The Human Host Defense Peptide LL-37 Induces Apoptosis in a Calpain- and Apoptosis-Inducing Factor–Dependent Manner Involving Bax Activity

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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 Jurkat T leukemia cells via apoptosis. A loss of mitochondrial membrane potential, DNA fragmentation, and phosphatidylserine externalization were detected following LL-37 exposure, whereas apoptosis was independent of caspase family members. The specific apoptotic pathway induced by LL-37 was defined through the utilization of Jurkat cells modified to express antiapoptotic proteins, as well as cells deficient in various proteins associated with apoptosis. Of interest, both Bcl-2–overexpressing cells and cells deficient in Bax and Bak proteins displayed a significant reduction in LL-37–induced apoptosis. In addition, Jurkat cells modified in the Fas receptor–associated pathway showed no reduction in apoptosis when exposed to LL-37. Analysis of the involvement of apoptosis-inducing factor (AIF) in LL-37–mediated apoptosis revealed that AIF transferred from the mitochondria to the nucleus of cells exposed to LL-37, where it may lead to large-scale DNA fragmentation and chromatin condensation. AIF knockdown analysis resulted in LL-37–resistant cells. This suggests that AIF is mandatory in LL-37–mediated killing. Lastly, chelation or inhibition of Ca^{2+} or calpains inhibited LL-37–mediated killing. Further analysis revealed that calpains were required for LL-37–mediated Bax translocation to mitochondria. Together, these data show that LL-37–induced apoptosis is mediated via the mitochondria-associated pathway in a caspase-independent and calpain- and AIF-dependent manner that involves Bax activation and translocation to mitochondria. (Mol Cancer Res 2009;7(5):689–702)

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Introduction

Cationic host defense peptides play an important role in innate immunity and are evolutionarily conserved throughout the animal and plant kingdoms (1). Many of these peptides display immunomodulatory functions, influencing processes such as cytokine and chemokine release and cell proliferation (2). Certain host defense peptides have been shown to display antitumorigenic activity (3-5), believed to be due to their membrane-destabilizing and apoptosis-inducing effects. LL-37 is a human cationic, amphipathic host defense peptide released by proteinase 3 proteolytic processing of the COOH-terminal domain of the human cathelicidin, human cationic antimicrobial protein-18 (6). LL-37 is found in the secondary granules of neutrophils (7), is endogenously expressed by a variety of cells including keratinocytes and epithelial cells, and is released in response to multiple inflammatory stimuli (8, 9). On release, LL-37 acts as an effector molecule of innate immunity, displaying anti-infective and immunomodulatory activities, including the induction of neutrophil, $CD4^+$ T-cell and monocyte chemotaxis (10), mast cell activation (11), and neutrophil survival (12, 13). However, a concentration of 100 μg/mL of LL-37 resulted in the death of 60% untransformed human airway epithelial cells (14). These variations in the cytotoxic nature of LL-37 may potentially be related to peptide-mediated augmentation of innate immunity (13). The ability of LL-37 to promote or inhibit apoptosis depending on the cell type, and the knowledge of other cationic host defense peptides that can kill cancer cells, has led researchers to try and understand the effect(s) of LL-37 on human tumor cells.

The mitochondria-associated pathway of apoptosis becomes activated by a variety of stimuli, including growth factor withdrawal and chemotherapeutic drug treatment (15), which can result in mitochondrial transmembrane potential dissipation ($\Delta\Psi_m$) and the release of proapoptotic proteins, including cytochrome *c*, into the cytosol. The mitochondrial-associated pathway is primarily regulated by the Bcl-2 family of proteins, which control $\Delta\Psi_m$ through their ability to interact with mitochondrial permeability transition pores that span the inner and outer mitochondrial membranes (16). The death receptor pathway becomes activated on ligand-induced trimerization of cell surface death receptors of the tumor necrosis factor receptor superfamily (i.e., Fas), which results in the proteolytic cleavage and dimerization of caspase-8 into its active form, leading to the activation of downstream executioner caspase-3, caspase-6, and caspase-7. Activated executioner caspases have a number of substrates, including proteins involved in membrane

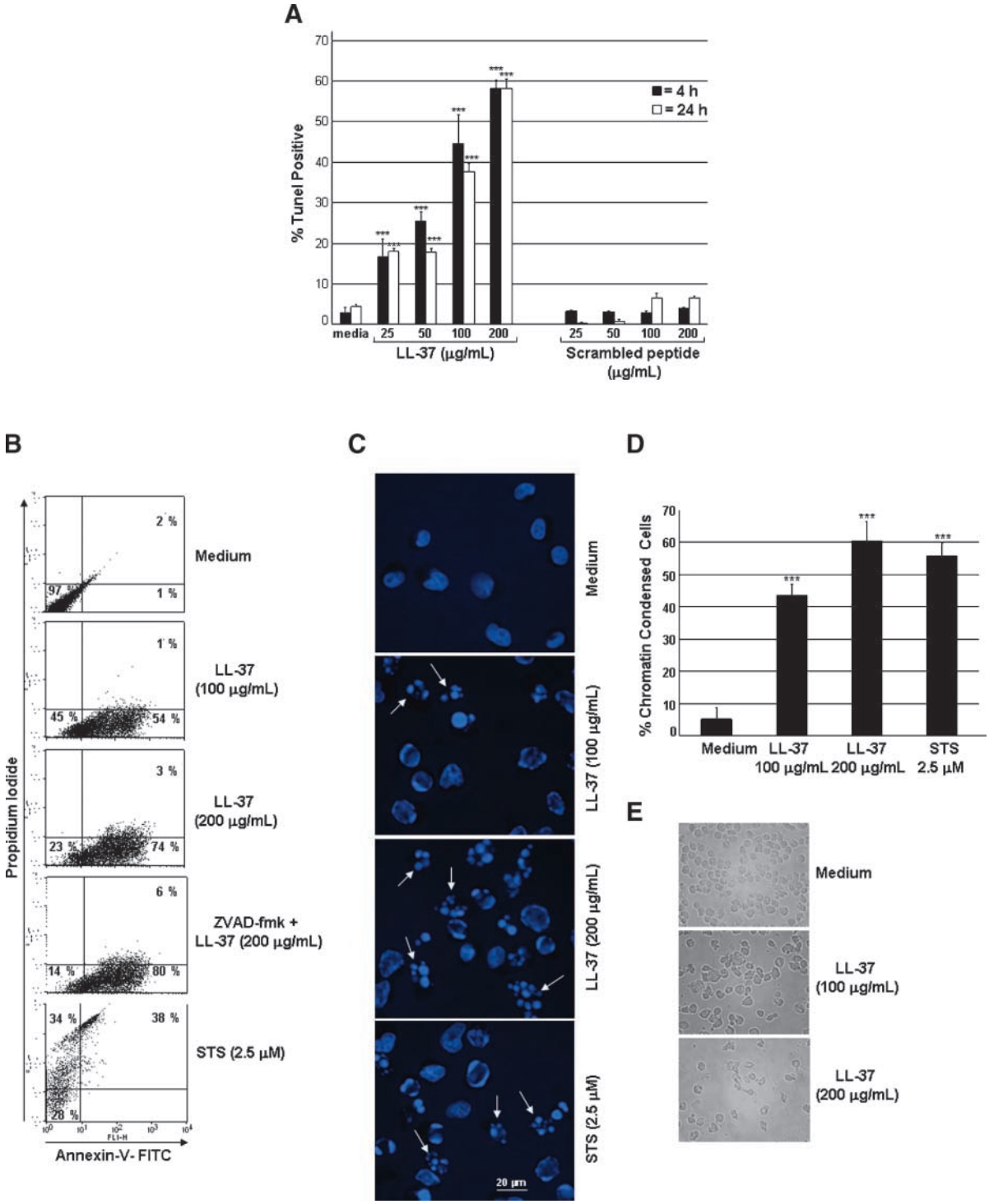


FIGURE 1. LL-37 kills Jurkat cells through the induction of apoptosis. **A.** Jurkat cells (2×10^5 per treatment) were treated with increasing concentrations of LL-37 or scrambled peptide (25-200 μg/mL) for 4 and 24 h. Cells were then stained with TUNEL-PE (1 h, 37°C) and analyzed by flow cytometry. Columns, mean percent cell death ($n = 3$); bars, SD. ***, $P < 0.001$, compared with media control. **B.** Jurkat cells (2×10^5 per treatment) were exposed to LL-37 (100 and 200 μg/mL), staurosporine (2.5 μmol/L), or LL-37 (200 μg/mL) + Z-VAD-fmk (50 μmol/L) for 4 h; stained with Annexin V-FITC and PI (30 min, room temperature); and analyzed by flow cytometry. Data are representative of three independent experiments. **C.** Jurkat cells (5×10^4 per treatment) were treated with LL-37 (100 and 200 μg/mL) or staurosporine (*STS*; 2.5 μmol/L) for 2 h. 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 done (10 min, room temperature) and slides were analyzed by confocal microscopy (×400 magnification). **D.** The percentage of cells showing chromatin condensation was determined by Hoechst staining and counting (500 cells per slide). Columns, mean percent chromatin condensed cells ($n = 3$); bars, SD. ***, $P < 0.001$, compared with media control. **E.** Jurkat cells (5×10^4 per treatment) were treated with LL-37 (100 and 200 μg/mL) for 2 h. Cells were then fixed with 2% paraformaldehyde, mounted on silinated microscope slides, and air-dried overnight. Cells were mounted and slides were analyzed by light microscopy (×400 magnification).

stability (i.e., gelsolin), cellular shape (i.e., fodrin), and nuclear structure.

