The Human Cathelicidin, LL-37, Induces Granzyme-mediated Apoptosis in Regulatory T Cells

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Summary: 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 and nonstimulated $CD4^+CD25^+FoxP3^+$ T cells (regulatory T cells; Tregs) through apoptosis, while having no cytotoxic effect on $CD4^+CD25^-$ T cells at the same LL-37 concentrations. Of interest, Tregs were much more sensitive to LL-37 than many other cells, dying at 10-fold lower concentrations than other cell types tested. LL-37 exposure resulted in DNA fragmentation, chromatin condensation, and apoptotic body formation, all indicative of an apoptotic form of cell death. The importance of granzyme family members in the apoptosis of Tregs after LL-37 treatment was analyzed by using C57Bl/6 lymphocytes obtained from mice that were homozygous for null mutations in the granzyme B gene, and both the granzyme A and B genes. Granzyme A and granzyme B were both shown to play a role in LL-37-induced apoptosis of Tregs. Further analysis showed that apoptosis occurred primarily through caspase-dependent apoptosis at high LL-37 concentrations. However, grA-dependent/caspase-independent cell death was also observed. This suggests that LL-37 induces apoptosis in Tregs through multiple different mechanisms, initiated by the LL-37-induced leakage of granzymes from cytolytic granules. Our results imply that LL-37 administered at the site of a tumor could influence the adaptive antitumor immune response by killing Tregs and thus inhibiting their suppressor activity.

Key Words: regulatory T cells, granzyme, host defense peptide, apoptosis

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Cationic host defense peptides play an important role in innate immunity,[1] 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-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 results in the death of 60% untransformed human airway epithelial cells.[10] These variations on the cytotoxic nature of LL-37 may potentially be related to peptide-mediated augmentation of innate immunity.[8]

$CD4^+CD25^+$ (Forkhead box P3) $FoxP3^+$ regulatory T cells (Tregs) are often associated with peripheral immune tolerance, which is involved in controlling detrimental immune responses to self-antigens, and preventing excessive inflammatory responses to foreign antigens. They also play a significant role in suppressing immunity to tumor-associated antigens.[11] Adoptively transferred Tregs have been shown to block tumor-reactive effector T cells in various murine model studies, whereas the in vivo removal of Tregs is known to enhance antitumor immunity.[12,13] Tregs can suppress the proliferation of numerous cell types, including bystander T cells, through both soluble and cell-associated molecules, such as interleukin-10, anticytotoxic T-lymphocyte antigen-4, and transforming growth factor-β.[14] They also exhibit granzyme B (grB)-mediated suppressive mechanisms, which result in the selective killing of bystander effector T cells[15] and antigen presenting B cells.[16] In addition, grB has been shown to be important within the tumor microenvironment for Treg-mediated suppression of tumor clearance.[17] Activated Tregs also express high levels of granzyme A and can display perforin-dependent cytotoxicity.[18]

Granule-mediated cell death involves a family of granule-associated serine proteases, known as granzymes. Granzyme A (grA) and grB are stored within cytotoxic granules as active enzymes, being released by effector cells such as Tregs, and taken up by target cells 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,[19] Ku70,[20] and NADH dehydrogenase (ubiquinone) iron-sulfur protein 3.[21] In contrast, grB is a serine protease that cleaves proapoptotic 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

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subsequent effecter caspase family members often results in the degradation of molecules that lead to the characteristic morphology associated with apoptosis, including inhibitor of caspase-activated DNase, poly ADP ribose polymerase, and proteins involved in membrane stability (ie, gelsolin), cellular shape (ie, fodrin), and nuclear structures.[26–28]

In this study, we have determined that low concentrations of the host-defense peptide, LL-37, can kill Tregs through a granzyme-mediated mechanism, involving the activation of caspase family members. At similar peptide concentrations, LL-37 has no cytotoxic effect on $CD4^+CD25^-$ or $CD4^+CD25^+FoxP3^-$ T cells. The potential for enhancing the antitumor T-cell response, through an LL-37-mediated reduction in suppressive Tregs, may be a promising treatment option for cancer patients.

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], or both grA and grB 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% (v/v) heat-inactivated fetal calf serum (Hyclone, Logan, UT), 100 μg/mL streptomycin, 100 units/mL penicillin, 2 mmol/L L-glutamine, and 5 mmol/L *N*-2-hydroxyl piperazine-*N*′-2-ehane sulfonic acid buffer (pH 7.4) (Invitrogen, Burlington, ON, Canada), from this point on referred to as RHF.

