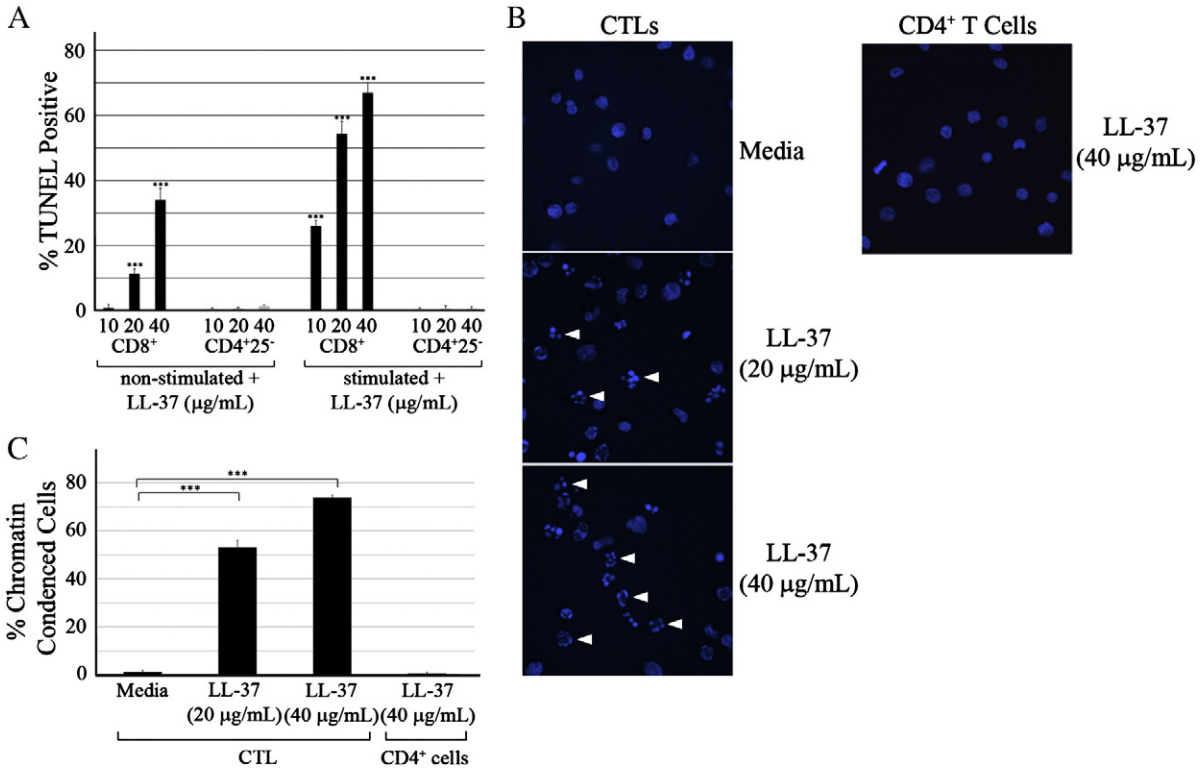


Fig. 1 – LL-37 induces apoptosis in $CD8^+$ CTLs (A) Stimulated and non-stimulated $CD8^+$ and $CD4^+CD25^-$ T cells (2×10^5/treatment) were treated with increasing concentrations of LL-37 (10, 20, and 40 μg/mL) for 4 h. Cells were then stained with TUNEL–FITC (1 h, 37 °C) and analyzed by flow cytometry. Data are shown as mean % cell death ± SD, $n=3$ (* denotes $p<0.001$ when compared to media control). (B) Stimulated $CD8^+$ (CTLs) and $CD4^+CD25^-$ T cells (5×10^4 cells/treatment) were exposed to LL-37 (20 and 40 μg/mL) for 2 h. Cells were then fixed with 2% PFA, mounted on silinated microscope slides and air dried o/n. Staining with Hoechst 33342 trihydrochloride dye (10 μg/mL) was performed (10 min, room temperature) and slides were analyzed by confocal microscopy, 400× magnification. (C) The percentage of cells showing chromatin condensation was determined by Hoechst staining and counting (500 cells/slide). Data are shown as mean % chromatin condensed cells ± SD, $n=3$ (*** denotes $p<0.001$ when compared to media control).**

grB knockout CTLs showed moderate resistance to LL-37 (Figs. 2 and 3), and $grA^{-/-}$ cells were not completely resistant to LL-37-induced apoptosis; therefore, LL-37 may elicit death in CTLs through more than one apoptotic pathway.