Other cytosolic cysteine proteases, known as calpain I and calpain II, require intracellular calcium (Ca^{2+}) for activation, and may trigger apoptosis and necrosis through the proteolysis of cellular substrates, including cytoskeletal and membrane proteins (17-19). Calpains can also activate proapoptotic Bcl-2 family members, resulting in outer mitochondrial membrane permeabilization and the release of proapoptotic proteins into the cytosol (see refs. 20, 21 for review). Bax protein is required for the release of apoptosis-inducing factor (AIF) in a number of death models, including AIF-mediated DNA damage–related programmed cell death (22) and camptothecin-mediated programmed cell death (23). AIF is a caspase-independent, 57-kDa proapoptotic flavoprotein that translocates from the mitochondria to the nucleus during many forms of cell death, following the required proteolytic cleavage of the 62-kDa mitochondrial protein into the soluble, proapoptotic 57-kDa truncated form (tAIF; ref. 24). Once in the nucleus, AIF can lead to large-scale DNA fragmentation and chromatin condensation (25, 26) in a caspase-independent manner (for review, ref. 27). In addition, there is a requirement for AIF in oxidative phosphorylation and for the assembly and/or stabilization of respiratory complex I (28). In turn, AIF translocation to the nucleus could result in a reduction of respiratory chain complex I activity, leading to high lactate production and increased dependency on ATP generation. This may contribute to the apoptotic effects observed with AIF release from mitochondria (28, 29). One model of AIF-mediated programmed cell death revealed that AIF release required the cooperative action of both calpains and Bax, the former cleaving AIF to tAIF and the latter releasing it from the mitochondria (22, 30). Cleavage of AIF by calpains most likely begins after mitochondrial membrane permeabilization, in that AIF cleavage is inhibited by Bcl-2 and Bcl-xL overexpression (24).

LL-37 has been shown to have antitumorigenic activity against a number of human cancer cell lines, including oral squamous cell carcinoma SAS-H1 cells and Jurkat T leukemia cells (31, 32). However, the downstream mechanism(s) of LL-37–induced cell death have not been fully examined. Therefore, in this study, the specific pathways of apoptosis were examined to further understand how LL-37 is inducing cell death. We show that LL-37 kills Jurkat human T leukemia cells through a caspase-independent form of apoptosis. LL-37 induces a calpain-mediated Bax protein translocation to mitochondria, destabilization of the mitochondrial membrane, and AIF-dependent apoptosis.

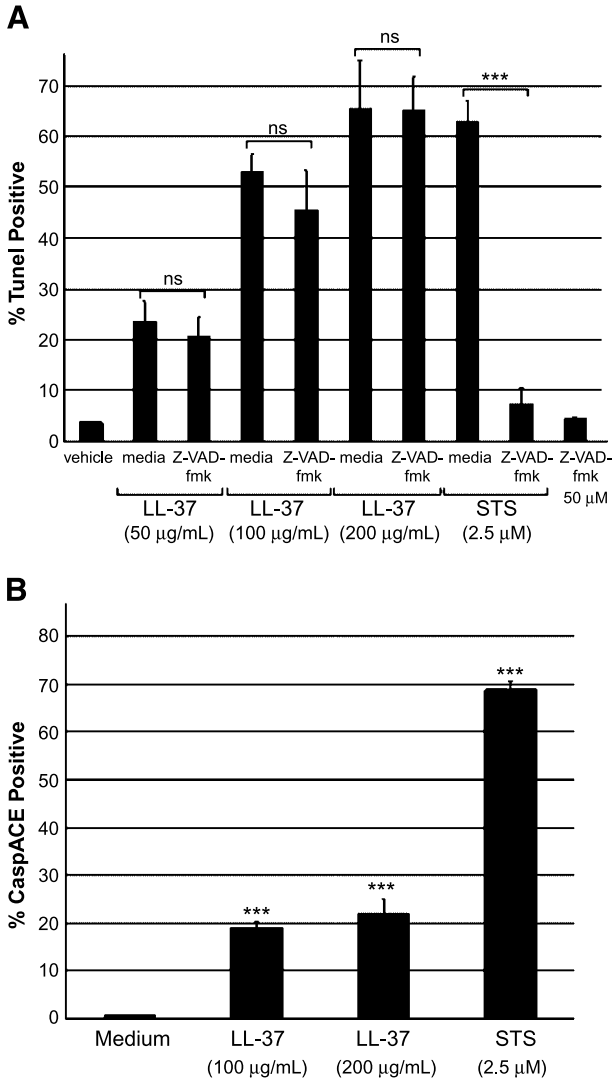
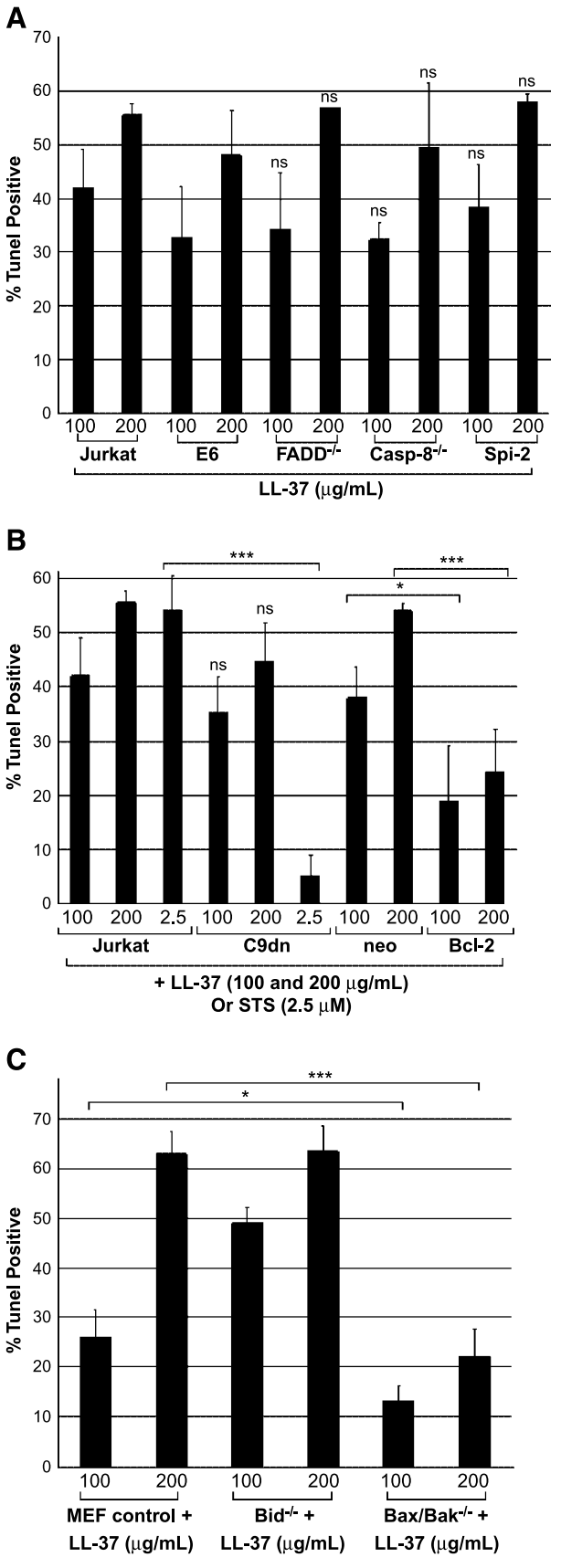


FIGURE 2. LL-37 kills Jurkat cells in a caspase-independent manner. **A.** Jurkat cells (2×10^5 per treatment) were exposed to LL-37 (100 and 200 μg/mL), staurosporine (2.5 μmol/L), or DMSO vehicle control for 4 h at 37°C in the absence or presence of Z-VAD-fmk (50 μmol/L, 1 h pretreatment). At this time, cells were stained with TUNEL-PE (1 h, 37°C) and flow cytometric analysis was done. Columns, mean percent cell death ($n = 3$); bars, SD. ns, not significant; ***, $P < 0.001$, compared with media control. **B.** Jurkat cells (2×10^5 per treatment) were exposed to LL-37 (100 and 200 μg/mL) or staurosporine (2.5 μmol/L) for 4 h at 37°C. At this time, cells were exposed to CaspACE FITC-VAD-fmk (1:500; 20 min, 37°C) and flow cytometric analysis was done. Columns, mean percent cell death ($n = 3$); bars, SD. ***, $P < 0.001$, compared with media control.