Synthetic LL-37 (amino acid sequence: NH_2-LL GDFFRKSKEKIGKEFKRIVQ-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, 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). Terminal transferase dUTP nick end labeling (TUNEL) in situ cell detection kits (fluorescein) were from Roche Diagnostics (Laval, PQ). PE-Cy7-conjugated rat anti-mouse CD4 (L3T4), and PE-conjugated rat anti-mouse CD25 were from BD Biosciences (Mississauga, ON). APC anti-mouse Foxp3 (clone FJK-16s) and the APC anti-mouse Foxp3 staining set were from eBioscience (San Diego, CA). Purified anti-mouse CD28, and purified anti-mouse CD3e were from Cedarlane Laboratories Ltd (Burlington, ON).

Regulatory T-cell Isolation

The isolation of naturally occurring regulatory T cells ($CD4^+CD25^+$) and $CD4^+CD25^-$ T cells from mouse spleen cell suspension was performed using the MACS $CD4^+CD25^+$ regulatory T-cell isolation kit (Miltenyi Biotec, Auburn, CA), a MidiMACS separator and a MiniMACS separator. In brief, spleens were passed through 30 μM nylon mesh, followed by red blood cell depletion using red blood cell lysis buffer [10 mM $KHCO_3$, 150 mM NH_4Cl, and 0.1 mM ethylene diaminetetra-acetic acid (pH 8.0) in distilled H_2O] (5 min, 4°C). Cells were washed 2× with ice-cold isolation buffer (0.5% BSA, and 2 mM ethylene diaminetetra-acetic acid in phosphate-buffered saline (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), anti-biotin microbeads (20 μL/10^7 total cell), and CD25-PE antibody (10 μL/10^7 total cells, 15 min, 4°C, 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 LD MACS separator column. The unlabelled, enriched $CD4^+$ T-cell fraction was collected after column passage and washes, centrifuged at 300*g* (10 min, 4°C), and resuspended in isolation buffer (90 μL/10^7 total cells) containing anti-PE microbeads (10 μL/10^7 total cells). Cells were incubated in the dark (15 min, 4°C), 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 MS MACS separator column. The enriched $CD4^+CD25^-$ T-cell fraction was collected after column passage and washes. The enriched $CD4^+CD25^+$ T-cell fraction was collected by removing the column from the separator, and quickly flushing out the fraction containing magnetically labeled $CD4^+CD25^+$ cells by firmly applying a plunger. The magnetic separation procedure was repeated 1× using a new MS column. The purity of the enriched $CD4a^+$ T-cell fraction was analyzed using anti-CD4-PECy7 and flow cytometry, and was determined to be greater than 90% (data not shown). Cell debris, dead cells, and non-$CD4^+$ cells were excluded from the analysis based on scatter signals and propidium iodide fluorescence (data not shown).

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 interleukin-2 for 48 hours (37°C).

DNA Fragmentation Assay

TUNEL assay was used to measure DNA fragmentation in cells undergoing cell death.[29] In brief, cells (2×10^5 cells/well) were exposed to media or LL-37 (5, 10, 20, 40, and 100 μg/mL) in triplicate wells of a 96-well round-bottom tissue culture plate (Becton Dickinson Labware, Franklin Lakes, NJ) for 4 hours 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_2H-PO_4 $2H_2O$, and KH_2PO_4) (PBS) (o/n, 4°C). After fixation, cells were permeabilized by exposure to 0.1% saponin for 15 minutes at room temperature. Cells were then resuspended in 20 μL of TUNEL-FITC pre-mix and incubated for 1 hours at 37°C. Cells were then washed (2×), and resuspended in IF buffer (1% BSA in PBS), and analyzed by flow cytometry.

Variations of this experiment were performed. Initial analysis of apoptosis in $CD4^+CD25^+Foxp3^+$ Tregs, $CD4^+CD25^-$, and $CD4^+CD25^+Foxp3^-$ T cells was performed using TUNEL-FITC and antibodies against CD4, CD25, and Foxp3. The addition of broad-spectrum caspase inhibitors (Q-VD-OPh, 20 μM or Z-VAD-fmk, 50 μM) 1 hour before LL-37 addition was used to determine the requirement of caspase involvement in LL-37-mediated cell death. Total cell counts were performed before and after LL-37 treatment; cells were not lost due to washes or lysis in control or LL-37-treated samples (data not shown). Data are shown as mean percentage of cell death ± SD, n = 3 independent experiments, each performed in triplicate.

Hoechst Staining

Murine $CD4^+CD25^+Foxp3^+$ Tregs (5×10^4 cells/treatment) were treated with media, or LL-37 (20 and 40 μg/mL, 37°C, 5% CO_2) for 2 hours. Cells were washed with PBS after 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 minutes at room temperature with Hoechst 33342 trihydrochloride dye (10 μg/mL in PBS). Slides were washed 4× 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 nonoverlapping fields. Total cell counts were performed before and after LL-37 treatment; cells were not lost due to washes or lysis in control or LL-37-treated samples (data not shown).

