The Antimicrobial Peptide LL-37 Alters Human Osteoblast Ca Handling and Induces Ca(2+)-Independent Apoptosis.

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The antimicrobial peptide LL-37 alters human osteoblast Ca^{2+} handling and induces Ca^{2+}-independent apoptosis

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Short title: **LL-37-induced apoptosis and Ca^{2+} signaling**

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Abstract

The human antimicrobial peptide cathelicidin LL-37 has, besides its antimicrobial properties, also been shown to regulate apoptosis in a cell type specific manner. Mechanisms involved in LL-37-regulated apoptotic signaling are not identified. Here, we show that LL-37 reduces human osteoblast-like MG63 cell number and cell viability in the µM concentration-range with an IC_{50} value of about 5 µM. Treatment with 4 µM LL-37 increased the number of Annexin V positive cells and stimulated activation of caspase 3 showing that LL-37 promotes apoptosis. Treatment with 4 µM LL-37 caused an acute and sustained rise in intracellular Ca^{2+} concentration assessed by laser-scanning confocal microscopy of Fluo 4-AM loaded MG63 cells. LL-37 increased Ca^{2+} also in the presence of the respective L and T-type voltage-sensitive Ca^{2+} channel blockers nifedipine and $NiCl_2$. LL-37 had no effect on Ca^{2+} in cells incubated with Ca^{2+}-free solution. LL-37 (4 and 8 µM) reduced MG63 cell number both in the presence and absence of Ca^{2+} in the medium. In conclusion, LL-37 reduces osteoblast cell number by promoting apoptosis, and furthermore, LL-37 stimulates Ca^{2+} inflow via a mechanism independent of voltage-sensitive Ca^{2+} channels. Interestingly, LL-37-induced lowering of cell number seems to be mediated via a mechanism independent of Ca^{2+}.

Key words: Annexin V; Apoptosis; Calcium; Caspase 3; LL-37; Osteoblast

Introduction

The human antimicrobial peptide cathelicidin LL-37 is stored as the proprotein form hCAP-18 in neutrophils and epithelial cells until activated by the serine protease proteinase 3 and subsequently released [1, 2]. LL-37 is an amphiphilic α-helical cationic peptide possessing hydrophobic as well as hydrophilic properties [3]. LL-37 exerts a direct antimicrobial activity through disrupting the cell wall of both gram-negative and gram-positive bacteria causing bacterial cell lysis and by neutralizing lipopolysaccharide [4-6]. The cationic LL-37 molecule binds to negatively charged microbial membrane lipids thereby showing membrane selectivity [3]. Furthermore, LL-37 potentiates chemokine production by microbial stimuli in keratinocytes and other epithelial cells, suggesting that it enhances the immune defense of the skin [7].

LL-37 has, besides its antimicrobial and immune modulator activities, also been shown to affect various cellular functions, such as phagocytosis [8], cell differentiation [9] and apoptosis [10-16]. Importantly, the effects of LL-37 on apoptosis seem to be cell type specific; LL-37 promotes apoptosis in vascular smooth muscle cells [10], periodontal ligament cells [11], neutrophils [12], T cells [13] and airway epithelium [14] but suppresses apoptosis in keratinocytes [15] and dermal fibroblasts [16]. For neutrophils, LL-37 has been reported to exert both pro- and anti-apoptotic effects [12, 17].

In periodontitis, which is a progressive inflammatory disease, the end-stage is characterized by destruction of the alveolar bone leading to loss of teeth. Interestingly, the LL-37

concentrations are high (μM) in the gingival crevicular fluid from patients suffering from chronic periodontitis [18], suggesting that this peptide may influence the disease process. The bone forming osteoblasts represent a very important cell type in both periodontal health and disease responsible for maintaining the alveolar bone mass [19].

The signaling pathways and mechanisms involved in LL-37 regulated apoptosis are not completely understood [3]. The Ca^{2+} ion is thought to play a role in cell killing and apoptosis and dysfunctional regulation of Ca^{2+} homeostasis can result in apoptosis [20, 21], suggesting that Ca^{2+} may be involved in LL-37-induced pro-apoptotic signaling. LL-37 has been reported to interact with different cellular proteins such as the purinergic $P2X_7$ receptor [17, 22] and the epidermal growth factor receptor [23-25], but LL-37 interacts also with DNA [26]. In the present study, we investigate effects of LL-37 on human osteoblast-like MG63 cell viability and Ca^{2+} signaling demonstrating that LL-37 induces apoptosis also in this cell type. Additionally, we demonstrate that LL-37 causes a rapid and sustained rise in the intracellular Ca^{2+} concentration through inflow of Ca^{2+} from the extracellular space, suggesting that LL-37 alters Ca^{2+} handling. Interestingly, the LL-37-induced attenuation of osteoblast cell number and cell viability seems to be mediated through a mechanism independent of Ca^{2+}.