Results

LL-37 Kills Jurkat Cells by Initiating Apoptosis

Apoptosis is a form of cellular death that is characterized by a number of specific morphologic features, including DNA fragmentation, chromatin condensation, and phosphatidylserine externalization. Figure 1A shows that Jurkat human T leukemia cells exposed to increasing concentrations of LL-37 (25, 50, 100, and 200 μg/mL, equivalent to 5.5, 11, 22, and 44 μmol/L, respectively; 4 and 24 hours) displayed significant DNA fragmentation as assessed by terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) DNA staining. Because DNA fragmentation is an indicator of apoptotic cell death, these data showed that Jurkat cells were undergoing apoptosis. In contrast, Jurkat cells exposed to a scrambled LL-37 amino acid sequence were not affected (Fig. 1A). This result shows that the specific order of the amino acid sequence, and not the overall peptide charge, is necessary for LL-37–induced cell death. The mode of cell death was further analyzed by measuring FITC-conjugated Annexin V and propidium iodide (PI) costaining. Jurkat cells exposed to LL-37 (100 and



200 μg/mL, 4 hours) showed elevated Annexin V staining (54% and 74%, respectively) compared with the medium control (1%; Fig. 1B), whereas PI staining was negligible. Treatment with staurosporine (2.5 μmol/L, 4 hours) showed high levels of PI staining indicative of late-stage apoptosis (Fig. 1B). The lack of PI staining in LL-37–treated cells, which specifically stains necrotic and late-stage apoptotic cells, supports the observation that Jurkat cells exposed to LL-37 were killed by apoptosis and not necrosis. Hoechst 33342 staining (Fig. 1C and D) revealed chromatin condensation and apoptotic body formation in Jurkat cells treated with LL-37 (100 and 200 μg/mL, 2 hours) or staurosporine positive control (2.5 μmol/L, 2 hours), further confirming an apoptotic form of cell death. Finally, the morphology of LL-37–treated Jurkat cells shows cell shrinkage and membrane changes (Fig. 1E).

Caspase Family Members Are Not Required for LL-37–Induced Cell Death

Apoptosis was analyzed using TUNEL or Annexin V/PI staining in the absence or presence of the pan-caspase inhibitors Z-VAD-fmk (50 μmol/L) or Q-VD-OPh (20 μmol/L). Neither Z-VAD-fmk (Figs. 1B and 2A) nor Q-VD-OPh (data not shown) had an inhibitory effect on LL-37–induced apoptosis of Jurkat cells. This is in contrast to the staurosporine positive control (2.5 μmol/L), where killing activity was significantly reduced (from 58 ± 6% to 5 ± 5%) by equivalent Z-VAD-fmk concentrations. Caspase inhibitors were dissolved in DMSO. Vehicle control analysis showed no cytotoxic effect on Jurkat cells. Caspase activity assays were done using the CaspACE FITC-VAD-fmk assay system as previously described (33). Caspases were activated following LL-37 treatment (100 and 200 μg/mL, Fig. 2B; 19 ± 1% and 22 ± 3%, respectively), whereas staurosporine exposure led to much higher levels (69 ± 2%) of active caspases. It is no surprise that low levels of caspase activation were observed following LL-37 exposure, in that the loss of mitochondrial membrane potential could result in caspase activation through cytochrome *c* release. In addition, calpains can also lead to caspase activation. Although caspase family members may become activated following LL-37 exposure, they do not seem to be necessary for apoptosis induction in this system.

A variety of Jurkat cell clones were used to further define the specific apoptotic pathway induced by LL-37. Fas-associated death domain knockout ($FADD^{-/-}$), caspase-8 knockout ($caspase\text{-}8^{-/-}$), and Spi-2 cells were treated with LL-37 (100 and 200 μg/mL, 4 hours; Fig. 3A) to definitively analyze whether caspase-8 activation or the death-receptor-associated pathway of apoptosis is required for LL-37–induced killing.

FIGURE 3. Bcl-2 family members play a significant role in LL-37–mediated apoptosis, whereas the Fas death receptor pathway is not involved. Jurkat clone lines that had modifications in death receptor apoptotic pathway proteins (**A**) or mitochondrial-associated apoptotic pathway proteins (**B**) were set up (2×10^5 per treatment) in the absence or presence of LL-37 (100 and 200 μg/mL) for 4 h or staurosporine (2.5 μmol/L) for 2 h at 37°C. At this time, cells were stained with TUNEL-PE (1 h, 37°C) and flow cytometric analysis was done. Columns, mean percent cell death ($n = 3$); bars, SD. ns, not significant; *, $P < 0.01$; ***, $P < 0.001$, compared with control cells. **C.** MEF clone lines that were Bid or Bax/Bak knockouts were set up as in **A** and **B**. Columns, mean percent cell death ($n = 3$); bars, SD. *, $P < 0.01$; ***, $P < 0.001$, compared with control cells.

CrmA/Spi-2 has been used extensively to clarify apoptotic cascades due to its ability to effectively inhibit both caspase-1 and caspase-8 (34). There was no significant difference between the percentage of TUNEL-positive cells observed with these lines versus Jurkat control cells or E6 control cells (control line for FADD$^{-/-}$), which suggests that the death receptor pathway is not involved in LL-37–induced apoptosis. TUNEL-positive staining of media control values was negligible and deducted from all treatment values.

LL-37–Induced Cell Death Is Independent of Caspase-9 and Requires the Activity of Bcl-2 Family Members

Jurkat cells expressing dominant negative caspase-9 (J-DN-c9) were used to definitively analyze whether caspase-9 or the mitochondrial apoptotic pathway was involved in LL-37–mediated apoptosis. There was no significant difference between the percentage of TUNEL-positive cells observed with J-DN-c9 versus Jurkat control cells (Fig. 3B), which confirms that caspase-9 and the classic mitochondria-associated pathway leading to, and requiring, caspase activation are not involved in LL-37–induced death. In contrast, J-DN-c9 cells exposed to staurosporine were significantly less sensitive (5 ± 6%) to drug treatment compared with Jurkat control cells (54 ± 7%; $P < 0.001$; Fig. 3B). TUNEL-positive staining of media control values was negligible and deducted from all treatment values.

Bcl-2 is an antiapoptotic protein that regulates apoptosis by maintaining mitochondrial transmembrane potential (16). A Jurkat line modified to overexpress antiapoptotic Bcl-2 and double knockout Bax$^{-/-}$/Bak$^{-/-}$ or single knockout Bid$^{-/-}$ mouse embryonic fibroblasts (MEF) were used to determine whether Bcl-2 family members are involved in LL-37–mediated apoptosis. Of interest, both the Bcl-2–overexpressing Jurkat line (Fig. 3B) and MEFs lacking Bax and Bak (Fig. 3C) were significantly less affected (LL-37 100 μg/mL, $P < 0.01$; LL-37 200 μg/mL, $P < 0.001$) by LL-37 exposure versus medium control–treated cells. In contrast, MEFs lacking Bid were as sensitive to LL-37 exposure as control MEFs (Fig. 3C), which suggests that activated, truncated Bid is not involved in LL-37–mediated apoptosis. TUNEL-positive staining of media control values was negligible and deducted from all treatment values.

LL-37 Induces a Loss in Mitochondrial Transmembrane Potential Associated with AIF Translocation to the Nucleus

To further examine the activation of the mitochondria-associated pathway of apoptosis, changes in $\Delta\Psi_m$ were analyzed following LL-37 exposure. $DiOC_6$ enters the mitochondria in healthy cells but leaches into the cytosol of the cell on $\Delta\Psi_m$ dissipation, resulting in decreased fluorescence intensity. LL-37 (100 and 200 μg/mL) led to significant dissipation in $\Delta\Psi_m$ in Jurkat cells, as determined by flow cytometry (Fig. 4). This dissipation was substantially blocked in Jurkat cells overexpressing Bcl-2 (Fig. 4), which suggests that Bcl-2 family members are involved in the loss of $\Delta\Psi_m$ observed following LL-37 exposure. LL-37 does, however, induce a slight loss in mitochondrial transmembrane potential in Bcl-2–overexpressing Jurkat cells. It is possible that proapoptotic Bcl-2 family members, such as Bax, are not completely blocked by Bcl-2 overexpression; it may also be due to a contribution from a Bcl-2–independent pathway, perhaps through leakage of lysosomal enzymes as indicated by cathepsin B inhibitor studies, but clearly, the Bcl-2/Bax pathway is playing the major role.

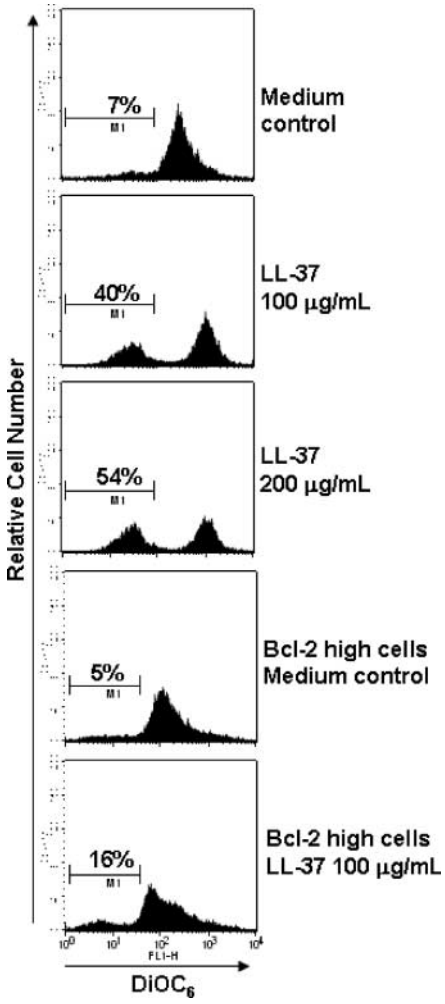


FIGURE 4. LL-37 treatment resulted in a loss in mitochondrial transmembrane potential and the release of AIF from the inner mitochondrial matrix and relocalization in the nucleus. AIF knockdown blocks LL-37–mediated apoptosis. Jurkat or Bcl-2–overexpressing Jurkat cells (2×10^5 per treatment) were set up with media or LL-37 (100 and/or 200 μg/mL) for 4 h at 37°C. $DiOC_6$ (40 nmol/L final concentration) was added 15 min before the assay end point and for a further 15 min (30 min total, 37°C). A loss in mitochondrial transmembrane potential was then analyzed by flow cytometry. Data are representative of three independent experiments.