Analysis of chromatin condensation, membrane changes, and apoptotic body formation using Hoechst 33342 trihydrochloride dye was performed at room temperature on an LSM510 laser scanning confocal microscope mounted on a Zeiss Axiovert 100 M microscope fitted with a Plan Neofluar objective 60/1.4 and analyzed with LSM5 software (Zeiss).

RESULTS

LL-37 Induces Apoptosis in Regulatory T Cells

Apoptosis is a form of cellular death that is characterized by specific morphological features, including DNA fragmentation, and chromatin condensation. Figure 1A shows that stimulated and nonstimulated murine $CD4^+25^+FoxP3^+$ Tregs exposed to increasing concentrations of LL-37 (5, 10, 20, 40, and 100 μg/mL, equivalent to 1, 2, 4.4, 8.8, and 20 μM, respectively, 4 h) displayed significant DNA fragmentation as assessed by TUNEL-FITC DNA staining. Media control values were negligible and were subtracted from the treatment values before graphing. As DNA fragmentation is an indicator of apoptotic cell death, these data suggest that the Tregs were undergoing apoptosis after LL-37 exposure. Both stimulated and nonstimulated $CD4^+25^-$, and $CD4^+25^{+low}FoxP3^-$ T cells were not affected by LL-37, which suggests that the apoptosis-inducing activity of LL-37 is specific to $FoxP3^+$ Tregs. LL-37 treatment led to apoptosis in nonstimulated Tregs, but not to the same extent as in activated cells. Hoechst 33342 staining (Figs. 1B, C) showed chromatin condensation and apoptotic body formation in Tregs treated with LL-37 (52% ± 5 and 74% ± 3, respectively, 20 and 40 μg/mL, 2 h), further confirming that LL-37 is inducing an apoptotic form of cell death in this cell type.

Tregs From grB and AB Knockout Mice Are Resistant to LL-37-induced Apoptosis

Tregs have been shown to express granzymes,[17,18] and these enzymes have been shown to induce apoptosis by caspase activation.[23,30] We decided to test whether granzymes might be involved in our system using $CD4^+CD25^+Foxp3^+$ Tregs isolated from granzyme knockout mice. Apoptosis in wild type (WT), $grB^{-/-}$, and $grAB^{-/-}$ Tregs was analyzed using TUNEL-FITC staining in the absence or presence of LL-37 (10, 20, and 40 μg/mL, 4 h) (Fig. 2). Tregs that were knockout in the grB and grAB genes were significantly less sensitive to apoptosis induced by LL-37. These data suggest that both grA and grB, which mediate apoptosis by caspase-independent[31,32] and caspase-dependent mechanisms, respectively, are both involved in LL-37-induced killing of Tregs. At the same time, granzyme ko Tregs were not completely resistant to LL-37-induced apoptosis at higher LL-37 concentrations.

Caspase Family Members Contribute to LL-37-induced Treg Apoptosis

Caspases are known to be critical as effectors of apoptosis.[26,28] We, therefore, tested if they were important in the death of $CD4^+CD25^+Foxp3^+$ Tregs. Apoptosis was analyzed using TUNEL-FITC staining in the absence or presence of the pan-caspase inhibitors, Q-VD-OPh (20 μM, 1 h pretreatment) or Z-VAD-fmk (50 μM, 1 h pretreatment). Both Q-VD-OPh and Z-VAD-fmk (Fig. 3) had an inhibitory effect on LL-37-induced apoptosis in WT, as well as $gr^{-/-}$ Tregs (LL-37 40 μg/mL, 4 h). Caspase inhibitors were dissolved in dimethylsulfoxide. Vehicle control analysis showed no cytotoxic effect on T cells.

DISCUSSION

Regulatory T cells are known to play a significant role in the induction of immune tolerance to self-antigens, and may be primarily responsible for inhibiting antitumor immune responses. In this study, we determined that LL-37 is cytotoxic to naturally occurring murine $CD4^+CD25^+FoxP3^+$ Tregs at concentrations ranging from 5 to 100 μg/mL, while having no cytotoxic effect on murine $CD4^+CD25^-$ T cells. It is interesting to note that, we saw significant fragmentation at 20 and 40 μg/mL with activated Tregs. In addition, preliminary experiments with human Tregs have determined that these cells act just like murine Tregs at 20 μg/mL LL-37, displaying 40% cell death, whereas $CD4^+CD25^-$ T cells showed 3% cell death (data not shown). In other cell types, this level of fragmentation required 100 to 200 μg/mL.[33] It was determined that Tregs exposed to LL-37 died through the induction of apoptosis, characterized by DNA fragmentation and chromatin condensation (Figs. 1A–C). Apoptosis was observed as early as 4 hours 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 $FoxP3^+$ Tregs, while having no cytotoxic effect on stimulated and nonstimulated $CD4^+CD25^-$ or $CD4^+CD25^{+low}FoxP3^-$ cells (Fig. 1A). In addition, LL-37 had less effect on nonstimulated Tregs versus stimulated cells (Fig. 1A). Earlier studies have shown that naive T-cell populations display less granzyme expression versus stimulated