Materials and methods

Cell culture and experimental procedure

The human osteoblast-like MG63 cell line and the primary human osteoblast cell line hFOB 1.19 from American Type Tissue Culture Collection (ATCC, Manassas, VA, USA) were cultured in DMEM/Ham's F12 (1:1) cell culture medium (Life Technologies, Invitrogen, Carlsbad, CA, USA) supplemented with antibiotics (penicillin 50 U/ml, streptomycin 50 µg/ml) and 10% fetal calf serum in accordance with instructions from ATCC. The cells were kept in a water-jacketed cell incubator at 37 °C under 5% CO_2 in air. The MG63 cells express markers for osteogenic differentiation such as alkaline phosphatase (ALP) enzyme activity and form mineralized nodules showing that they are representative for native osteoblasts [27, 28]. The cells were trypsinized (0.25% trypsin/EDTA) and reseeded upon reaching confluence. Medium was exchanged every second day. Experiments were performed on sub-confluent cells (80% confluence). The cells were used for experiments in passages 3-10. Incubation with LL-37 caused similar effects irrespective of passage number. Before experiments fetal calf serum was omitted in order to standardize the experimental conditions. For experiments assessing the importance of Ca^{2+} for LL-37-induced effects on cell number, MG63 cells were incubated in either DMEM cell culture medium containing 1.8 mM Ca^{2+} or Ca^{2+}-free DMEM medium, both from Life Technologies. The Ca^{2+}-free DMEM medium was further supplemented with 1.8 mM $MgCl_2$ to achieve iso-osmolar conditions and to supplement with divalent cations to compensate for those lost by the omission of Ca^{2+}. In order to achieve culture conditions with low Ca^{2+} concentration, the Ca^{2+} containing DMEM medium was supplemented with 1.8 mM of the Ca^{2+} chelator ethylene glycol-bis(β-

aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA, Sigma Chemicals, St Louis, MO, USA).

Assessment of cell number and cell viability

Number of cells was calculated in a Bürker chamber as appropriate. Cell viability was assessed using trypan blue exclusion test. The cells were incubated for 2 min with 0.4% trypan blue (Sigma) and then washed three times in 0.9% NaCl. Cells containing trypan blue was counted as a measure of dead/dying cells. Cell morphology was assessed in digital photographs from a Nikon TMS microscope equipped with digital camera (Pixelink, Nikon, Nikon Nordic AB, Solna, Sweden).

Determination of active caspase 3

After washing in phosphate buffered saline (PBS), cellular proteins were extracted using a protein extraction buffer provided in the active caspase 3 (aCasp-3) ELISA kit from R&D (R&D Systems, Minneapolis, MN, USA). The amount of active caspase 3 was determined by ELISA according to manufacturer's instructions, and normalized to total protein in each sample measured by a Bio-Rad protein assay kit (BioRad, Hercules, CA, USA). Each sample was analyzed in duplicate.

Annexin V flow cytometry

Apoptosis was assessed by flow cytometry using Annexin V staining. Cells were washed with PBS and then incubated with FITC-labeled Annexin V and the fluorescent viability dye 7-

aminoactinomycin D (7-AAD) using FITC Annexin V apoptosis detection kit 1 (BD, Franklin Lakes, NJ, USA) according to manufacturer's instructions. The proportion of Annexin V positive/negative and 7-AAD positive/negative cells was determined by flow cytometry using an Accuri C6 flow cytometer (BD). Cells in early stages of apoptosis are Annexin V positive and 7-AAD negative, whereas cells in later stages of apoptosis and necrosis stain positive for both Annexin V and 7-AAD. Gates for Annexin V and 7-AAD positive cells were set using fluorescence minus one control. 20 000 events were recorded in each sample.

Measurement of intracellular Ca^{2+} concentration

For determination of intracellular Ca^{2+} concentration, the cells were cultured on glass bottom cell culture Petri dishes (MatTek, Ashland, MA, USA). The cells were washed with PBS, incubated with the Ca^{2+} sensitive fluorescent dye Fluo 4-AM (3 µM, Invitrogen) for 40 min at room temperature, and then washed carefully. During the Ca^{2+} measurements the cells were incubated in a HEPES buffered salt solution containing 2.5 mM Ca^{2+}. This Ca^{2+} concentration is somewhat higher than the plasma concentration of Ca^{2+} in healthy adults. We used HEPES buffered salt solution with 2.5 mM Ca^{2+} only in these acute experiments. The composition of the HEPES buffered salt solution was (mM): NaCl 135.5, KCl 5.9, $CaCl_2$ 2.5, $MgCl_2$ 1.2, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES) 11.6 and glucose 11.5. In some experiments measurement of Ca^{2+} was performed under Ca^{2+}-free conditions, i.e. in HEPES buffered salt solution without $CaCl_2$ and with addition of 2 mM EGTA (Sigma). The experiments were performed at room temperature. Fluorescence was recorded using a laser-scanning confocal microscope (LSM 510 PASCAL, Carl Zeiss AG, Göttingen, Germany). The excitation and emission wavelengths were 488 and 505 nm, respectively. The Ca^{2+} measurements were performed on sub-confluent (80% confluence) cells as an integrated

signal from all cells (about 300 cells) within the visual field at x100 magnification. The confocal pinhole setting was kept identical for all experiments.