To further analyze whether LL-37 was leading to a complete disintegration of mitochondria or initiating a specific mitochondrio-tropic effect, we examined the location of cytochrome *c* oxidase subunit IV (Cox IV), an integral inner mitochondrial membrane protein that is often unaffected by mitochondrial transmembrane potential, therefore remaining intact in mitochondria during cytochrome *c* and AIF release (35). We have determined that LL-37 does not induce the release of Cox IV from mitochondria (Fig. 5), which suggests that LL-37 is inducing a specific mitochondrio-tropic effect and not simply a complete mitochondrial disintegration.

Because LL-37 induced apoptosis in a caspase-independent manner, caspase-independent mechanisms of apoptosis, downstream of $\Delta\Psi_m$ dissipation, were analyzed. Nuclear, mitochondrial, and cytosolic cellular fractions were isolated from control and LL-37–treated Jurkat T leukemia cells to access the localization of caspase-independent, proapoptotic AIF following LL-37 treatment. Figure 5 shows that AIF transferred from the mitochondria to the nucleus of Jurkat cells exposed to LL-37 (200 μg/mL, 2 hours). Heat shock protein 60 (Hsp60), a mitochondria-associated protein, and poly(ADP-ribose) polymerase, a nuclear specific chromatin-associated enzyme, were used to show the purity of the cellular fractions. In addition to AIF release, and as previously shown by Aarbiou et al. (32), we have confirmed that LL-37 induces the release of cytochrome *c* from mitochondria into the cytosol of Jurkat cells (Fig. 5). This is not surprising in that cytochrome *c* is often released from mitochondria following a dissipation of $\Delta\Psi_m$.

Purified mitochondria treated with LL-37 (25 and 50 μg/mL, 15 minutes) also resulted in cytochrome *c* release (Fig. 5). The ability of LL-37 to induce the release of cytochrome *c* from isolated mitochondria suggests that LL-37 may interact directly with mitochondria leading to a dissipation of $\Delta\Psi_m$. This has been shown for other cationic peptides, such as LfcinB and magainin 2 (36), which can enter the cytosol of human tumor cells. This may not be the case for LL-37 *in vitro* in that it is not known whether LL-37 can enter Jurkat T leukemia cells. LL-37 had no effect on AIF localization because little protein was observed in the supernatant of LL-37–treated mitochondria (Fig. 5). These data suggest that LL-37 alone is not sufficient to induce AIF release, even if it were able to enter the cell cytosol. This is not unexpected because AIF requires both mitochondrial membrane destabilization and direct lysis to exit mitochondria. These data support the hypothesis that there is a requirement for intracellular protein activation to release AIF from mitochondria following LL-37 exposure. Ongoing studies in our laboratory will analyze whether LL-37 crosses the plasma membrane of Jurkat cells to interact with specific intracellular organelles.

AIF Knockdown Cells Are Resistant to LL-37–Mediated Killing

SiRNA knockdown protocols were not successful in the transfection of Jurkat cells; therefore, AIF siRNA knockdown

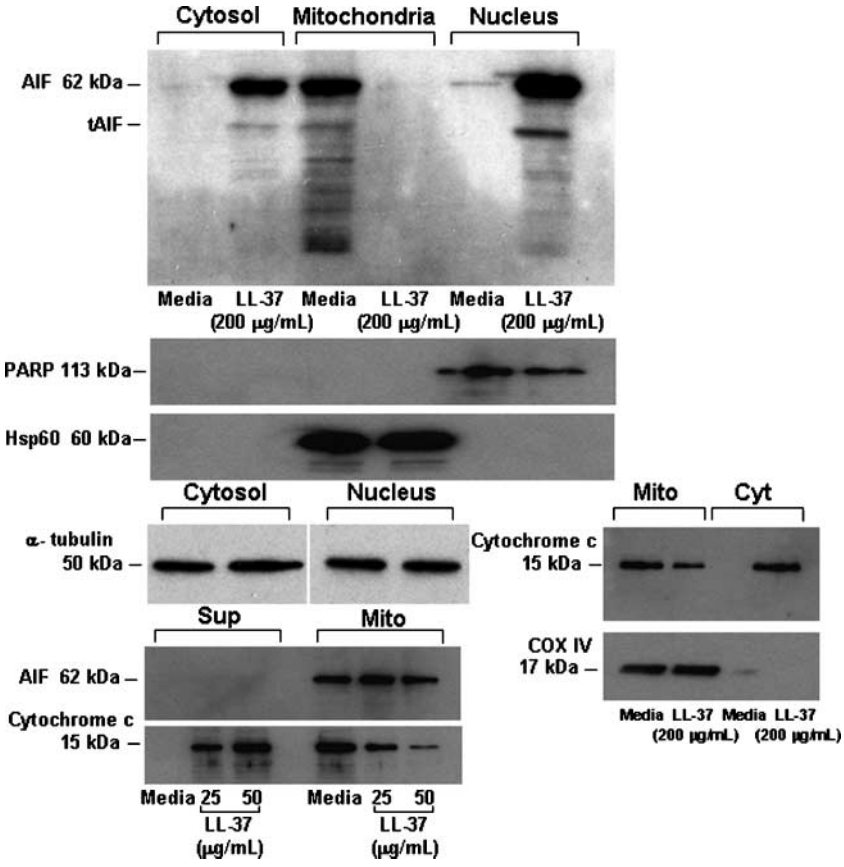


FIGURE 5. Jurkat cells (1×10^6 per treatment) were set up with media or LL-37 (200 μg/mL) for 2 h at 37°C. Cellular fractions (cytosolic, mitochondrial, and nuclear) were then isolated using the Qproteome cell compartment kit. Samples were boiled in SDS sample buffer and 20 μg of total protein were loaded into each well of a 10% SDS-polyacrylamide gel for separation by electrophoresis. Immunoblot was probed with mouse anti-human AIF (1:1,000), mouse anti–Cox IV (1:2,000), or mouse anti–cytochrome *c* (1:1,000), and then goat anti-mouse horseradish peroxidase (1:3,000). Data are representative of three independent experiments. Protein loading and cell compartment contamination were assessed using anti–α-tubulin (1:1,000), mitochondria-specific anti-Hsp60 (1:1,000), and nuclear-specific anti–poly(ADP-ribose) polymerase (1:1,000).