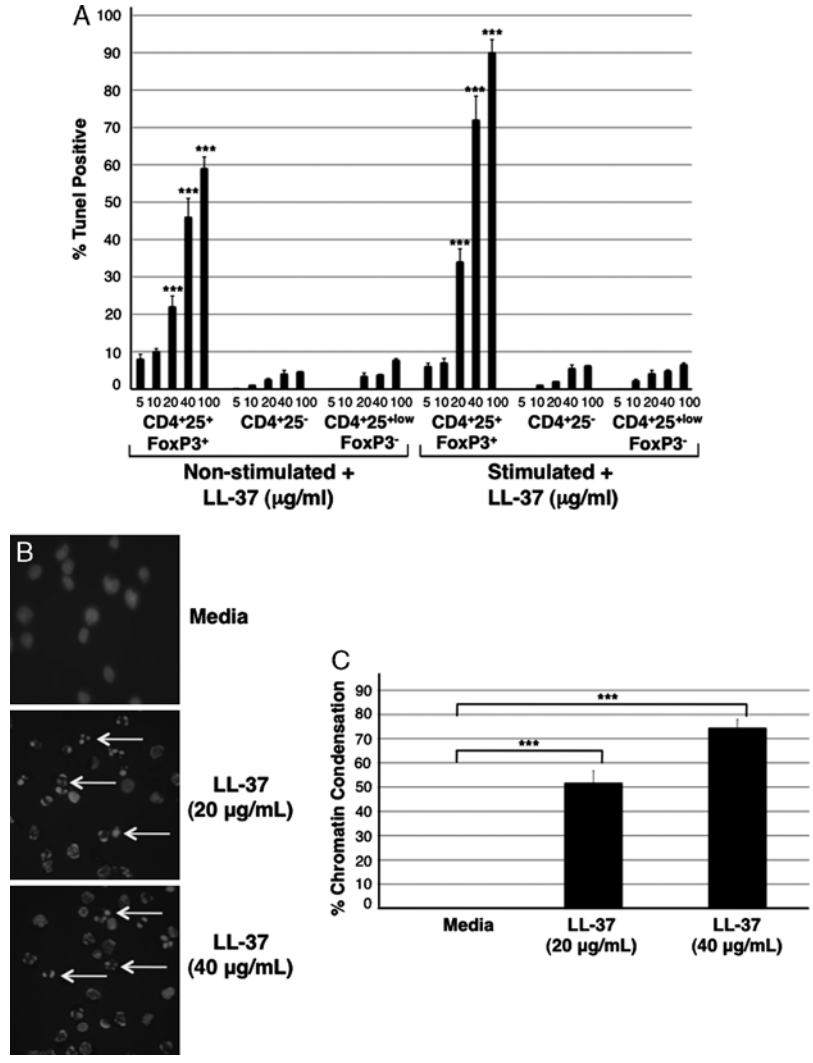

FIGURE 1. LL-37 induces apoptosis in $CD4^+CD25^+Foxp3^+$ T cells. A, Stimulated and nonstimulated $CD4^+CD25^+Foxp3^+$ and $CD4^+CD25^-$ T cells (2×10^5/treatment) were treated with increasing concentrations of LL-37 (5, 10, 20, 40, and 100 μg/mL) for 4 hours. Cells were then stained with terminal transferase dUTP nick end labeling (TUNEL)-FITC (1 h, 37°C) and analyzed by flow cytometry. Data are shown as mean percentage of cell death (± SD, n = 3) (***$P < 0.001$ when compared with media control). B, Stimulated $CD4^+CD25^+Foxp3^+$ regulatory T cells (5×10^4 cells/treatment) were exposed to LL-37 (20 and 40 μg/mL) for 2 hours. Cells were then fixed with 2% paraformaldehyde, mounted on silinated microscope slides and air dried overnight. Staining with Hoechst 33342 trihydrochloride dye (10 μg/mL) was performed (10 min, RT) and slides were analyzed by confocal microscopy (magnification: ×400). Arrows indicate apoptotic cells C, The percentage of cells showing chromatin condensation was determined by Hoechst staining and counting (500 cells/slide). Data are shown as mean percentage of chromatin condensed cells (± SD, n = 3) (***$P < 0.001$ when compared with media control).

cells.[34] Therefore, the involvement of granzymes in LL-37-induced Treg apoptosis was investigated.