Drugs

LL-37 was purchased from Bachem AG (Bubendorf, Switzerland) and dissolved in dimethyl sulfoxide (DMSO) according to manufacturer's instructions. The L-type Ca^{2+} channel blocker nifedipine (Sigma), the thromboxane A2 analogue U46619 (Tocris Bioscience, Bristol, United Kingdom) and the $P2X_7$ receptor antagonist AZ 11645373 (Tocris) were dissolved in DMSO. The controls received vehicle, DMSO, as appropriate. The final concentration of DMSO was 0.1%.

Statistics

Values are presented as means ± S.E.M. Statistical significance was calculated using ANOVA and Student's two-tailed t-test for unpaired comparisons with Bonferroni correction for post hoc analysis as appropriate. P values less than 0.05 were regarded to denote statistical significance.

Results

LL-37 attenuates MG63 cell number and cell viability

Treatment with LL-37 for 24 h reduced the number of human MG63 osteoblasts by 40 and 75% at 4 and 8 µM, respectively (Fig. 1a). Lower concentrations of LL-37 (0.1-2 µM) had no effect on the number of cells (Fig. 1a). LL-37 caused a concentration-dependent decrease in cell number with an IC_{50} value of about 5 µM. Treatment with 4 µM LL-37 for 1 and 3 days reduced MG63 cell number by about 40% (Fig. 1b). A lower concentration (0.1 µM) of LL-37 had no effect on cell number neither at 1 nor 3 days of treatment (Fig. 1b). Staining with trypan blue, assessing cell viability, showed that treatment with LL-37 (8 µM) for 24 h reduced cell number and caused alteration of cell morphology characterized by shrinkage of the cells representing classical signs of apoptosis (Fig. 2). Furthermore, almost all cells remaining after treatment with 8 µM LL-37 contained trypan blue indicating that they represent dead/dying cells (Fig. 2). No trypan blue positive cells were observed in response to a lower concentration (1 µM) of LL-37 (Fig. 2). The positive trypan blue staining observed in response to 8 µM LL-37 in MG63 cells was confirmed also in another human osteoblast cell line, hFOB 1.19 cells (Fig. 3), demonstrating that µM concentrations of LL-37 reduce cell viability in two different human osteoblast cell lines.

LL-37 increases the number of Annexin V positive cells and elevates active caspase 3 in MG63 cells

Treatment with LL-37 (4 µM) for 6 h increased the proportion of Annexin V positive MG63 cells by about 70% demonstrated by flow cytometry (Fig. 4a). The well-known pro-apoptotic

agent staurosporine (0.5 µM) was included as a positive control [29]. Treatment with 0.5 µM staurosporine for 6 h increased the number of Annexin V positive cells by about 9 times (Fig. 4a). Staurosporine (0.5 µM) but not LL-37 (4 µM) increased the proportion of Annexin V positive cells also at a shorter time-point, i.e. 2 h (Fig. 4a). Stimulation with LL-37 (4 µM) for 24 h increased the amount of active caspase 3 between 2 and 3 times in MG63 cells (Fig. 4b). The positive control staurosporine (0.5 µM) increased active caspase 3 by about 2-fold (Fig. 4b). Taken together these data show that LL-37 is pro-apoptotic for osteoblasts.

LL-37 increases MG63 intracellular Ca^{2+} concentration

Defective cellular Ca^{2+} handling is thought to be involved in pro-apoptotic signaling [21], and therefore we assessed the effects of LL-37 on the intracellular Ca^{2+} concentration. Treatment with LL-37 (4 µM) caused an acute (within about 60 s) and sustained rise in intracellular Ca^{2+} concentration demonstrated by laser-scanning confocal microscopy of Fluo 4-AM labeled MG63 cells incubated in Ca^{2+} containing (2.5 mM) HEPES-buffered salt solution (Figs. 5a and b). A lower concentration of LL-37 (0.4 µM) also elevated Ca^{2+} but the Ca^{2+} response to 0.4 µM LL-37 was small compared to that of 4 µM (Fig. 5b). Addition of vehicle control (0.1% DMSO) had no effect on Ca^{2+} (Fig. 5a). LL-37 (0.4 and 4 µM) had no effect on intracellular Ca^{2+} concentration in Ca^{2+}-free solution (Fig. 5c), suggesting that the LL-37-induced increase in Ca^{2+} depends on inflow of extracellular Ca^{2+}. Treatment with 100 nM and 1 µM of the L-type voltage-sensitive Ca^{2+} channel blocker nifedipine had no effect on the LL-37-induced Ca^{2+} response (4 µM LL-37), suggesting that LL-37 acts via another mechanism than by stimulation of Ca^{2+} inflow through L-type Ca^{2+} channels (Figs. 6 and 7a). The T-type Ca^{2+} channel blocker $NiCl_2$ (100 µM) had no effect on the LL-37-induced (4 µM) Ca^{2+} response (Fig. 7b), suggesting that inflow of Ca^{2+} by LL-37 is mediated through another

pathway than via T-type Ca^{2+} channels. Furthermore, inclusion of the $P2X_7$ receptor antagonist AZ 11645373 (10 µM) had no impact on the rise in Ca^{2+} evoked by 4 µM LL-37 (Fig. 7c). The thromboxane A2 analogue U46619 was included as positive control causing a rapid and powerful rise in intracellular Ca^{2+} concentration (Fig. 7d).