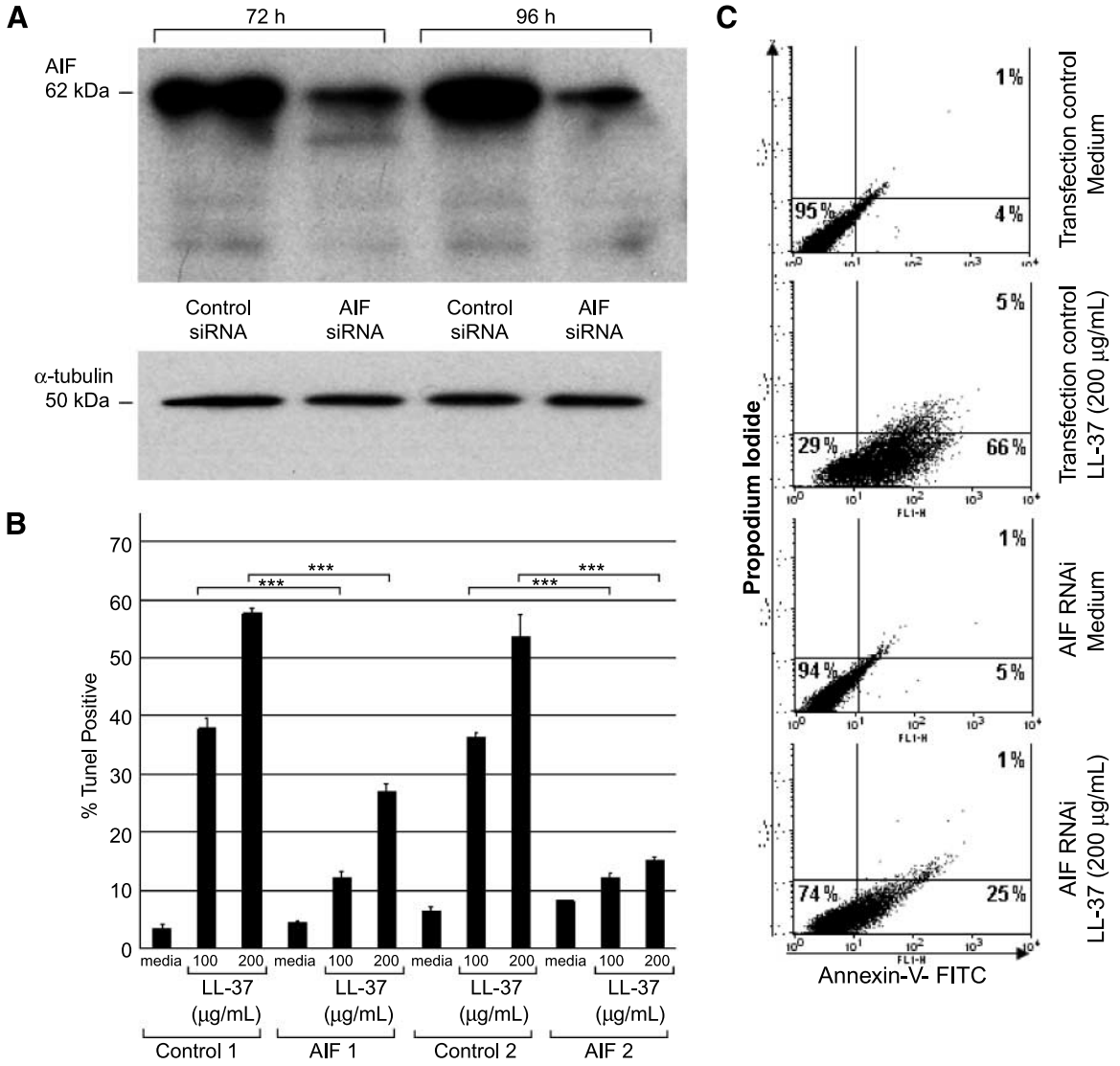
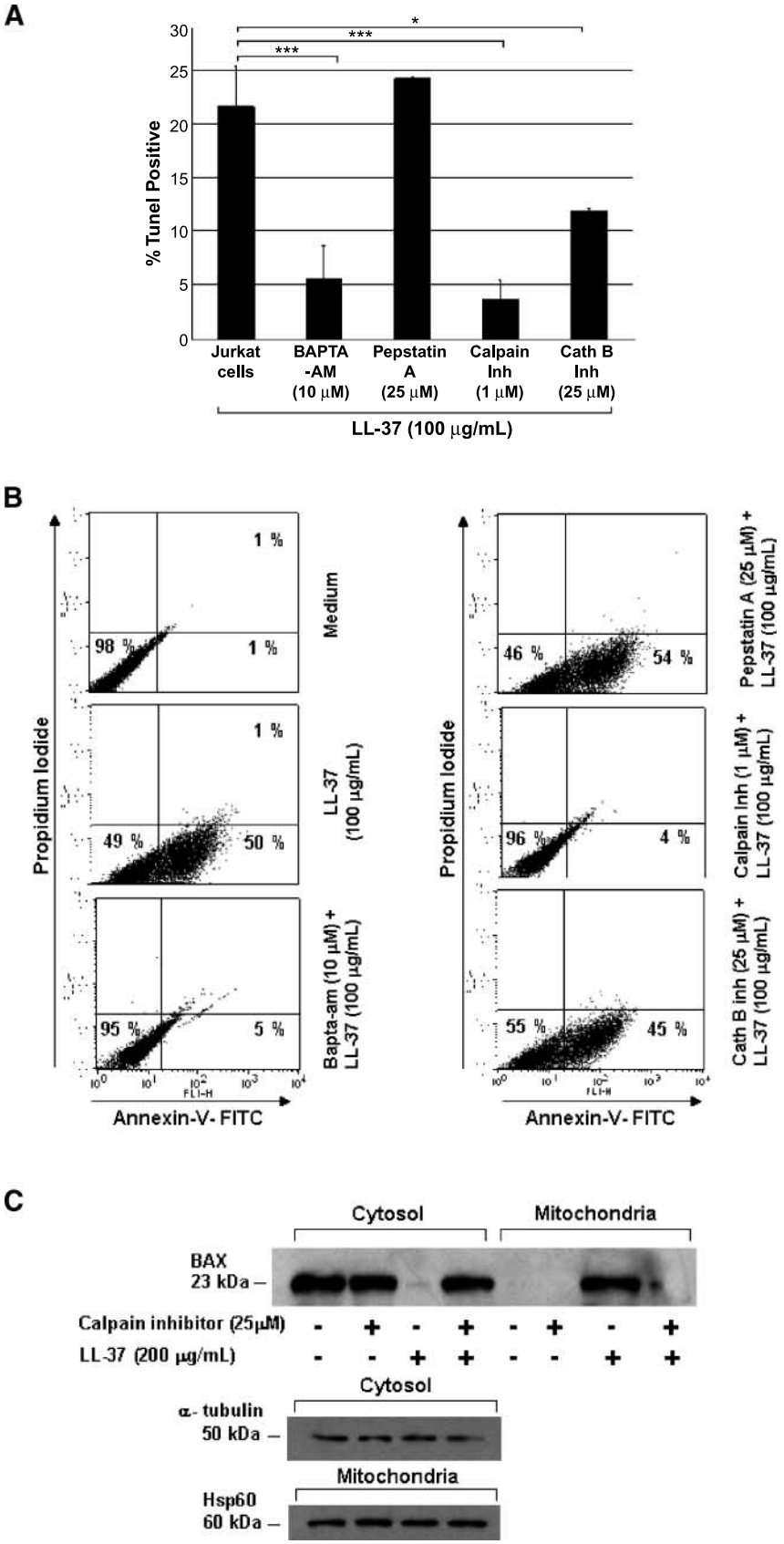


FIGURE 6. **A.** HeLa cells (2×10^5 per treatment) were transfected with AIF ON-TARGET*plus* siRNA or ON-TARGET*plus* si*CONTROL* nontargeting siRNA (negative control) using Dharma*FECT* transfection reagent 1. Cells were incubated at 37°C in 5% CO_2 for 72 and 96 h, and total protein was isolated using radioimmunoprecipitation assay buffer. Samples were boiled in SDS sample buffer and 20 μg of total protein were loaded into each well of a 10% SDS-polyacrylamide gel for separation by electrophoresis. Immunoblot was probed with mouse anti-human AIF (1:1,000) and goat anti-mouse horseradish peroxidase (1:3,000). Data are representative of three independent experiments. **B.** SiRNA-transfected HeLa cells (2×10^5 per treatment, 72 and 96 h) were exposed to LL-37 (100 and 200 μg/mL) for 4 h at 37°C. Control 1/AIF 1: 72-h transfected cells; control 2/AIF 2: 96-h transfected cells. At this time, cells were stained with TUNEL-PE (1 h, 37°C) and flow cytometric analysis was done. Columns, mean percent cell death ($n = 3$); bars, SD. ***, $P < 0.001$, compared with media control. **C.** SiRNA-transfected HeLa cells (2×10^5 per treatment, 96 h) were exposed to LL-37 (200 μg/mL) for 4 h at 37°C. At this time, cells were stained with Annexin V-FITC and PI (30 min, room temperature), and analyzed by flow cytometry. Data are representative of three independent experiments.

analysis was done using HeLa cells, which are easily transfected with siRNA. By doing so, these data allowed us to analyzed apoptosis in multiple cell types following LL-37 exposure. TUNEL and AnnexinV/PI analysis done in HeLa cells showed caspase-independent apoptosis following LL-37 treatment similar to that observed in Jurkat cells (data not shown). HeLa were transfected with AIF ON-TARGET*plus* siRNA using Dharma*FECT* transfection reagents. Following transfection, cells were analyzed for AIF knockdown by immunoblot (Fig. 6A). AIF protein expression was drastically decreased at both the 72- and 96-hour time points. TUNEL-PE analysis was done with AIF siRNA–transfected cells, which showed significantly less sensitivity ($P < 0.001$) to LL-37 (100 and 200 μg/mL) versus siRNA negative control–transfected cells (Fig. 6B). This decrease in LL-37 sensitivity was further confirmed by Annexin V/PI staining (Fig. 6C), wherein AIF-transfected cells showed a drastic reduction in sensitivity to LL-37 (200 μg/mL) compared with siRNA control cells.

Intracellular Ca^{2+} and Calpains Are Required for LL-37–Mediated Apoptosis

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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Jurkat cells
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LL-37 (100 μg/mL)
B
Propidium Iodide
Annexin-V- FITC
1 %
98 %
1 %
Medium
1 %
49 %
50 %
LL-37 (100 μg/mL)
95 %
5 %
Bapta-am (10 μM) + LL-37 (100 μg/mL)
46 %
54 %
Pepstatin A (25 μM) + LL-37 (100 μg/mL)
96 %
4 %
Calpain Inh (1 μM) + LL-37 (100 μg/mL)
55 %
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Cath B inh (25 μM) + LL-37 (100 μg/mL)
C
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23 kDa –
Calpain inhibitor (25μM)
LL-37 (200 μg/mL)
Cytosol
α- tubulin
50 kDa –
Mitochondria
Hsp60
60 kDa –

release from the mitochondria (37, 38). Jurkat cells that were pre-exposed to the Ca^{2+}-chelating agent BAPTA-AM (10 μmol/L) or the calpain inhibitor Ac-LLnL-CHO (1 μmol/L) were not killed when exposed to LL-37 (100 μg/mL; Fig. 7A and B). These data suggest that calpain is required for LL-37–mediated apoptosis. A specific cathepsin B inhibitor (25 μmol/L) significantly ($P < 0.01$) inhibited LL-37–mediated killing in TUNEL analysis (Fig. 7A); however, the same inhibitor showed little effect in Annexin V/PI analysis (Fig. 7B). This suggests that the phosphatidylserine externalization observed following LL-37 treatment may be independent of cathepsin B activation, whereas DNA fragmentation is partially blocked by inhibition. All inhibitors were dissolved in DMSO and diluted to a concentration that had no cytotoxic effect on Jurkat cells (vehicle control values were deducted from treatment values before graphing).