Caspase family members are not required for, but do contribute to, LL-37-induced CTL apoptosis

Apoptosis was analyzed using TUNEL–FITC staining in the absence or presence of the pan-caspase inhibitors, Q-VD-OPh (20 μM, 1 h pre-treatment) or Z-VAD-fmk (50 μM, 1 h pre-treatment). Both Q-VD-OPh (Fig. 3) and Z-VAD-fmk (data not shown) had an inhibitory effect on LL-37-induced apoptosis in wt, as well as $gr^{-/-}$ $CD8^+$ CTLs (LL-37 20 μg/mL, 4 h). However, caspase-independent apoptosis is decidedly predominant, as confirmed by both the apoptosis occurring in wt cells in the presence of Q-VD-OPh, as well as the inhibitory effect of $grA^{-/-}$ on LL-37-induced apoptosis. Staurosporine (STS, 2.5 μM, 4 h), a known inducer of caspase-dependent apoptosis, was used as a positive control for caspase inhibitor function [33]. Caspase inhibitors were dissolved in DMSO. Vehicle control analysis showed no cytotoxic effect on T cells (vehicle control values were deducted from treatment values prior to graphing).

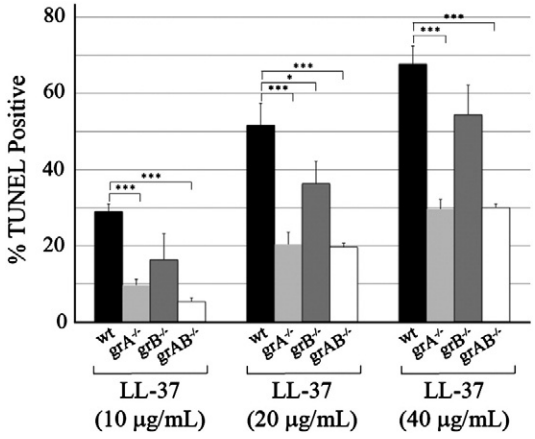


Fig. 2 – Granzyme $A^{-/-}$ and granzyme $AB^{-/-}$ CTLs are resistant to LL-37-induced apoptosis. Wild type (wt), $grA^{-/-}$, $grB^{-/-}$, or $grAB^{-/-}$ $CD8^+$ CTLs (2×10^5/treatment) were exposed to LL-37 (10, 20, and 40 μg/mL) for 4 h at 37 °C. At this time, cells were stained with TUNEL–FITC (1 h, 37 °C) and flow cytometric analysis was performed. Data are shown as mean % cell death ± SD, $n=3$ (* and * denote $p<0.01$ and $p<0.001$, respectively, when compared to media control).**

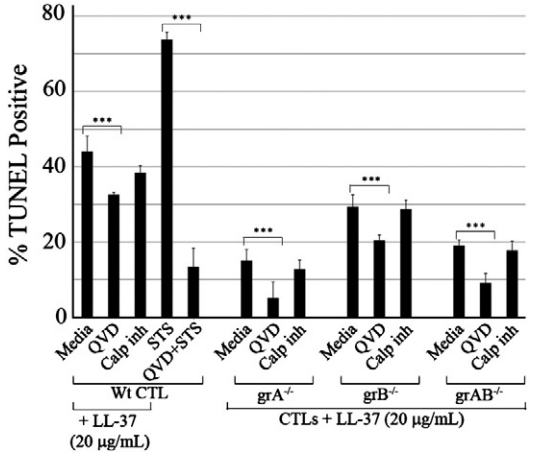


Fig. 3 – Caspase-independent and caspase-dependent pathways are involved in LL-37-mediated apoptosis of CTLs. Wt, grA$^{-/-}$, grB$^{-/-}$, or grAB$^{-/-}$ CD8$^+$ CTLs (2×10^5/treatment) were set up in the absence or presence of broad-spectrum caspase inhibitor, Q-VD-OPh (20 μM, 1 h pre-treatment) or calpain inhibitor 1 (1 μM, 2 h pre-treatment). At this time, cells were exposed to LL-37 (20 μg/mL, 4 h) or STS (2.5 μM, 4 h), stained with TUNEL–FITC (1 h, 37 °C), and flow cytometric analysis was performed. Data are shown as mean % cell death ± SD, $n = 3$ (* denotes $p < 0.001$ when compared to DMSO vehicle control).**

Calpains are not required for LL-37-mediated apoptosis in CTLs

The Ca^{2+}-dependent calpains are a family of cysteine proteases that can orchestrate apoptosis by cleaving various cellular substrates, including Bax, which results in $\Delta\Psi_m$ and AIF release from the mitochondria [34,35]. LL-37 requires calpain activation in order to kill Jurkat T leukemia cells *in vitro* [29]. Therefore, the involvement of calpains in LL-37-induced CTL apoptosis was analyzed. Pre-treatment with the calpain inhibitor, Ac-LLnL-CHO (1 μM), had no effect on apoptosis induced by LL-37 (20 μg/mL) in CTLs from wt, or gr$^{-/-}$ mice (Fig. 3). These data suggest that calpain is not required for LL-37-mediated CTL apoptosis. Calpain inhibitor was dissolved in DMSO and diluted to a concentration that had no cytotoxic effect on T cells (vehicle control values were deducted from treatment values prior to graphing).