LL-37 reduces MG63 cell number both in the presence and absence of extracellular Ca^{2+}

Treatment with LL-37 (8 µM) for 24 h reduced the number of MG63 cells by about 55% for cells cultured in DMEM culture medium containing 1.8 mM Ca^{2+} and by about 85% for cells cultured in Ca^{2+}-free DMEM culture medium (Fig. 8a). In fact, the LL-37-induced reduction of cell number was more powerful ($P<0.01$) in the absence than in the presence of extracellular Ca^{2+} (Fig. 8a). A lower concentration of LL-37 (4 µM) reduced cell cumber by about 15% in the presence (86 ± 2% for LL-37-treated cells vs. 100 ± 5% for control cells, $P<0.05$, n=3 in each group) and by about 35% in the absence (66 ± 7% for LL-37-treated cells vs. 100 ± 16% for control cells, $P<0.05$, n=3 in each group) of extracellular Ca^{2+}. Omitting Ca^{2+} for 24 h reduced slightly, but not significantly, the number of cells as demonstrated when cell-counts are plotted as absolute data (Fig. 8b). The MG63 cells showed similar morphology in the presence and absence of Ca^{2+} (Fig. 8c). Treatment with LL-37 (4 µM) for 24 h attenuated cell number by about 60% when the Ca^{2+} chelator agent EGTA (1.8 mM) was included in the Ca^{2+}-containing DMEM culture medium in order to lower Ca^{2+} (Fig. 9). LL-37 (4 µM) reduced cell number by about 60% also when extra Ca^{2+} (final Ca^{2+} concentration 3.6 mM) was included in the EGTA containing medium (Fig. 9). For these experiments EGTA was administered in an equimolar concentration to extracellular Ca^{2+} (1.8 mM) in order to bind most of the Ca^{2+} ions. Thus, LL-37-induced lowering of osteoblast cell number is

observed both when the cells are cultured under Ca^{2+}-free conditions and when Ca^{2+} ions are absorbed with EGTA.

Discussion

In the present study, we demonstrate that the human antimicrobial peptide LL-37 induces apoptosis in human osteoblast-like MG63 cells and that this effect is associated with elevated intracellular Ca^{2+} concentration. LL-37 causes, in the µM concentration range, an acute and sustained rise in intracellular Ca^{2+} in Ca^{2+}-containing but not in Ca^{2+}-free solution showing that the LL-37-induced increase in intracellular Ca^{2+} is due to an inflow of Ca^{2+} along its gradient from the extracellular space to the cytosol. The increase in Ca^{2+} by LL-37 was much stronger at 4 than at 0.4 µM, suggesting a concentration-dependent effect, although it is difficult to conclude firmly the concentration-dependence since we have investigated only two concentrations of LL-37. Membrane depolarization results in an inflow of Ca^{2+} from the extracellular space causing a sustained and long-lasting increase in intracellular Ca^{2+} [30] similar to that observed in response to LL-37 in the present study. The pattern of LL-37-induced rise in intracellular Ca^{2+} concentration is thus compatible with inflow of Ca^{2+} from the extracellular space. Osteoblasts (MC3T3-E1 cells and MG63 cells) have been reported to express voltage-sensitive Ca^{2+} channels [31, 32]. Our data show that neither the L-type Ca^{2+} channel blocker nifedipine nor the T-type Ca^{2+} channel blocker $NiCl_2$ have impact on the LL-37 evoked rise in Ca^{2+} in MG63 cells, suggesting that the inflow of Ca^{2+} triggered by LL-37 is through another mechanism than via voltage-sensitive L-type and T-type Ca^{2+} channels. We used relevant concentrations of nifedipine (0.1 and 1 µM), which fully inhibit inflow of Ca^{2+} via L-type Ca^{2+}channels in cultured vascular smooth muscle cells [30], and $NiCl_2$ (100 µM) inhibiting T-type but not L-type Ca^{2+} channels [33]. Furthermore, inclusion of the selective and highly potent $P2X_7$ receptor antagonist AZ 11645373 [34] had no effect on the rise in Ca^{2+} evoked by LL-37, suggesting an alternative mechanism. Based on our findings presented

here, we suggest that the LL-37-induced inflow of extracellular Ca^{2+} represents a novel signaling pathway for LL-37 that may involve LL-37-induced permeabilization of the cell-membrane causing formation of trans-membrane pores and/or that LL-37 acts as a detergent. These data implicate that LL-37 may work through a similar mechanism in human osteoblasts as in LL-37-induced permeabilization of the bacterial cell wall [3].