Calpain Inhibition Blocks Bax Activation and Translocation to Mitochondria following LL-37 Treatment

Various mediators of apoptosis can trigger the activation of Bax and its relocation to the mitochondria, where it can induce $\Delta\Psi_m$ dissipation (39, 40). Because $Bax^{-/-}/Bak^{-/-}$ cells displayed significantly less susceptibility to LL-37–mediated apoptosis, Bax translocation to the mitochondria was analyzed following LL-37 treatment (Fig. 7B). Jurkat cells were exposed to LL-37 (200 μg/mL) in the absence or presence of calpain inhibitor (Ac-LLnL-CHO, 1 μmol/L) to analyze the involvement of calpains in Bax translocation following LL-37 treatment. Calpains have been previously shown to trigger Bax activation downstream of death induction (38, 41). Figure 7B shows that Bax localizes to the mitochondrial fraction in Jurkat cells exposed to LL-37, whereas this relocation is blocked by pretreatment of cells with calpain inhibitor.

Discussion

LL-37 is a cationic host defense peptide that has both anti-infective and immunomodulatory activity (10, 11). LL-37 is expressed at ~5 μg/mL in bronchoalveolar lavage of healthy infants and is up-regulated by inflammation to ~30 μg/mL in bronchoalveolar lavage from infants with pulmonary infections (42). LL-37 expression is also up-regulated in other inflammatory conditions, such as psoriatic skin lesions, where levels are ~1.5 mg/mL due to *in vivo* processing (43). An association between cancer and inflammation has long been appreciated (44, 45). Therefore, the concentrations of LL-37 used in this study are believed to be physiologically relevant. In addition, tumor cells were not rendered necrotic when exposed to LL-37 concentrations as high as 200 μg/mL, as PI staining of these cells was negligible (Fig. 1B).

In this study, the mechanism of cell death induced by LL-37 in Jurkat T leukemia cells was characterized to examine its potential use as an anticancer treatment option. LL-37 killed Jurkat cells through the activation of a form of caspase-independent apoptosis, requiring the calpain activation and using calpain-dependent translocation of Bax to the mitochondria, which can result in mitochondrial membrane potential loss and AIF release. AIF expression was necessary for LL-37–mediated apoptosis, in that HeLa cells lacking AIF were resistant to LL-37. Apoptosis was observed as early as 4 hours after LL-37 treatment. This time point was used for subsequent experiments because apoptosis was not significantly greater at a later time point.

Although caspases were activated following exposure to LL-37 (Fig. 2B), it was shown that caspase inhibition had no effect on LL-37–mediated apoptosis (Figs. 1B and 2A). This is consistent with the knowledge that cytochrome *c*, necessary in the cleavage and activation of caspase-9 (46), is released from mitochondria into the cytosol following LL-37 treatment (Fig. 5; ref. 32). However, in contrast to our findings, it was previously shown that caspase family members were not significantly activated following LL-37 treatment (31), which may be due to the use of different analytic methods for caspase activation. Our results show that caspase family members, although activated, are not required for LL-37–mediated cell death, in that multiple broad-spectrum caspase inhibitors had no effect on cell death, and caspase knockout cell lines displayed similar sensitivities to LL-37 compared with control cells. In addition, knockdown of caspase-independent AIF significantly blocked apoptosis (Fig. 6B).

AIF is a caspase-independent proapoptotic protein that is found in the intermembrane space of the mitochondria in healthy cells. However, it can be released into the cytosol following $\Delta\Psi_m$ where it may enter the nucleus, resulting in large-scale (~50 kb) DNA fragmentation and chromatin condensation (25). Caspase family members were once thought to be necessary for chromatin condensation and DNA fragmentation during apoptosis; however, caspase-independent proapoptotic proteins, such as AIF and endonuclease G, can also induce morphologic changes commonly associated with apoptosis (25, 47, 48). Following LL-37 exposure, both DNA fragmentation and chromatin condensation were observed by TUNEL and Hoechst staining, respectively (Fig. 1A and C). In addition, DNA fragmentation was observed in the presence of caspase inhibitors (Figs. 1B and 2A). Together, these results suggest that LL-37 treatment generates signature morphologic characteristics of apoptosis in a caspase-independent manner.

In this study, it was shown that Bax/Bak knockout and Bcl-2 overexpression significantly reduced apoptosis following

FIGURE 7. LL-37 requires intracellular Ca^{2+} and calpains to kill Jurkat cells. Bax translocation to mitochondria following LL-37 treatment depends on calpain activation. **A.** Jurkat cells (2×10^5 per treatment) were set up with BAPTA-AM (10 μmol/L), pepstatin A (25 μmol/L), calpain inhibitor I (1 μmol/L), and cathepsin B inhibitor (25 μmol/L) for 2 h. At this time, LL-37 (100 μg/mL) was added for 4 h at 37°C. Cells were then stained with TUNEL-PE (1 h, 37°C) and flow cytometric analysis was done. Columns, mean percent cell death ($n = 3$); bars, SD. *, $P < 0.01$; ***, $P < 0.001$, compared with LL-37 control. **B.** Jurkat cells (2×10^5 per treatment) were set up with BAPTA-AM (10 μmol/L), pepstatin A (25 μmol/L), calpain inhibitor I (1 μmol/L), and cathepsin B inhibitor (25 μmol/L) for 2 h. At this time, LL-37 (100 μg/mL) was added for 4 h at 37°C, stained with Annexin V-FITC and PI (30 min, room temperature), and analyzed by flow cytometry. Data are representative of three independent experiments. **C.** Jurkat cells (1×10^6 per treatment) were exposed to LL-37 (200 μg/mL) in the absence or presence of calpain inhibitor I (1 μmol/L, 2 h pretreatment) for 4 h at 37°C. Cellular fractions (cytosolic and mitochondrial) were isolated using the Qproteome cell compartment kit. Samples were boiled in SDS sample buffer and 20 μg of total protein were loaded into each well of a 10% SDS-polyacrylamide gel for separation by electrophoresis. Immunoblot was probed with rabbit anti-human Bax (N20; 1:1,000) and goat anti-rabbit horseradish peroxidase (1:3,000). Data are representative of three independent experiments.

LL-37 treatment (Fig. 3B and C), indicating that Bcl-2 family members are integral in LL-37–mediated apoptosis. In addition, the dissipation of $\Delta\Psi_m$ was clearly decreased in Bcl-2–overexpressing cells treated with LL-37 (Fig. 4). This is similar to human granulysin, another cationic antimicrobial peptide that induces apoptosis of Jurkat cells, resulting in the caspase-independent dissipation of $\Delta\Psi_m$, cytochrome *c* release, and AIF translocation to the nucleus that can be inhibited by Bcl-2 overexpression (49, 50). Overexpression of Bcl-2 is known to inhibit Bax conformational change, oligomerization, and mitochondrial translocation (51); therefore, Bcl-2 may have inhibited Bax activity at the mitochondrial membrane following LL-37 treatment. In this study, LL-37 exposure induced a pronounced dissipation of $\Delta\Psi_m$ (Fig. 4). Previous reports determined that Bax translocation and insertion into the mitochondrial membrane not only can lead to $\Delta\Psi_m$ dissipation but also can result in AIF release and AIF-mediated cell death (38, 52). Because Bax knockdown significantly reduced LL-37–mediated apoptosis, and AIF activity was necessary for LL-37–mediated killing (Fig. 6B), we hypothesized that LL-37 was inducing Bax translocation to mitochondria, where it can lead to $\Delta\Psi_m$ dissipation and AIF release. Using Western immunoblot analysis, it was determined that Bax indeed translocated to the mitochondria following LL-37 treatment (Fig. 7B). Further, a calpain-specific inhibitor, which was shown to block LL-37–mediated apoptosis (Fig. 7A), inhibited the mitochondrial translocation of Bax. This is consistent with a number of studies that determined that calpains can induce apoptosis through the activation of Bax, leading to dissipation of $\Delta\Psi_m$ and the release of AIF from mitochondria (38, 53). In addition, both calpains and proapoptotic Bcl-2 family members are required for significant AIF release in a number of model systems (22, 54). This is thought to be due to the direct cleavage of AIF by calpains, allowing for its release through the permeabilized organelle. One study did show that Bax was not sufficient to induce AIF release in the presence of high levels of the caspase inhibitor Z-VAD-fmk (35), and it was concluded that cytochrome *c* release and caspase family member activation were required upstream of AIF release. However, this study failed to analyze whether the high Z-VAD-fmk concentrations were having nonspecific inhibitory effects on other proteases such as calpains (55). Bax knockdown did not completely inhibit LL-37–mediated killing (Fig. 3C). This may be due to some residual Bax activity within the knockdown cells; however, it is also possible that other proapoptotic proteins may be involved in LL-37–mediated killing downstream of calpain activation.