GrB and grA are the 2 best characterized proapoptotic granzymes, leading to caspase-dependent and caspase-independent apoptosis, respectively. The requirement for grB and/or grA in LL-37-induced Treg apoptosis was analyzed using lymphocytes obtained from $grB^{-/-}$ and $grAB^{-/-}$ mice. $GrB^{-/-}$ and $grAB^{-/-}$ Tregs were significantly less sensitive to LL-37-induced apoptosis (Fig. 2). $GrAB^{-/-}$ Tregs were more resistant to cell death at lower LL-37 concentrations than Tregs that were ko for only $grB^{-/-}$, which suggests that, whereas grB is important in LL-37-mediated apoptosis of Tregs, grA or a combination of grA and grB is necessary for death at lower peptide concentrations. However, when exposed to 40 μg/mL LL-37, $grB^{-/-}$ and $grAB^{-/-}$ Tregs displayed similar resistance to LL-37, and whereas $grB^{-/-}$ and $grAB^{-/-}$ cells are significantly more resistant to death, these cells are killed at higher LL-37 peptide concentrations (Fig. 2). This may be due to the activation of cell death pathways that are not reliant on grA or B, such as the calpain-dependent mechanism previously described.[33] In this earlier study, higher concentrations of LL-37 (100 μg/mL) were required to induce apoptosis in cell lines such as Jurkat. Activation of calpain and the release of apoptosis inducing factor from mitochondria were found to be critical in the demise of the cell. However, some inhibition of death was observed in the presence of a lysosomal cathepsin inhibitor, indicating

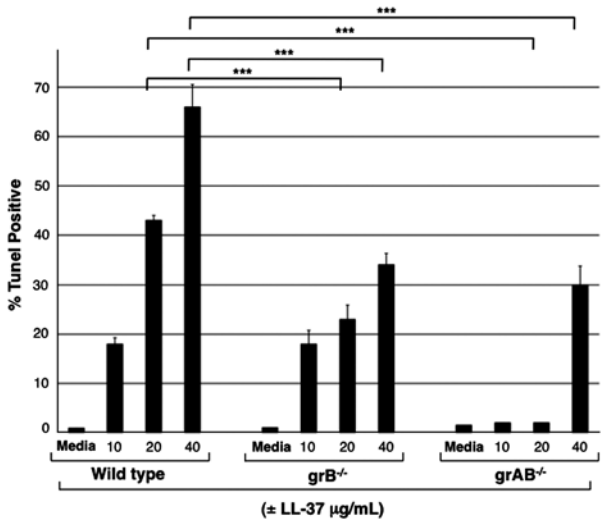

FIGURE 2. Granzyme $B^{-/-}$ and granzyme $AB^{-/-}$ regulatory T cells are resistant to LL-37-induced apoptosis. Wild type, $grB^{-/-}$, or $grAB^{-/-}$ stimulated $CD4^+CD25^+Foxp3^+$ regulatory T cells (2×10^5/treatment) were exposed to LL-37 (10, 20, and 40 μg/mL) for 4 hours at 37°C. At this time, cells were stained with terminal transferase dUTP nick end labeling (TUNEL)-FITC (1 × h, 37°C) and flow cytometric analysis was performed. Data are shown as mean percentage of cell death (± SD, n = 3) (***$P < 0.001$, when compared with media control).

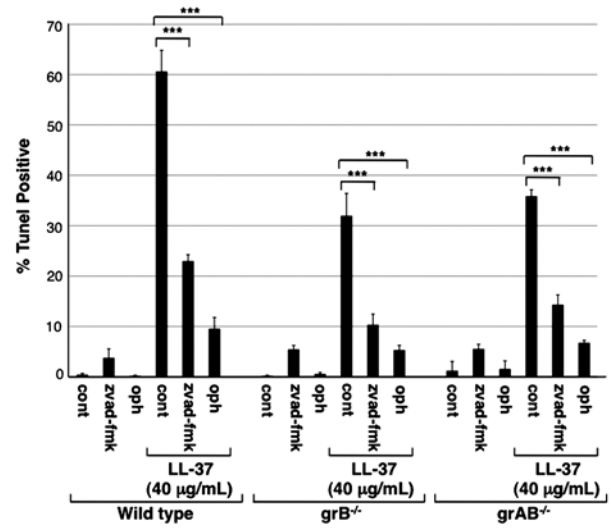

FIGURE 3. Caspase-dependent pathways are involved in LL-37-mediated apoptosis of regulatory T cells. Wild type, granzyme $B^{-/-}$ and granzyme $AB^{-/-}$ $CD4^+CD25^+Foxp3^+$ regulatory T cells (2×10^5/treatment) were set up in the absence or presence of the broad-spectrum caspase inhibitors, ZVAD-fmk (50 μM, 1 h pretreatment), or Q-VD-OPh (20 μM, 1 h pretreatment). At this time, cells were exposed to LL-37 (40 μg/mL, 4 h), stained with terminal transferase dUTP nick end labeling (TUNEL)-FITC (1 h, 37°C), and flow cytometric analysis was performed. Data are shown as mean percentage of cell death (± SD, n = 3) (***$P < 0.001$ when compared with dimethylsulfoxide vehicle control).