Here, we show for the first time that the antimicrobial peptide LL-37 reduces human MG63 osteoblast cell number by promoting apoptosis. LL-37-induced apoptosis was demonstrated by both enhanced proportion of Annexin V positive cells and by elevated active caspase 3 level in response to LL-37-treatment. Furthermore, LL-37-treated cells showed altered morphology such as cell shrinkage representing a classical sign of apoptosis. Previously, pro-apoptotic effects of LL-37 have been demonstrated in vascular smooth muscle cells, periodontal ligament cells, neutrophils, T cells and airway epithelial cells [10-14]. We demonstrate reduction of osteoblast cell number by LL-37 in the µM concentration-range, while no effect on cell number is observed at lower concentrations of LL-37. We have previously shown that LL-37 reduces lipopolysaccharide-induced MCP-1 and IL-6 expression at low concentrations (0.1 and 1 µM) and induces apoptosis only at high (>5 µM) concentrations in human periodontal ligament cells [11]. LL-37-induced pro-apoptosis is thus observed in different cell systems but only in the µM concentration-range. Very high levels of LL-37 have been demonstrated in lesional tissue from patients suffering from autoimmune diseases such as psoriasis, rosacea and ulcerative colitis [35-37]. For example, the median concentration of LL-37 is 304 µM in psoriatic skin lesions [35]. The LL-37 levels are elevated locally in chronic periodontitis, in fact they are well within the µM concentration-range [18], suggesting that LL-37-induced apoptosis of osteoblasts may have impact on alveolar bone

homeostasis in patients suffering from this disease. Thus, we may conclude that the LL-37-induced apoptosis of osteoblasts, observed in the µM concentration-range in the present study, is relevant for the in-vivo situation considering the very high levels of LL-37 observed in various autoimmune and inflammatory diseases.

Ca^{2+} governs many important cellular processes and is thought to be involved in apoptosis [21, 38]. A rise in intracellular Ca^{2+} may originate from intracellular stores such as the endoplasmic reticulum, but also from the extracellular space [21]. Here, we show that LL-37 elevates intracellular Ca^{2+} concentration in human MG63 osteoblasts through stimulation of Ca^{2+} inflow. The LL-37 evoked inflow of Ca^{2+} seems not to be critically important for the LL-37-induced attenuation of MG63 cell number, since LL-37 lowers cell number both in the presence and absence of Ca^{2+} in the cell culture medium and furthermore, LL-37 causes a rise in intracellular Ca^{2+} but no apoptosis at a low concentration (0.4 µM). Ca^{2+}-independent apoptosis has been described in different experimental systems [39, 40]. Interestingly, the LL-37 evoked inward flow of Ca^{2+}, occurring independent of LL-37-induced apoptosis, may represent an important mechanism regulating Ca^{2+}-dependent cellular processes governed by LL-37. Thus, we demonstrate here that LL-37 alters human osteoblast Ca^{2+} handling and induces Ca^{2+}-independent apoptosis.

In summary, we show that LL-37 alters cellular Ca^{2+} homeostasis by causing stimulation of Ca^{2+} inflow through a voltage-sensitive L- and T-type Ca^{2+} channel independent mechanism in human osteoblasts. Furthermore, we demonstrate that LL-37 induces apoptosis of osteoblasts via a mechanism that is independent of Ca^{2+}.

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Figure legends

Fig. 1. (a) Treatment with LL-37 for 24 h reduces MG63 osteoblast cell number in a concentration-dependent manner. LL-37 at 4 and 8 µM reduces osteoblast cell number by 40 and 75%, respectively, while lower concentrations of LL-37 lack effect. **(b)** Treatment with 4 µM LL-37 for 1 and 3 days reduces MG63 cell number by about 40%. A lower concentration (0.1 µM) of LL-37 has no effect on cell number neither at 1 nor 3 days of treatment. Values are means ± SEM of 4-15 observations in each group. *, ** and *** represent $P<0.05$, $P<0.01$ and $P<0.001$, respectively, compared to controls (ctrl).

Fig. 2. Staining with trypan blue, assessing cell viability, shows that treatment with 8 µM LL-37 for 24 h reduces MG63 cell number and causes alteration of cell morphology characterized by shrinkage of the cells. Furthermore, nearly all LL-37-treated cells contain trypan blue indicating that these cells represent dead/dying cells. No trypan blue positive cells were observed in response to a lower concentration (1 µM) of LL-37. Control cells (ctrl) show normal morphology with no trypan blue positive cells. Bars represent 25 µm.

Fig. 3. Treatment with LL-37 (8 µM) for 24 h causes accumulation of trypan blue in primary human osteoblast hFOB 1.19 cells. Control cells (ctrl) show no trypan blue positive cells. Trypan blue positive cells represent dead/dying cells. Bars represent 20 µm.