Release of the proapoptotic lysosomal cathepsins is also known to induce Bax activation and mitochondrial destabilization following exposure to a variety of stimuli (56, 57). Therefore, the involvement of cathepsins, as well as calpains, was accessed following LL-37 exposure. Specific inhibitors of calpain, as well as the cell-permeable Ca^{2+}-chelating agent BAPTA-AM (58), significantly reduced LL-37–mediated apoptosis in Jurkat cells (Fig. 7A). Because Ca^{2+} is known to be required for calpain activation (35), these data suggest that Ca^{2+} and calpains are necessary intermediary molecules in LL-37–induced killing. In addition, cathepsin B inhibition significantly reduced LL-37–mediated DNA fragmentation, although to a lesser extent than calpain inhibition (Fig. 7A). Taken together, these results suggest that LL-37 may activate multiple intermediary molecules and affect multiple organelles during death induction. The mechanism(s) by which lysosomal permeabilization occurs following apoptosis induction is not completely understood. Bcl-2 proteins have been shown to contribute to this process, as Bax translocation to lysosomes followed by lysosomal permeabilization has recently been reported (59). Activated calpains have also been implicated in lysosomal permeabilization, leading to cathepsin release (60, 61). Therefore, it is possible that LL-37 triggers lysosomal permeabilization through the activation of calpains, as indicated by the result that calpain inhibition blocks both Bax translocation to mitochondria and apoptosis (Fig. 7B). It is also possible that, once activated, Bax leads to cathepsin release from lysosomes. Future studies will be required to determine the specific effects of LL-37 on various organelles affecting this process, including lysosomes.

As previously mentioned, the release of proapoptotic AIF from the mitochondrial inner membrane space and relocation to the nucleus was observed (Fig. 5). Therefore, the requirement for AIF activity during LL-37–induced apoptosis was assessed by using gene knockdown by specific siRNA. AIF knockdown significantly inhibited LL-37–mediated apoptosis (Fig. 6B and C), which confirms that AIF release from mitochondria is necessary for LL-37–mediated apoptosis. HeLa cells that are knocked out for AIF show a reduction in complex I activity (28); however, the HeLa cells in our experiments are knocked down for AIF, and therefore cells are not completely deficient in AIF and may still have low levels of functional complex I. We did not observe significant cell death due to AIF knockdown (as compared with vector control–transfected cells; data not shown).

Use of LL-37 in the treatment of cancer patients is beyond the scope of this article. However, it is believed that there are many ways in which the antitumorigenic activity of this peptide may be exploited. The possibilities include, but are not limited to, the use of liposomal delivery systems (36), previously shown to significantly reduce bystander cytotoxicity when peptide is delivered at high concentrations. Another potential mechanism of LL-37 administration *in vivo* may entail the removal of the NH_2-terminal hydrophobic residues of the peptide, resulting in less cytotoxicity toward eukaryotic cells (62). In conclusion, we have identified the specific mechanism of LL-37–mediated apoptosis, as outlined in Fig. 8. Briefly, LL-37 induces a Ca^{2+}/calpain–dependent pathway of apoptosis, resulting in a calpain-dependent Bax translocation to mitochondria. AIF release from mitochondria and entrance into the nucleus was observed. Clearly, the ability of LL-37 to induce apoptosis in this manner has clinical potential. For instance, LL-37 may induce death in tumors where the caspase pathway is blocked by molecules such as inhibitor of apoptosis proteins. In addition, it will be interesting to evaluate how the synthetic class of host defense peptides that are conceptually based on endogenous peptides, such as LL-37 (63), impinge on this pathway.

Materials and Methods

Cell Culture and Reagents

Jurkat human T leukemia cells were obtained from the American Type Culture Collection. Cells were maintained at

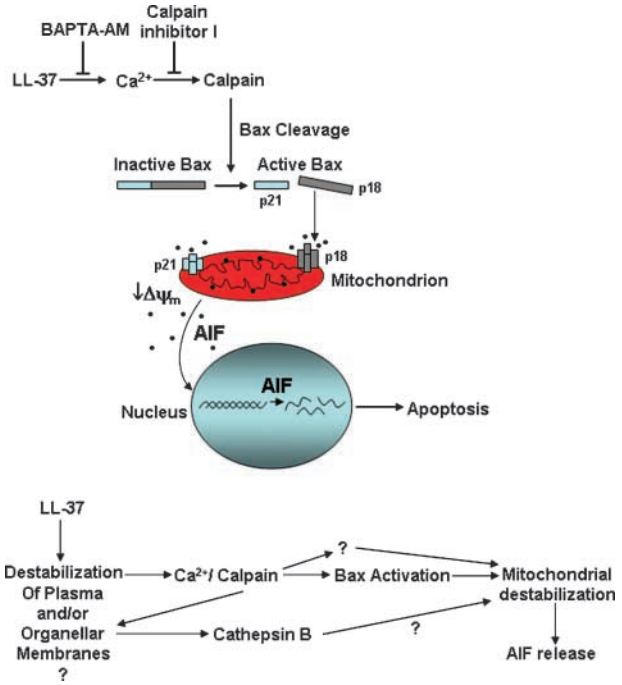

FIGURE 8. Proposed model of LL-37–induced apoptosis: LL-37 treatment results in increased Ca^{2+} levels, potentially through membrane-destabilizing effects. Ca^{2+}-dependent calpains mediate Bax translocation to mitochondria, where it may result in the dissipation of $\Delta\Psi_m$. Dissipation of $\Delta\Psi_m$ allows for the release of AIF into the cytosol and subsequent migration to the nucleus, where AIF triggers DNA fragmentation and chromatin condensation. Calpains may also lead to the release of cathepsin B from lysosomes, which can then induce apoptosis through a number of mechanisms.

37°C in a 5% CO_2 humidified atmosphere in RPMI 1640 (Sigma-Aldrich Canada) supplemented with 10% (v/v) heat-inactivated FCS (Hyclone), 100 μg/mL streptomycin, 100 units/mL penicillin, 2 mmol/L L-glutamine, and 5 mmol/L HEPES buffer (pH 7.4; Invitrogen). This will be now referred to as RHF. Jurkat stable transfectant lines were maintained in RHF supplemented with 0.8 mg/mL G418 (Invitrogen). These included cells overexpressing Bcl-2 (J-Bcl-2; ref. 64), dominant negative caspase-9 (J-DN-c9; ref. 65), and Spi-2 (66). FADD knockout and caspase-8 knockout cells were a gift from Dr. M. Barry (University of Alberta, Edmonton, Alberta, Canada), and C57Bl/6 MEFs were a gift from Dr. M. Michalak (University of Alberta, Edmonton, Alberta, Canada). MEFs that lack Bid ($Bid^{-/-}$) or Bax and Bak ($Bax/Bak^{-/-}$) were a gift from the late Dr. S.J. Korsmeyer (Department of Pathology and Medicine, Harvard Medical School, Cambridge, MA). Human cervical adenocarcinoma cells (HeLa) were obtained from American Type Culture Collection. MEFs and HeLa were maintained in DMEM with the same specifications as RHF. Stock flasks were passaged twice weekly or as required to maintain optimal cell growth. Synthetic LL-37 (amino acid sequence: NH_2-LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES-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). Scrambled LL-37 (NH_2-GLKLRFEFSKIKGEFLKTPEVRFR-DIKLKDNRISVQR-COOH) was synthesized in linear form by Innovagen. Lyophilized peptides were resuspended in endotoxin-free water (Sigma-Aldrich) and aliquots were stored at −20°C. Bovine serum albumin, paraformaldehyde, saponin, and Hoechst 33342 trihydrochloride dye were purchased from Sigma-Aldrich. Caspase inhibitors (Z-VAD-fmk and Q-VD-OPh) were purchased from Kamiya Biomedical Company. TUNEL *in situ* cell detection kits (TMR red) were purchased from Roche Diagnostics. Annexin V-FITC, PI, and 10× concentrated Annexin V binding buffer were from BD Biosciences. The CaspACE FITC-VAD-fmk *In Situ* Marker Assay Kit was from Promega. 3,3′-Dihexyloxacarbocyanine iodide ($DiOC_6$) and BAPTA-AM were purchased from Molecular Probes. Qproteome Cell Compartment kit and Qproteome mitochondria isolation kit were purchased from Qiagen. Mouse anti-human AIF, rabbit anti-human Bax (N20, total Bax), goat anti-human Hsp60 (N20), and mouse anti-human α-tubulin antibodies were purchased from Santa Cruz Biotechnology. Mouse anti-human Cox IV and mouse anti-human cytochrome *c* were purchased from Invitrogen. Purified mouse anti-human poly(ADP-ribose) polymerase antibody was purchased from BD Pharmingen. Horseradish peroxidase–conjugated goat anti-mouse, goat anti-rabbit, and rabbit anti-goat secondary antibodies were purchased from Bio-Rad. AIF ON-TARGET*plus* siRNA (PDCD8), ON-TARGET*plus* si*CONTROL* Non-targeting siRNA (negative control), and Dharma*FECT* transfection reagent 1 were from Dharmacon. Pepstatin A (cathepsin D inhibitor), [L-3-(propylcarbamyl)oxirane-2-carbonyl]-L-isoleucyl-L-proline (specific cathepsin B inhibitor), and Ac-LLnL-CHO (calpain inhibitor 1) were from Sigma-Aldrich.

DNA Fragmentation Assay

TUNEL assay was used to measure DNA fragmentation in cells undergoing cell death (67). Briefly, cells (2×10^5 per well) were exposed to media or LL-37 (25-200 μg/mL) in triplicate wells of a 96-well round-bottomed tissue culture plate (Becton Dickinson Labware) for designated time points at 37°C. At the end of the experimental time point, cells were resuspended in 2% paraformaldehyde in phosphate-buffered NaCl solution (NaCl, KCl, $Na_2HPO_4 \cdot 2H_2O$, and KH_2PO_4; PBS; overnight, 4°C). After 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-PE pre-mix and incubated for 1 h at 37°C. Cells were then washed once with immunofluorescence buffer, resuspended in immunofluorescence buffer (1% bovine serum albumin in PBS), and analyzed by flow cytometry.