Granzymes A and B are released from intracellular cytotoxic granules in CTLs exposed to LL-37

The previous results clearly pointed to a key role for granzymes in the demise of the CD8$^+$ cells. Normally, these enzymes are sequestered in cytosolic granules and thus do not initiate apoptosis in the cells that produce them. However, it was possible that the action of LL-37 caused the release of the granzymes into the cytosol of the producing cell. The release of grA and grB from cytotoxic granules of CTLs exposed to LL-37 (20 μg/mL, 1 h) was assessed using anti-grA or -grB antibodies and confocal microscopy (Fig. 4). When untreated CTL cells were stained with antibodies against either grA or grB, granules were observed in a predicted punctuated pattern (Fig. 4A *i*). This phenotype changed to a diffused signal phenotype when cells were treated with LL-37 (Fig. 4A *ii*). LL-37 treatment resulted in the release of both grA and grB (82% ± 3, 72% ± 6, respectively) from cytotoxic granules into the cytosol, as observed by diffuse granzyme positive staining within the cell, supporting the role of grA and grB in LL-37-induced apoptosis of CTLs (Figs. 4B and C).

In vitro serum concentration has no inhibitory effect on LL-37-induced apoptosis of CTLs

Stimulated and non-stimulated CD8$^+$ T cells were exposed to LL-37 under high (10%) and low (0.5%) serum concentrations (Fig. 5). T cells cultured in high and low serum conditions were similarly sensitive to LL-37-induced apoptosis. These data suggest that LL-37 would be effective at higher serum levels, such as those found *in vivo*. Future investigations will analyze the *in vivo* effects of LL-37 in various disease models involving CTLs.

Discussion

In this study, the mechanism of cell death induced by LL-37 in CD8$^+$ CTLs was characterized. It was determined that CTLs exposed to LL-37 died through the induction of apoptosis, characterized by DNA fragmentation and chromatin condensation (Fig. 1A, B, and C). Apoptosis was observed as early as 4 h post-LL-37 treatment. This time point was used for subsequent experiments as apoptosis was not significantly greater at a later time point. Of interest, LL-37 specifically killed CD8$^+$ CTLs, while having no cytotoxic effect on stimulated CD4$^+$ cells (Fig. 1A). In addition, LL-37 had no effect on non-stimulated CD4$^+$ and minimal effect on non-stimulated CD8$^+$ T cells (Fig. 1A). We believed that LL-37 was killing CTLs through granzyme activation and that the selective cytotoxicity displayed by LL-37 was due to a variance in granzyme expression by the CTLs. Previous studies have shown that naïve T cell populations display minimal to no granzyme expression [36]. Therefore, the involvement of granzymes in LL-37-induced CTL apoptosis was investigated.

GrA and grB are the two best characterized pro-apoptotic granzymes, leading to caspase-independent and caspase-dependent apoptosis, respectively. The requirement for grA and/or grB in LL-37-induced CTL apoptosis was analyzed using lymphocytes obtained from grA$^{-/-}$, grB$^{-/-}$, and grAB$^{-/-}$ mice. GrA$^{-/-}$ and grAB$^{-/-}$ CTLs were significantly less sensitive to LL-37-induced apoptosis, while grB$^{-/-}$ had a lesser effect on cell death following LL-37 exposure (Fig. 2). These results suggest that grA and downstream grA-mediated caspase-independent apoptosis is activated following LL-37 exposure. Recent reports have indicated that in many cases purified grA is not cytotoxic *in vitro* [37,38]. If this is accurate, then grA-mediated CTL-induced killing may depend on additional molecules present in intracellular granules of the cytotoxic cells (such as other granzymes and/or cathepsins) or a distinctive quality of CTL-delivered grA [38]. The lack of grA expression in stimulated CD4$^+$ T cells may be why these cells were not susceptible to LL-37 cytotoxicity. While significantly more resistant to death, grA$^{-/-}$ and grAB$^{-/-}$ cells are killed at higher LL-37 peptide concentrations (Fig. 2). This may be due to a calpain-dependent mechanism previously described [29]. The broad-spectrum caspase inhibitor, Q-VD-OPh, minimally but significantly inhibited apoptosis in wt CD8$^+$ CTLs treated with LL-37 (Fig. 3). These experiments were conducted at 20 ug/mL LL-37. At higher doses [above 40 ug/mL], the calpain-induced mechanism may become relevant. The caspase-dependent apoptosis observed may be due to the activity of caspase-activating granzymes other than grB. Similarly, the caspase-dependent cell death observed in grB$^{-/-}$ and