Fig. 4. (a) Treatment with 4 µM LL-37 for 6 h increases the proportion of apoptotic MG63 cells. Apoptosis was determined by flow cytometric analysis of Annexin V positive cells. Staurosporine (0.5 µM) was included as positive control. **(b)** Treatment with 4 µM LL-37 for 24 h increases MG63 cellular active caspase 3 (aCasp-3) level 2 to 3 times. The amount of aCasp-3 was determined by ELISA and normalized to total protein in each sample. Values are means ± SEM of 3-4 observations in each group. *, ** and *** represent $P<0.05$, $P<0.01$ and $P<0.001$, respectively, compared to controls (ctrl). N.S. = not significant.

Fig. 5. (a) Treatment with LL-37 (4 µM) causes an acute and sustained rise in intracellular Ca^{2+} concentration assessed by laser-scanning confocal microscopy of Fluo 4-AM loaded MG63 cells incubated in Ca^{2+} containing (2.5 mM) HEPES-buffered salt solution. No treatment (left panel) represents the Ca^{2+} signal in the presence of vehicle control (0.1% DMSO). Addition of 0.1% DMSO has no effect on Ca^{2+}. The Ca^{2+} indicator Fluo 4-AM fluorescence is shown in red. **(b)** Line trace showing that both 0.4 and 4 µM LL-37 elevates Ca^{2+}. **(c)** LL-37 (0.4 and 4 µM) has no effect on intracellular Ca^{2+} concentration in Ca^{2+}-free solution. Ca^{2+}-free conditions were achieved by removing $CaCl_2$ from the HEPES-buffered salt solution and by inclusion of 2 mM EGTA. Each experiment was repeated at least twice.

Fig. 6. LL-37 (4 µM) increases MG63 intracellular Ca^{2+} concentration monitored by laser-scanning confocal microscopy of Fluo 4-AM loaded cells in the presence of L-type Ca^{2+} channel blocker nifedipine (100 nM). Nifedipine was included at the arrow and present throughout the experiment. This trace shows one representative experiment out of two.

Fig. 7. Treatment with **(a)** 1 µM nifedipine, **(b)** 100 µM $NiCl_2$ and **(c)** 10 µM AZ11645373 has no effect on the LL-37-induced (4 µM) Ca^{2+} response in MG63 cells. **(d)** The thromboxane A2 analogue U46619 (10 µM) was included as positive control causing a rapid and powerful rise in intracellular Ca^{2+} concentration. The intracellular Ca^{2+} concentration was monitored by laser-scanning confocal microscopy of Fluo 4-AM loaded MG63 cells. Nifedipine, $NiCl_2$ and AZ11645373 were included at the arrow and present throughout the experiment. Each experiment was repeated at least twice.

Fig. 8. (a) Treatment with 8 µM LL-37 for 24 h reduces MG63 cell number by about 55% for cells cultured in DMEM culture medium containing 1.8 mM Ca^{2+} and by 85% for cells cultured in Ca^{2+}-free DMEM culture medium. **(b)** Omitting Ca^{2+} for 24 h reduces slightly, but not significantly, the number of cells as demonstrated when cell-count data are plotted as absolute data. **(c)** The MG63 cells show similar morphology in the presence and absence of Ca^{2+}. Bars in panel c represent 20 µm. Values are means ± SEM of 4-5 observations in each group. * and *** represent $P<0.05$ and $P<0.001$ compared to controls (ctrl) and ** represents $P<0.01$ for LL-37-treated groups as indicated.

Fig. 9. Treatment with 4 µM LL-37 for 24 h reduces cell number by about 60% for cells grown in culture medium (DMEM + 1.8 mM Ca^{2+}) containing either 1.8 mM EGTA alone or 1.8 mM EGTA in combination with Ca^{2+} in excess (3.6 mM Ca^{2+}). Values are means ± SEM of 3 observations in each group. ** represents $P<0.01$ compared to controls (ctrl).