some leakage of the enzymes from this organelle. We believe that when the potent apoptosis-inducing granzymes are present in the secretory lysosomes of Tregs, then this becomes the major pathway to death. The lack of granzyme expression in most $CD4^+CD25^-$ T cells may be why these cells were not susceptible to LL-37 cytotoxicity.[34] The broad-spectrum caspase inhibitors, Z-VAD-fmk and Q-VD-OPh, significantly inhibited apoptosis in Tregs treated with LL-37 (Fig. 3). The caspase-dependent apoptosis observed is most likely due to the activity of grB (in W T cells) or other caspase-activating granzymes (in $grB^{-/-}$ and $grAB^{-/-}$ cells), such as grK. Caspase-dependent apoptosis is decidedly predominant, as confirmed by both the inhibition of apoptosis observed in Treg cells (both WT and granzyme ko) in the presence of broad-spectrum caspase inhibitors, and the inhibitory effect of $grB^{-/-}$ on LL-37-induced apoptosis. However, it is also noted that grA, and therefore caspase-independent apoptosis, plays a role in Treg death after LL-37 treatment, in that caspase inhibitors do not completely block LL-37-mediated death, but ko of both grA and grB completely blocked Treg death at low LL-37 concentrations (Fig. 2).

LL-37 is a cationic host defense peptide that has both anti-infective and immunomodulatory activity.[9,35] It is expressed at approximately 5 μg/mL in bronchoalveolar lavage of healthy infants, and is upregulated by inflammation to approximately 30 μg/mL in bronchoalveolar lavage from infants with pulmonary infections.[10] Peptide expression is also upregulated in other inflammatory conditions, such as psoriatic skin lesions where levels are approximately 1.5 mg/mL due to in vivo processing.[36] Therefore, the concentrations of LL-37 used in this study are believed to be physiologically relevant. In addition, many studies have been performed to analyze the effects of LL-37 on untransformed cells, thus aiding in the characterization of an acceptable efficacy/safety ratio for LL-37.[7–9] LL-37 is not cytotoxic to a large variety of untransformed cell types at the concentrations used in our study. High concentrations of LL-37 (100 μg/mL and higher) are cytotoxic to airway epithelial cells.[10] However, much lower concentrations of LL-37 are effective at killing Tregs, therefore it is not believed that epithelial cells would be affected during Treg exposure. An earlier paper published by our laboratory analyzed the mechanism of apoptosis induced in Jurkat T leukemia cells exposed to LL-37.[33] We found that Jurkat cells were killed by higher concentrations of LL-37 (50 to 200 μg/mL), through a calpain-dependent and apoptosis inducing factor-dependent pathway requiring the Bcl-2 family member, BAX.

Use of LL-37 in the treatment of cancer patients is beyond the scope of this study. However, it is believed that there may be ways in which the anti-Treg activity of this peptide may be exploited. The possibilities include, but are not limited to, the use of liposomal-delivery systems,[37] earlier shown to significantly increase selectivity while reducing bystander cytotoxicity when the peptide is delivered at high concentrations to specific cell types. We examined the effect of LL-37 on $CD8^+$ T cells, a major subset of T cells that have an antitumor effector role. Activated $CD8^+$ T cells were sensitive to LL-37 exposure (data not shown). However, we do not believe the death of a few activated $CD8^+$ T-cell will have too much effect. Rather, the eradication of the Tregs may lead to the activation of multiple arms of the antitumor response, including the generation of new $CD8^+$ T-cell.

There is emerging evidence that shows a potential role for inflammatory molecules, including LL-37, in tumor

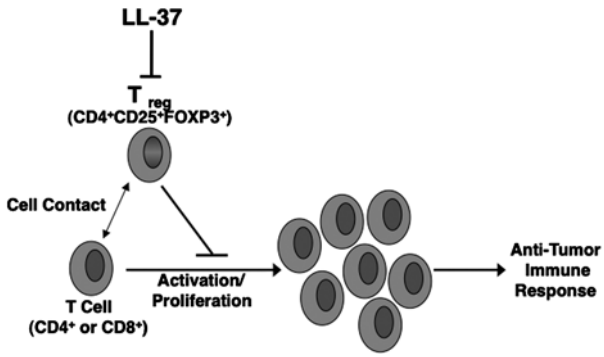

FIGURE 4. LL-37-induced regulatory T-cell apoptosis; potential application: LL-37 has the potential to be used to prevent Treg-mediated inhibition of $CD4^+/CD8^+$ T-cell activation/proliferation in cancer treatment.