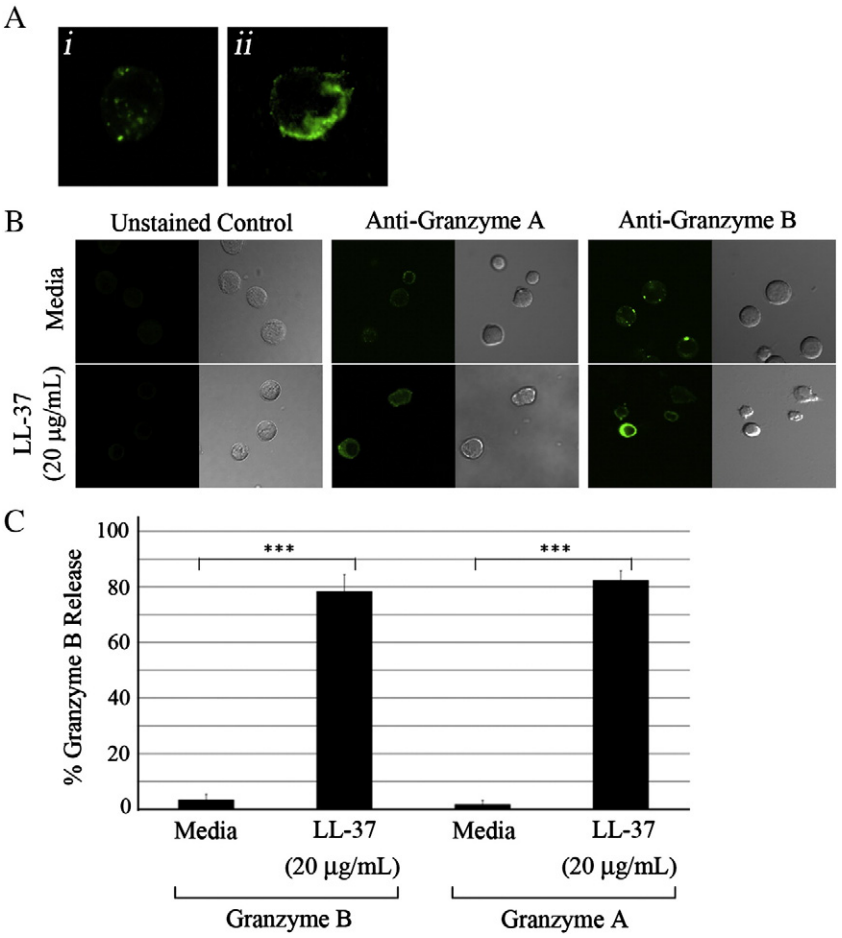


Fig. 4 – LL-37 treatment resulted in the release of grA and grB from intracellular cytotoxic granules. (A) $CD8^+$ CTLs were stained against grA and grB to monitor subcellular localization of these proteins. A punctuated granular pattern (A *i*) was interpreted as signal within the CTL granules, whereas diffused signals obtained as a result of LL-37 treatment (A *ii*) were interpreted as cytosolic localization of grA or grB. (B) Wt $CD8^+$ CTLs (5×10^4 cells/treatment) were set up with media or LL-37 (20 μg/mL, 30 min, 37 °C) on coverslips pre-treated with poly-L-lysine. Cells were then exposed to media alone or stained with goat anti-mouse grA (D-15, 1:50) or goat anti-mouse grB (1:500, 1 h, 37 °C), followed by Alexa Fluor 488 donkey anti-goat secondary antibody (1:300, 1 h, 37 °C). Confocal microscopy was performed at 600× magnification. Data are representative of 3 independent experiments. (C) The percentage of cells showing grA or grB release was determined by counting (500 cells/slide). Data are shown as mean % granzyme release ± SD, $n=3$ (* denotes $p<0.001$ when compared to media control).**

$grAB^{-/-}$ cells may be due to other caspase-activating granzymes, such as grK, which is expressed in both *in vivo*-derived and *in vitro*-propagated CTLs [39]. LL-37 has similar cytotoxic effects on un-stimulated wild type and mutant cells. Thus, the peptide can kill these cells in a grA/B independent manner also. However, the specific mode of cell death in the un-stimulated cells was not assessed.