Fig. 1

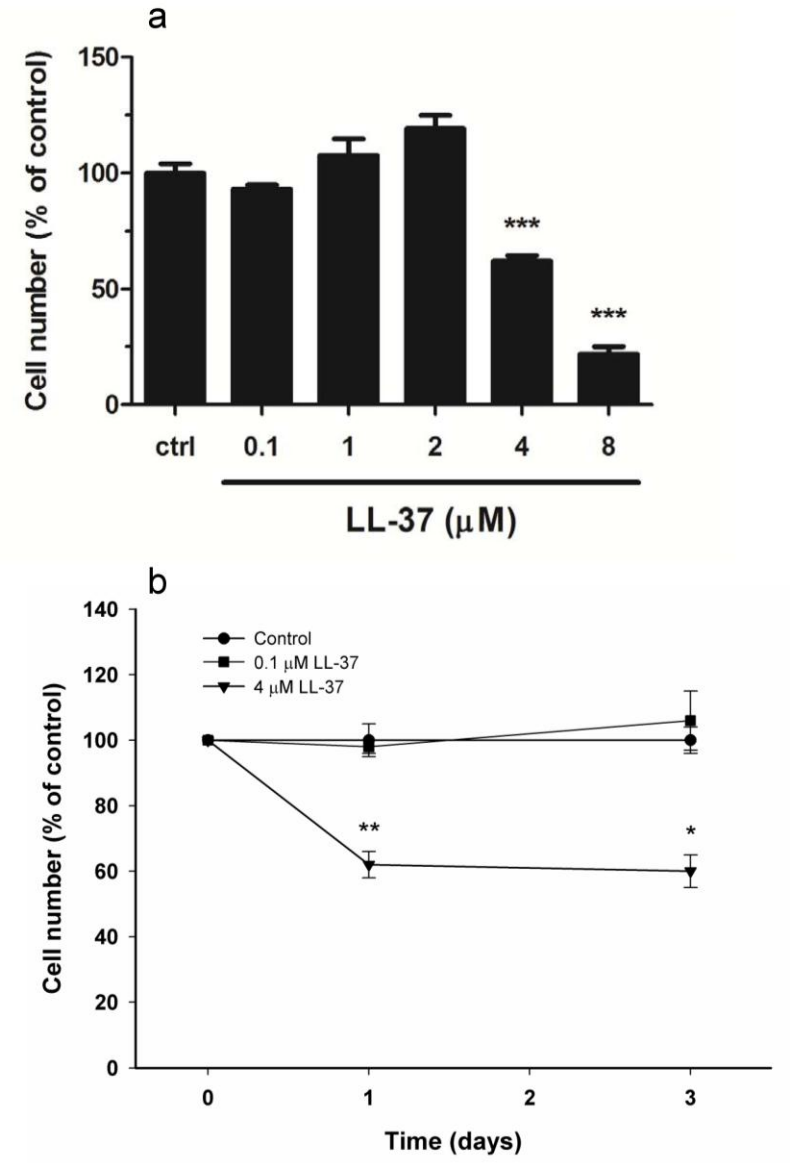

a
Cell number (% of control)
150
100
50
0
***
***
ctrl
0.1
1
2
4
8
LL-37 (μM)
b
Cell number (% of control)
140
120
100
80
60
40
20
0
Control
0.1 μM LL-37
4 μM LL-37
**
*
0
1
2
3
Time (days)