progression. LL-37 has been shown to promote ovarian cancer progression through the recruitment of proangiogenic cells and immunomodulatory factors,[38] while potentially acting as a growth factor for epithelial cells in breast cancer.[39] However, LL-37 can play a role in host defense against tumors,[40,41] and is known to suppress tumorigenesis in gastric cancer,[42] and epidermoid carcinoma.[43] Chuang et al[44] has determined that coadministration of CpG oligodeoxynucleotides and LL-37 produces synergistic antitumor effects against ovarian cancer in mice. Therefore, a multifunctional role for LL-37 in cancer progression, dependent on the tissue origin of the tumor, is becoming increasingly apparent, and this would need to be taken into consideration for future clinical application.

In conclusion, we have identified the specific mechanism of LL-37-mediated apoptosis in Tregs. This knowledge could be used as outlined in Figure 4. In brief, LL-37 induces apoptosis in Treg cells, resulting in the blockage of Treg suppressor activity, which would allow for an enhanced antitumor immune response. Future analysis will test the ability of LL-37 to kill Tregs in vivo using a murine EL4 model of tumorigenesis.

REFERENCES

1. Zasloff M. Antimicrobial peptides of multicellular organisms. *Nature*. 2002;415:389–395.
2. Brown KL, Hancock RE. Cationic host defense (antimicrobial) peptides. *Curr Opin Immunol*. 2006;18:24–30.
3. Søresen OE, Follin P, Johnsen AH, et al. Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3. *Blood*. 2001;97: 3951–3959.
4. Gudmundsson GH, Agerberth B, Odeberg J, et al. The human gene FALL39 and processing of the cathelin precursor to the antibacterial peptide LL-37 in granulocytes. *Eur J Biochem*. 1996;238:325–332.
5. Agerberth B, Charo J, Werr J, et al. The human antimicrobial and chemotactic peptides LL-37 and alpha-defensins are expressed by specific lymphocyte and monocyte populations. *Blood*. 2000;96:3086–3093.
6. Murakami M, Dorschner RA, Stern LJ, et al. Expression and secretion of cathelicidin antimicrobial peptides in murine mammary glands and human milk. *Pediatr Res*. 2005;57:10–15.
7. Nagaoka I, Tamura H, Hirata M. An antimicrobial cathelicidin peptide, human CAP18/LL-37, suppresses neutrophil apoptosis via the activation of formyl-peptide receptor-like 1 and P2X. *J Immunol*. 2006;176:3044–3052.
8. Barlow PG, Li X, Wilkinson TS, et al. The human cationic host defense peptide LL-37 mediates contrasting effects on apoptotic pathways in different primary cells of the innate immune system. *J Leuk Biol*. 2006;80:1–12.
9. Yang D, Chen Q, Schmidt AP, et al. 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*. 2000;192:1069–1074.
10. Schaller-Bals S, Schulze A, Bals R. Increased levels of antimicrobial peptides in tracheal aspirates of newborn infants during infection. *Am J Respir Crit Care Med*. 2002;165: 992–995.
11. Sakaguchi S. Naturally arising CD41 regulatory T cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol*. 2004;22:531–562.
12. Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med*. 2004;10: 942–949.
13. Somasundaram R, Jacob L, Swoboda R, et al. Inhibition of cytolytic T lymphocyte proliferation by autologous CD41/CD251 regulatory T cells in transforming growth factor-beta. *Cancer Res*. 2002;62:5267–5272.
14. Miyara M, Sakaguchi S. Natural regulatory T cells: mechanisms of suppression. *Trends Mol Med*. 2007;13:108–116.
15. Gondek DC, Lu LF, Quezada SA, et al. Cutting edge: contact-mediated suppression by $CD4^+CD25^+$ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *J Immunol*. 2005;174:1783–1786.
16. Zhao DM, Thornton AM, DiPaolo RJ, et al. Activated $CD4^+CD25^+$ T cells selectively kill B lymphocytes. *Blood*. 2006;107:3925–3932.
17. Cao X, Cai SF, Fehniger TA, et al. Granzyme B and perforin are important for regulatory T-cell-mediated suppression of tumor clearance. *Immunity*. 2007;27:635–646.
18. Grossman WJ, Verbsky JW, Barchet W, et al. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity*. 2004;21:589–601.
19. Zhu P, Zhang D, Chowdhury D, et al. Granzyme A, which causes single-stranded DNA damage, targets the double-strand break repair protein Ku70. *EMBO Rep*. 2006;7:431–437.
20. Zhu P, Martinvalet D, Chowdhury D, et al. The cytotoxic T lymphocyte protease granzyme A cleaves and inactivates poly(adenosine 5′-diphosphate-ribose) polymerase-1. *Blood*. 2009;114:1205–1216.
21. Martinvalet D, Dykxhoorn DM, Ferrini R, et al. Granzyme A cleaves a mitochondrial complex I protein to initiate caspase-independent cell death. *Cell*. 2008;133:681–692.
22. Murphy ME, Moult J, Bleackley RC, et al. Comparative molecular model building of two serine proteinases from cytotoxic T lymphocytes. *Proteins*. 1988;4:190–204.
23. Odake S, Kam CM, Narasimhan L, et al. Human and murine cytotoxic T lymphocyte serine proteases: subsite mapping with peptide thioester substrates and inhibition of enzyme activity and cytolysis by isocoumarins. *Biochemistry*. 1991;30:2217–2227.
24. Darmon AJ, Nicholson DW, Bleackley RC. Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B. *Nature*. 1995;377:446–448.
25. Gu Y, Sarnecki C, Fleming MA, et al. Processing and activation of CMH-1 by granzyme B. *J Biol Chem*. 1996;271: 10816–10820.
26. Brenner D, Mak TW. Mitochondrial cell death effectors. *Curr Opin Cell Biol*. 2009;21:871–877.
27. Danial NN, Korsmeyer SJ. Cell death: critical control points. *Cell*. 2004;116:205–219.
28. Suen DF, Norris KL, Youle RJ. Mitochondrial dynamics and apoptosis. *Genes Dev*. 2008;22:1577–1590.
29. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol*. 1992;119:493–501.