Variations of this experiment were done. The addition of broad-spectrum caspase inhibitors (Z-VAD-fmk, 50 μmol/L or Q-VD-OPh, 20 μmol/L) 1 h before LL-37 addition was used to determine the requirement of caspase involvement in LL-37–mediated cell death. The addition of a cathepsin D inhibitor (pepstatin A, 25 μmol/L), a specific calpain inhibitor (1 μmol/L), a specific cathepsin B inhibitor (25 μmol/L), and the Ca^{2+} chelating agent BAPTA-AM (10 μmol/L) was done 2 h before LL-37 exposure to determine the requirement for Ca^{2+}, calpain and/or cathepsin involvement in LL-37–mediated apoptosis. Total cell counts were done 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).

Annexin V Staining

Annexin V staining was used to measure phosphatidylserine headgroup externalization on cells undergoing apoptosis. Jurkat cells (2×10^5 per well) were plated in triplicate in a 96-well round-bottomed plate and exposed to media, LL-37 (100 and 200 μg/mL), or staurosporine (2.5 μmol/L) for 4 h at 37°C. Following incubation, cells were washed thrice with PBS and resuspended in 15 μL of 1× binding buffer. Annexin V pre-mix was then added to each well for 30 min at room temperature. To distinguish apoptotic cells from necrotic cells, PI (1 μg/mL), which enters and stains leaky necrotic cells, was added in conjunction with Annexin V. Cells were then washed once with PBS and resuspended in 200 μL of 2% paraformaldehyde in PBS, and flow cytometric analysis was done.

Variations of this experiment was done. The addition of broad-spectrum caspase inhibitors (Z-VAD-fmk, 50 μmol/L or Q-VD-OPh, 20 μmol/L) 1 h before LL-37 addition was used to determine the requirement of caspase involvement in LL-37–mediated cell death. The addition of a cathepsin D inhibitor (pepstatin A, 25 μmol/L), a specific calpain inhibitor (1 μmol/L), a specific cathepsin B inhibitor (25 μmol/L), and the Ca^{2+} chelating agent BAPTA-AM (10 μmol/L) was done 2 h before LL-37 exposure to determine the requirement for Ca^{2+}, calpain and/or cathepsin involvement in LL-37–mediated apoptosis. Total cell counts were done before and following LL-37 treatment; cells were not lost due to washes or lysis in control or LL-37–treated samples (data not shown).

Hoechst Staining and Cell Morphology Analysis

Jurkat cells (5×10^4 per treatment) were treated with media, LL-37 (100 and 200 μg/mL; 37°C, 5% CO_2), or staurosporine (2.5 μmol/L) for 2 h. Cells were washed with PBS following LL-37 treatment, resuspended in 50 μL of 4% paraformaldehyde in PBS, and placed on microscope slides to air-dry overnight (Corning). Staining was then done for 10 min at room temperature with Hoechst 33342 trihydrochloride dye (10 μg/mL in PBS). Slides were washed four times with distilled water and allowed to air-dry in the dark. Chromatin condensation and nuclear fragmentation were then assessed by counting 500 cells from nonoverlapping fields by confocal microscopy. Total cell counts were done 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). For morphology analysis, cells were mounted on slides without additional staining and light microscopy was done.

Caspase Activity Assay

The CaspACE FITC-VAD-fmk *in situ* assay was done according to the manufacturer's protocol. Briefly, Jurkat cells (2×10^5 per well) were plated in triplicate in a 96-well round-bottomed plate and exposed to media or LL-37 (100 and 200 μg/mL) for 4 h at 37°C. Following incubation, cells were washed twice with PBS, and each sample of cells was resuspended in 100 μL of assay buffer (1:500 dilution of FITC-VAD-fmk in PBS) and incubated for 20 min in the dark at 37°C. The cell suspension was spun down, washed, resuspended in 500 μL PBS, and then evaluated immediately by flow cytometry.

Analysis of Mitochondrial Transmembrane Potential

Flow cytometric analysis of cells stained with $DiOC_6$ was used to measure changes in mitochondrial transmembrane potential (68). $DiOC_6$ was stored at −20°C as a 1 mmol/L stock in DMSO. Cells (2×10^5 per treatment) were exposed to media or LL-37 (100 and 200 μg/mL) for 4 h in triplicate wells of a 96-well round-bottomed tissue culture plate (37°C, 5% CO_2). $DiOC_6$ was then added for 30 min to untreated or LL-37–treated cells at a final concentration of 40 nmol/L. Cells were analyzed by flow cytometry using the FL1 channel. $DiOC_6$ enters healthy, intact mitochondria and is released into the cytosol on mitochondrial transmembrane potential loss. This is observed as a left-ward shift in fluorescence intensity.

Mitochondria Isolation

The isolation of intact mitochondria was done according to the manufacturer's protocol. Briefly, Jurkat T leukemia cells (2×10^7 per treatment) were centrifuged at 500 × *g* (10 min, 4°C), washed with 0.9% sodium chloride solution, and resuspended in 2 mL ice-cold lysis buffer (10 min, 4°C) on an end-over-end shaker. The lysate was then centrifuged at 1,000 × *g* (10 min, 4°C); the supernatant was removed; and the cell pellet was resuspended in 1.5 mL of ice-cold disruption buffer. Cell disruption was completed by using a blunt-ended needle and a syringe (10×). The lysate was centrifuged at 1,000 × *g* (10 min, 4°C); the supernatant was transferred to a clean 1.5-mL tube and centrifuged at 6,000 × *g* (10 min, 4°C). The supernatant was removed and the mitochondrial pellet was washed with Mitochondria Storage Buffer. The pellet was then centrifuged at 6,000 × *g* (20 min, 4°C), resuspended in Mitochondria Storage Buffer, and incubated in the absence or presence of LL-37 (100 μg/mL, 15 min). At this time, the samples were centrifuged at 6,000 × *g* (20 min, 4°C); mitochondrial pellets were resuspended in ice-cold lysis buffer (25 mmol/L Tris at pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 0.25% sodium doxycholate, and 0.1% SDS); and Western blot analysis was done on the supernatants and mitochondrial pellets (5 μg/well) as described below.

Immunoblotting

Cells (1×10^6) were exposed to media or LL-37 (200 μg/mL) for 2 h at 37°C in a 5% CO_2 humidified atmosphere and then washed in PBS. Jurkat cells were pretreated with calpain inhibitor (25 μmol/L) for 2 h before LL-37 exposure for Bax translocation analysis. For cellular compartment isolation, the total cell pellet was resuspended in extraction buffers provided with the Qproteome cell compartment isolation kit (Qiagen). Final cell pellets containing cytosolic, membrane, or nuclear proteins, as well as RNAi-transfected pellets, were resuspended in equal volumes of radioimmunoprecipitation assay buffer [10 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 2 mmol/L EDTA, 1% NP40, 0.5% Na-deoxycholate] and 2× SDS sample buffer and boiled for 5 min at 100°C. Protein concentrations were determined by the bicinchoninic acid protein assay (Pierce Biotechnology), and 20 μg of total protein were loaded into each well of a 10% SDS-polyacrylamide gel for separation by electrophoresis. Protein bands were transferred onto nitrocellulose membranes. The resulting blots were blocked for 1 h at room temperature with TBS-Tween (3 mol/L NaCl, 10 mol/L Tris-HCl, 0.1%

Tween 20) containing 5% powdered skim milk and probed overnight with specific primary antibodies including mouse anti-AIF antibody (1:1,000 dilution), rabbit anti-Bax (N20, 1:1,000), mouse anti–Cox IV (1:2,000), and mouse anti–cytochrome *c* (1:1,000). Protein loading was assessed using anti–α-tubulin (1:1,000), mitochondria-associated anti-Hsp60 (1:1,000), and nuclear-specific anti–poly(ADP-ribose) polymerase (1:1,000). Blots were washed with TBS-Tween 20 and probed for 1 h with horseradish peroxidase–conjugated antimouse or antirabbit IgG at 1:3,000 dilution. Following additional washes with TBS-Tween, the protein bands were visualized using an ECL detection system (Bio-Rad Laboratories Ltd.).

Transfection of HeLa Cells with AIF SiRNA

Human cervical epithelial adenocarcinoma cells (HeLa) were transfected with AIF ON-TARGET*plus* siRNA or ON-TARGET*plus* si*CONTROL* nontargeting siRNA (negative control) using Dharma*FECT* transfection reagent 1 according to the manufacturer's protocol. Briefly, HeLa cells (2.5×10^5 per well) were plated into multiple wells of a 12-well tissue culture plate and grown overnight (37°C, 5% CO_2). AIF and nontargeting siRNA solutions were diluted to a 2 μmol/L working concentration in 1× siRNA buffer. In separate tubes, 2 μmol/L siRNA (tube 1; 50 μL) and Dharma*FECT* reagent 1 (tube 2; 3 μL) were diluted with serum-free medium (total volume of 100 μL/tube). The contents of each tube were mixed gently by pipetting up and down and incubated for 5 min at room temperature. The contents of tube 1 were then added to tube 2, mixed by pipetting, and incubated for 20 min at room temperature. At this time, antibiotic-free complete medium was added to the mix for the desired transfection volume of 1 mL. Culture medium was removed from the wells of the 12-well plate and 1 mL of the appropriate transfection mix was added to each well. Cells were incubated at 37°C in 5% CO_2 for 72 and 96 h (for protein analysis).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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