Apoptosis was observed to be independent of calpains as a specific calpain inhibitor had no effect on LL-37-induced death (Fig. 3). This result was in contrast to our previous analysis of LL-37 cytotoxicity, which showed that LL-37 kills Jurkat T leukemia cells through a calpain-dependent pathway [29]. These data suggest that LL-37 activates diverse apoptotic pathways depending on the target cell involved. Thus, in Jurkat cells, the major mechanism involves calpain activation, although a minor contribution due to cathepsin release from lysosomes was observed [29]. In contrast, the leakage of potently cytotoxic enzymes, such as granzymes, from intracellular granules becomes the predominant mode of cell death in cells that contain these enzymes, including CTL.

Exposure to LL-37 resulted in the significant release of grA and grB from intracellular cytotoxic granules (Fig. 4). During target cell attack by CTLs, the trafficking of granzymes to the target is designed to minimize cytosolic granzyme leakage. In contrast, LL-37 treatment leads to the release of granzymes directly into the CTL cytosol, where they can then initiate apoptosis. The release of the powerful apoptotic enzyme grB would be expected to have a major death-inducing effect, yet it appears to be less important than grA. However, PI-9, a potent grB inhibitor belonging to the serpin family, has been found in the cytosol of CTL [40]. Thus, it is likely that this protein may significantly dampen the cytolytic activities of grB. In contrast, no intracellular inhibitors are known to inactivate grA. Therefore, the release of grA into the cytosol following LL-37 exposure and the subsequent activation of caspase-independent apoptosis is most likely unconstrained by intracellular inhibitors.

LL-37 is a cationic host defense peptide that has both anti-infective and immunomodulatory activity [9,41]. It is expressed at ~5 μg/mL in bronchoalveolar lavage (BAL) of healthy infants and is

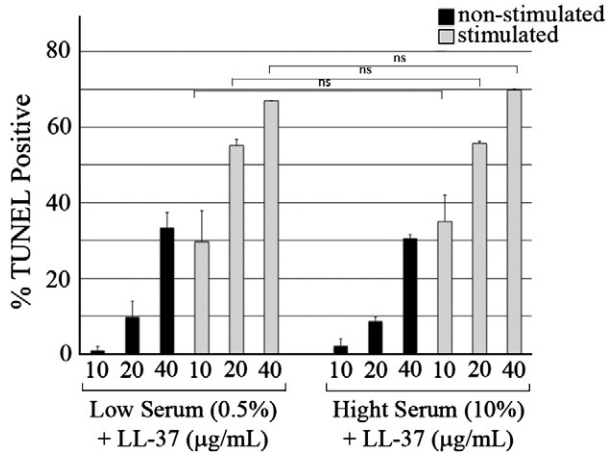


Fig. 5 – High serum concentration has no effect on LL-37-induced apoptosis in CD8$^+$ CTLs. Stimulated and non-stimulated CD8$^+$ T cells (2×10^5/treatment) were treated with increasing concentrations of LL-37 (10, 20, and 40 μg/mL) for 4 h in the presence of low (0.5%) and high (10%) serum concentrations. Cells were then stained with TUNEL–FITC (1 h, 37 °C) and analyzed by flow cytometry. Data are shown as mean % cell death ± SD, $n=3$ (ns denotes not significant when compared to media control).

upregulated by inflammation to ~30 μg/mL in BAL from infants with pulmonary infections [10]. Peptide expression is also upregulated in other inflammatory conditions, such as psoriatic skin lesions where levels are ~1.5 mg/mL due to *in vivo* processing [42] or increased PMN influx and degranulation. Therefore, the concentrations of LL-37 used in this study are believed to be physiologically relevant.

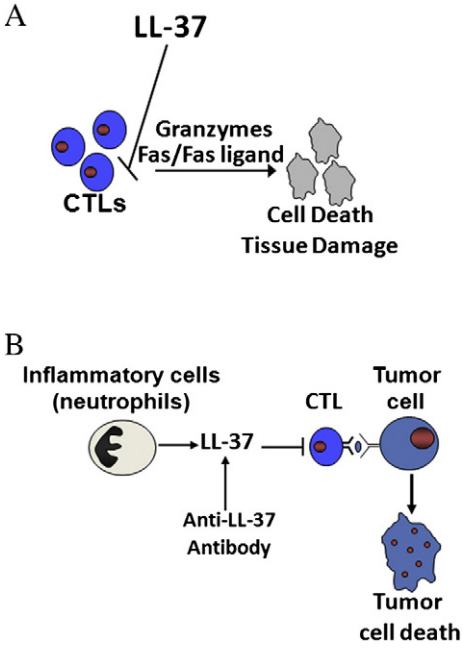


Fig. 6 – LL-37-induced CTL apoptosis; potential applications: (A) LL-37 may be used to treat autoimmune diseases that have prevalent CTL involvement. (B) Anti-LL-37 antibody treatment at the tumor site may block LL-37-induced CTL death.