30. Zhao T, Zhang H, Guo Y, et al. Granzyme K directly processes Bid to release cytochrome C and endonulease G leading to mitochondria-dependent cell death. *J Biol Chem*. 2007;282: 12104–12111.
31. Beresford PJ, Xia Z, Greenberg AH, et al. Granzyme A loading induces rapid cytolysis and a novel form of DNA damage independently of caspase activation. *Immunity*. 1999;10:585–594.
32. Shresta S, Graubert TA, Thomas DA, et al. Granzyme A initiates an alternative pathway for granule-mediated apoptosis. *Immunity*. 1999;10:595–605.
33. Mader JS, Mokherjee N, Hancock REW, et al. The human host defense peptide LL-37 induces apoptosis in a calpain- and apoptosis-inducing factor-dependent manner involving Bax activity. *Mol Cancer Res*. 2009;7:689–702.
34. Chattopadhyay PK, Betts MR, Price DA, et al. The cytolytic enzymes granyzme A, granzyme B, and perforin: expression patterns, cell distribution, and their relationship to cell maturity and bright CD57 expression. *J Leuk Biol*. 2009;85: 88–97.
35. Niyonsaba F, Someya A, Hirata M, et al. 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*. 2001;31:1066–1075.
36. Ong PY, Ohtake T, Brandt C, et al. Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *N Engl J Med*. 2002;347:1151–1160.
37. Mader JS, Richardson A, Salsman J, et al. Bovine lactoferricin causes apoptosis in Jurkat T-leukemia cells by sequential permeabilization of the cell membrane and targeting of mitochondria. *Exp Cell Res*. 2007;313:2634–2650.
38. Coffelt SB, Marini FC, Watson K, et al. The pro-inflammatory peptide LL-37 promotes ovarian tumor progression through recruitment of multipotent mesenchymal stromal cells. *PNAS*. 2009;106:3806–3811.
39. Heilborn JD, Nilsson MF, Chamorro Jimenez CI, et al. Antimicrobial protein hCAP18/LL-37 is highly expressed in breast cancer and is a putative growth factor for epithelial cells. *Int J Cancer*. 2005;114:713–719.
40. Winder D, Gunzburg WH, Erfle V, et al. Expression of antimicrobial peptides has an antitumour effect in human cells. *Biochem Biophys Res Commun*. 1998;242:608–612.
41. Ohtake T, Fujimoto Y, Ikuta K, et al. Proline-rich antimicrobial peptide, PR-39 gene transduction altered invasive activity and actin structure in human hepatocellular carcinoma cells. *Br J Cancer*. 1999;81:393–403.
42. Wu WK, Sung JJ, To KF, et al. The host defense peptide LL-37 activates the tumor suppressing bone morphogenetic protein signaling via inhibition of proteasome in gastric cancer cells. *J Cell Physiol*. 2010;223:178–186.
43. Li X, Li Y, Han H, et al. Solution structures of human LL-37 fragments and NMR-based identification of a minimal membrane-targeting antimicrobial and anticancer region. *J Am Chem Soc*. 2006;128:5776–5785.
44. Chuang CM, Monie A, Wu A, et al. Treatment with LL-37 peptide enhances the antitumor effects induced by CpG oligodeoxynucleotides against ovarian cancer. *Hum Gene Ther*. 2009;20:303–313.