LL-37 induces apoptosis in CTLs in the presence of high (10%) serum concentrations (Fig. 5). This result is important for prospective *in vivo* analysis of the anti-CTL activity of LL-37. Future studies will look to determine whether LL-37 could be used to specifically kill CTLs to treat conditions where CTLs are detrimental to the host, such as that observed in many autoimmune diseases [43–45] (Fig. 6). The specific anti-CTL activity of LL-37 is advantageous in that the majority of therapies used to treat chronic autoimmune diseases have employed nonspecific immunosuppression, which results in a large number of adverse side effects. A most interesting ramification of our results is that they suggest a novel way in which inflammatory cells, such as neutrophils, could attenuate the killing activity of CTL. This would represent a novel level of crosstalk between inflammatory and adaptive immune responses. Thus, another potential application for our results could be to construct and administer an anti-LL-37 antibody at the tumor site. Both CTLs and LL-37-producing neutrophils are often abundant at tumor sites. Therefore, blocking the cytotoxic activity of LL-37 may be a means of increasing anti-tumor CTL effects (Fig. 6).

Acknowledgments

We acknowledge the support of Genome Canada, Genome BC and Genome Prairie for the 'Pathogenomics of Innate Immunity' research program, the Canadian Institute of Health Research (CIHR), the Canadian Cancer Society, and the Foundation for National Institutes of Health through the Grand Challenges in Global Health initiative. R. C.B. is a Medical Scientist of the AHFMR, J.M. was supported by a postdoctoral award from AHFMR. The authors have no conflicting financial interests.

REFERENCES

[1] M. Zasloff, Antimicrobial peptides of multicellular organisms, Nature 415 (2002) 389–395.
[2] K.L. Brown, R.E. Hancock, Cationic host defense (antimicrobial) peptides, Curr. Opin. Immunol. 18 (2006) 24–30.
[3] O.E. Søresen, P. Follin, A.H. Johnsen, J. Calafat, G.S. Tjabringa, P.S. Hiemstra, N. Borregaard, Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3, Blood 97 (2001) 3951–3959.
[4] G.H. Gudmundsson, B. Agerberth, J. Odeberg, T. Bergman, B. Olsson, R. Salcedo, The human gene FALL39 and processing of the cathelin precursor to the antibacterial peptide LL-37 in granulocytes, Eur. J. Biochem. 238 (1996) 325–332.
[5] B. Agerberth, J. Charo, J. Werr, B. Olsson, F. Idali, L. Lindbom, R. Kiessling, H. Jörnvall, H. Wigzell, G.H. Gudmundsson, The human antimicrobial and chemotactic peptides LL-37 and alpha-defensins are expressed by specific lymphocyte and monocyte populations, Blood 96 (2000) 3086–3093.
[6] M. Murakami, R.A. Dorschner, L.J. Stern, K.H. Lin, R.L. Gallo, Expression and secretion of cathelicidin antimicrobial peptides in murine mammary glands and human milk, Pediatr. Res. 57 (2005) 10–15.
[7] I. Nagaoka, H. Tamura, M. Hirata, An antimicrobial cathelicidin peptide, human CAP18/LL-37, suppresses neutrophil apoptosis via the activation of formyl-peptide receptor-like 1 and P2X, J. Immunol. 176 (2006) 3044–3052.
[8] P.G. Barlow, X. Li, T.S. Wilkinson, D.M. Bowdish, Y.E. Lau, C. Cosseau, C. Haslett, A.J. Simpson, R.E.W. Hancock, D.J. Davidson, The human cationic host defense peptide LL-37 mediates contrasting effects on apoptotic pathways in different primary cells of the innate immune system, J. Leukoc. Biol. 80 (2006) 1–12.

[9] D. Yang, Q. Chen, A.P. Schmidt, G.M. Anderson, J.M. Wang, J.J. Wooters, LL-37, the neutrophil granule-and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells, J. Exp. Med. 192 (2000) 1069–1074.
[10] Y.E. Lau, D.M.E. Bowdish, C. Cosseau, R.E.W. Hancock, D.J. Davidson, Apoptosis of airway epithelial cells: human serum sensitive induction by the cathelicidin LL-37, Am. J. Respir. Cell Mol. Biol. 34 (2006) 399–409.
[11] Å. Bjorstad, G. Askarieh, K.L. Brown, K. Christenson, H. Forsman, K. Önnheim, H.-N. Li, S. Teneberg, O. Maier, D. Hoekstra, C. Dahlgren, D. J. Davidson, J. Bylund, The host defense peptide LL-37 selectively permeabilizes apoptotic leukocytes, Antimicrob. Agents Chemother. 53 (2009) 1027–1038.
[12] H.-N. Li, P.G. Barlow, J. Bylund, A. Mackellar, Å. Bjorstad, J. Conlon, P.S. Hiemstra, C. Haslett, M. Gray, A.J. Simpson, A.G. Rossi, D.J. Davidson, Secondary necrosis of apoptotic neutrophils induced by the human cathelicidin LL-38 is not proinflammatory to phagocytosing macrophages, J. Leukoc. Biol. 86 (2009) 891–902.
[13] J.A. Trapani, M.J. Smyth, Functional significance of the perforin/granzyme cell death pathway, Nat. Rev. Immunol. 2 (2002) 735–747.
[14] G. Menasche, J. Feldmann, A. Fischer, G. de Saint Basile, Primary hemophagocytic syndromes point to a direct link between lymphocyte cytotoxicity and homeostasis, Immunol. Rev. 203 (2005) 165–179.
[15] D.T. Shen, J.S. Ma, J. Mather, S. Vukmanovic, S. Radoja, Activation of primary T lymphocytes results in lysosome development and polarized granule exocytosis in CD4+ and CD8+ subsets, whereas expression of lytic molecules confers cytotoxicity to CD8+ T cells, J. Leukoc. Biol. 80 (2006) 827–837.
[16] R. Bleackley, A molecular view of cytotoxic T lymphocyte induced killing, Biochem. Cell Biol. 83 (2005) 747–751.
[17] L. Chávez-Galán, M.C. Arenas-Del Angel, E. Zenteno, R. Chávez, R. Lascurain, Cell death mechanisms induced by cytotoxic lymphocytes, Cell. Mol. Immunol. 6 (2009) 15–25.
[18] P. Zhu, D. Zhang, D. Chowdhury, D. Martinvalet, D. Keefe, L. Shi, J. Lieberman, Granzyme A, which causes single-stranded DNA damage, targets the double-strand break repair protein Ku70, EMBO Rep. 7 (2006) 431–437.
[19] P. Zhu, D. Martinvalet, D. Chowdhury, D. Zhang, A. Schlesinger, J. Lieberman, The cytotoxic T lymphocyte protease granzyme A cleaves and inactivates poly(adenosine 5′-diphosphate-ribose) polymerase-1, Blood 114 (2009) 1205–1216.
[20] D. Martinvalet, D.M. Dykxhoorn, R. Ferrini, J. Lieberman, Granzyme A cleaves a mitochondrial complex I protein to initiate caspase-independent cell death, Cell 133 (2008) 681–692.
[21] Z. Fan, P.J. Beresford, D.Y. Oh, D. Zhang, J. Lieberman, Tumor suppressor NM23-H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor, Cell 112 (2003) 659–672.
[22] M.E. Murphy, J. Moult, R.C. Bleackley, H. Gershenfeld, I.L. Weissman, M.N. James, Comparative molecular model building of two serine proteinases from cytotoxic T lymphocytes, Proteins 4 (1988) 190–204.
[23] S. Odake, C.M. Kam, L. Narasimhan, M. Poe, J.T. Blake, O. Krahenbuhi, J. Tschoop, J.C. Powers, Human and murine cytotoxic T lymphocyte serine proteases: subsite mapping with peptide thioester substrates and inhibition of enzyme activity and cytolysis by isocoumarins, Biochemistry 30 (1991) 2217–2227.
[24] A.J. Darmon, D.W. Nicholson, R.C. Bleackley, Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B, Nature 377 (1995) 446–448.
[25] Y. Gu, C. Sarnecki, M.A. Fleming, J.A. Lippke, R.C. Bleackley, M.S.S. Su, Processing and activation of CMH-1 by granzyme B, J. Biol. Chem. 271 (1996) 10816–10820.
[26] D. Brenner, T.W. Mak, Mitochondrial cell death effectors, Curr. Opin. Cell Biol. 21 (2009) 871–877.
[27] N.N. Danial, S.J. Korsmeyer, Cell death: critical control points, Cell 116 (2004) 205–219.
[28] D.F. Suen, K.L. Norris, R.J. Youle, Mitochondrial dynamics and apoptosis, Genes Dev. 22 (2008) 1577–1590.
[29] J.S. Mader, N. Mokherjee, R. Hancock, R. Bleackley, The human host defense peptide LL-37 induces apoptosis in a calpain- and apoptosis-inducing factor-dependent manner involving Bax activity, Mol. Cancer Res. 7 (2009) 689–702.
[30] Y. Gavrieli, Y. Sherman, S.A. Ben-Sasson, Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation, J. Cell Biol. 119 (1992) 493–501.
[31] P.J. Beresford, Z. Xia, A.H. Greenberg, J. Lieberman, Granzyme A loading induces rapid cytolysis and a novel form of DNA damage independently of caspase activation, Immunity 10 (1999) 585–594.
[32] S. Shresta, T.A. Graubert, D.A. Thomas, S.Z. Raptis, T.J. Ley, Granzyme A initiates an alternative pathway for granule-mediated apoptosis, Immunity 10 (1999) 595–605.
[33] S. Hakimelahi, H.R. Parker, A.J. Gilchrist, M. Barry, Z. Li, R.C. Bleackley, M. Pasdar, Plakoglobin regulates the expression of the anti-apoptotic protein Bcl-2, J. Biol. Chem. 275 (2000) 10905–10911.
[34] M.K. Squier, A.C. Miller, A.M. Malkinson, J.J. Cohen, Calpain activation in apoptosis, J. Cell. Physiol. 159 (1994) 229–237.
[35] R.S. Moubarak, V.J. Yuste, C. Artus, A. Bouharrour, P.A. Greer, J. Menissier-de Murcia, S.A. Susin, Sequential activation of poly (ADP-ribose) polymerase 1, calpains, and Bax is essential in apoptosis-inducing factor-mediated programmed necrosis, Mol. Cell. Biol. 27 (2007) 4844–4862.
[36] P.K. Chattopadhyay, M.R. Betts, D.A. Price, E. Gostick, H. Horton, M. Roederer, S.C. De Rosa, The cytolytic enzymes granyzme A, granzyme B, and perforin: expression patterns, cell distribution, and their relationship to cell maturity and bright CD57 expression, J. Leukoc. Biol. 85 (2009) 88–97.
[37] S.S. Metkar, C. Menaa, J. Pardo, B. Wang, R. Wallich, M. Freudenberg, S. Kim, S.M. Raja, L. Shi, M.M. Simon, C.J. Froelich, Human and mouse granzyme A induce a proinflammatory cytokine response, Immunology 29 (2008) 720–733.
[38] J. Pardo, J.I. Aguilo, A. Anel, P. Martin, L. Joeckel, C. Borner, R. Wallich, A. Müllbacher, C.J. Froelich, M.M. Simon, The biology of cytotoxic granule exocytosis pathway: granzymes have evolved to induce cell death and inflammation, Microbes Infect. 11 (2009) 452–459.
[39] T. Zhao, H. Zhang, Y. Guo, Z. Fan, Granzyme K directly processes Bid to release cytochrome C and endonulease G leading to mitochondria-dependent cell death, J. Biol. Chem. 282 (2007) 12104–12111.
[40] D. Kaiserman, P.I. Bird, Control of granzymes by serpins, Cell Death Differ. 17 (4) (2009) 586–595, doi:10.1038/cdd.2009.169.
[41] F. Niyonsaba, A. Someya, M. Hirata, H. Ogawa, I. Nagaoka, Evaluation of the effects of peptide antibiotics human beta-defensins-1/-2 and LL-37 on histamine release and prostaglandin D(2) production from mast cells, Eur. J. Immunol. 31 (2001) 1066–1075.
[42] P.Y. Ong, T. Ohtake, C. Brandt, I. Strickland, M. Boguniewicz, T. Ganz, R. Gallo, D. Leung, Endogenous antimicrobial peptides and skin infections in atopic dermatitis, N. Engl. J. Med. 347 (2002) 1151–1160.
[43] P. Blanco, V. Pitard, J.F. Viallard, J.L. Taupin, J.L. Pellegrin, J.F. Moreau, Increase in activated CD8+ T lymphocytes expressing perforin and granzyme B correlates with disease activity in patients with systemic lupus erythematosus, Arthritis Rheum. 52 (2005) 201–211.
[44] P. Blanco, J.F. Viallard, J.L. Pellegrin, J.F. Moreau, Cytotoxic T lymphocytes and autoimmunity, Curr. Opin. Rheumatol. 17 (2005) 731–734.
[45] L. Couzi, P. Merville, C. Deminiere, J.F. Moreau, C. Combe, J.L. Pellegrin, J.F. Viallard, P. Blanco, Predominance of CD8+ T lymphocytes among periglomerular infiltrating cells and link to the prognosis of class III and class IV lupus nephritis, Arthritis Rheum. 56 (2007) 2362–2370.