Fig. 2

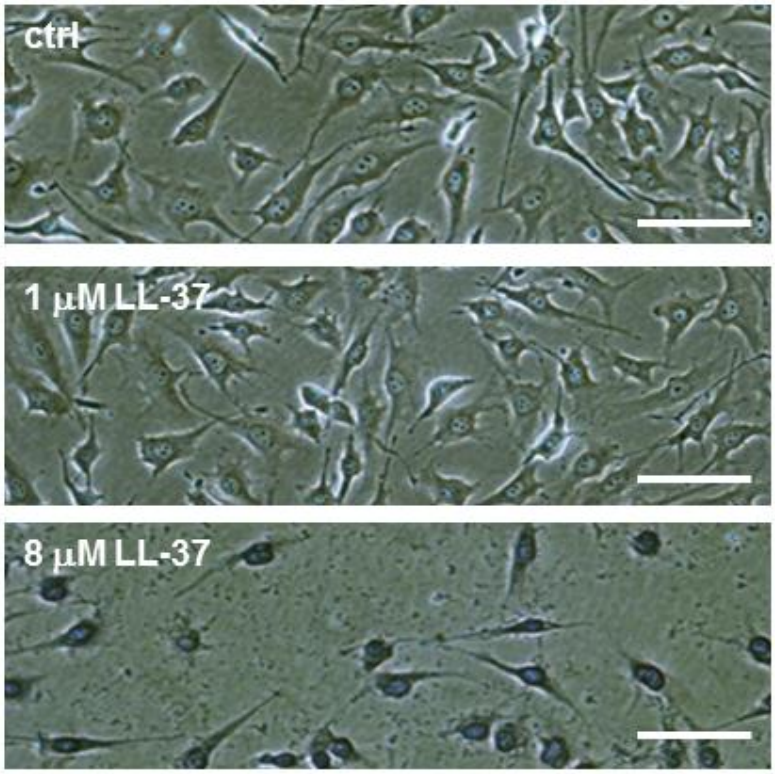


Fig. 3

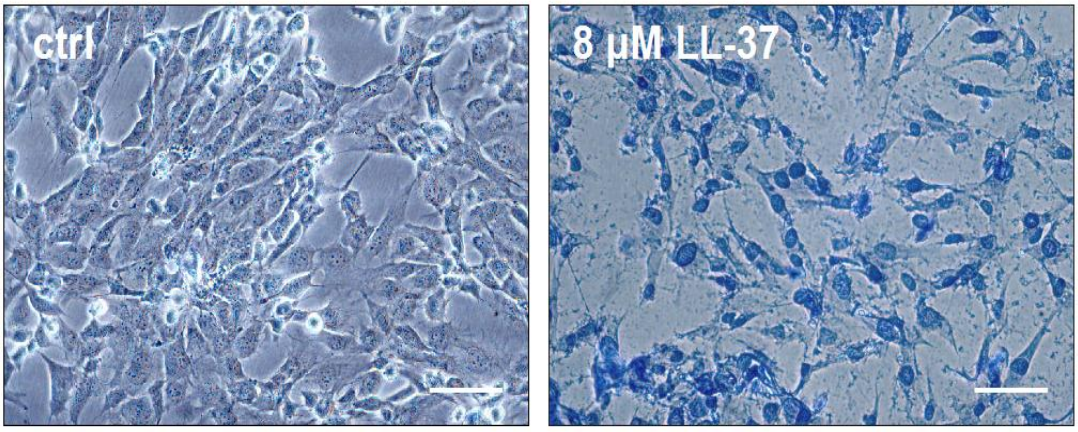


Fig. 4

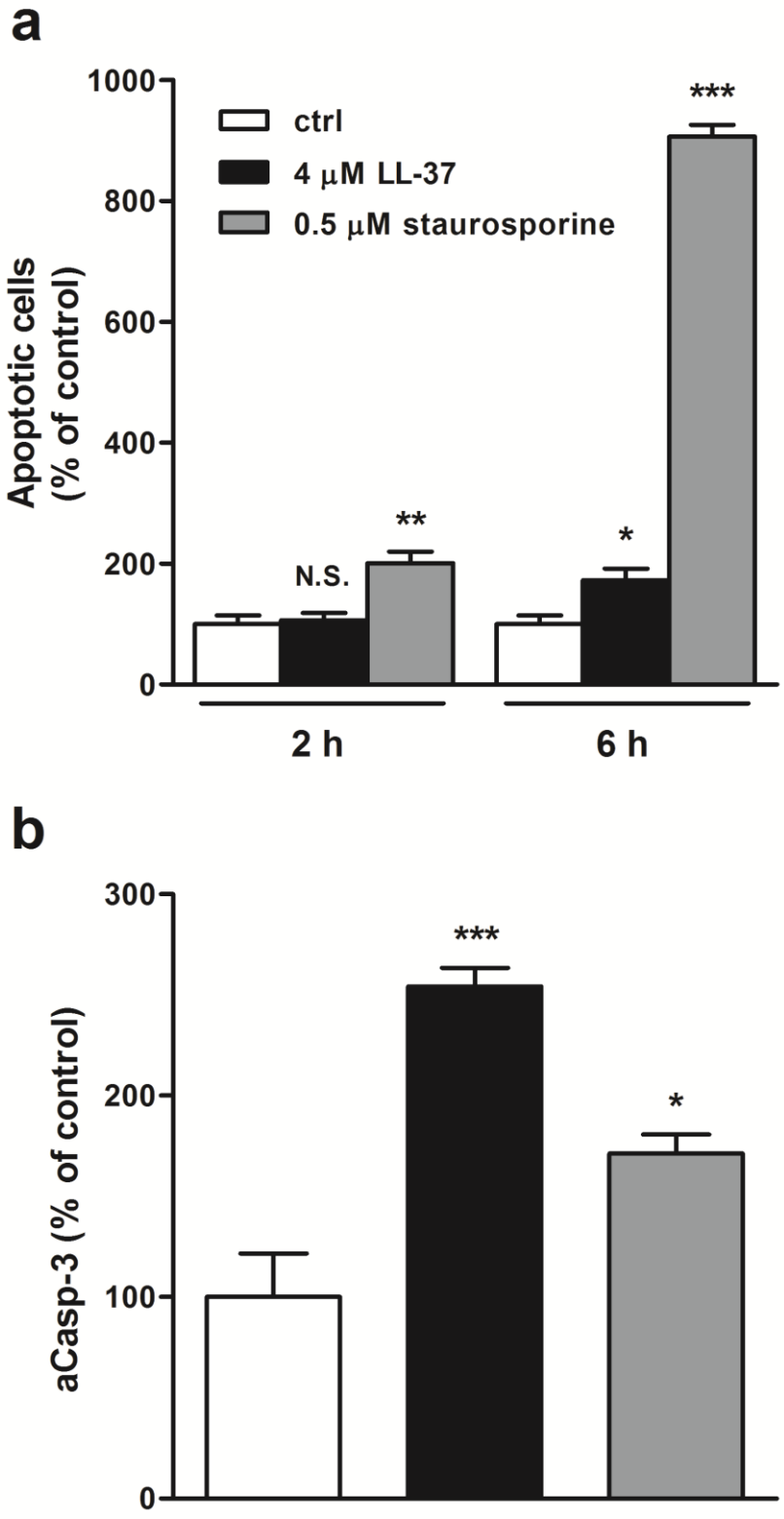

a
1000
800
600
400
200
0
Apoptotic cells
(% of control)
ctrl
4 μM LL-37
0.5 μM staurosporine
***
**
*
N.S.
2 h
6 h
b
300
200
100
0
aCasp-3 (% of control)
***
*

Fig. 5

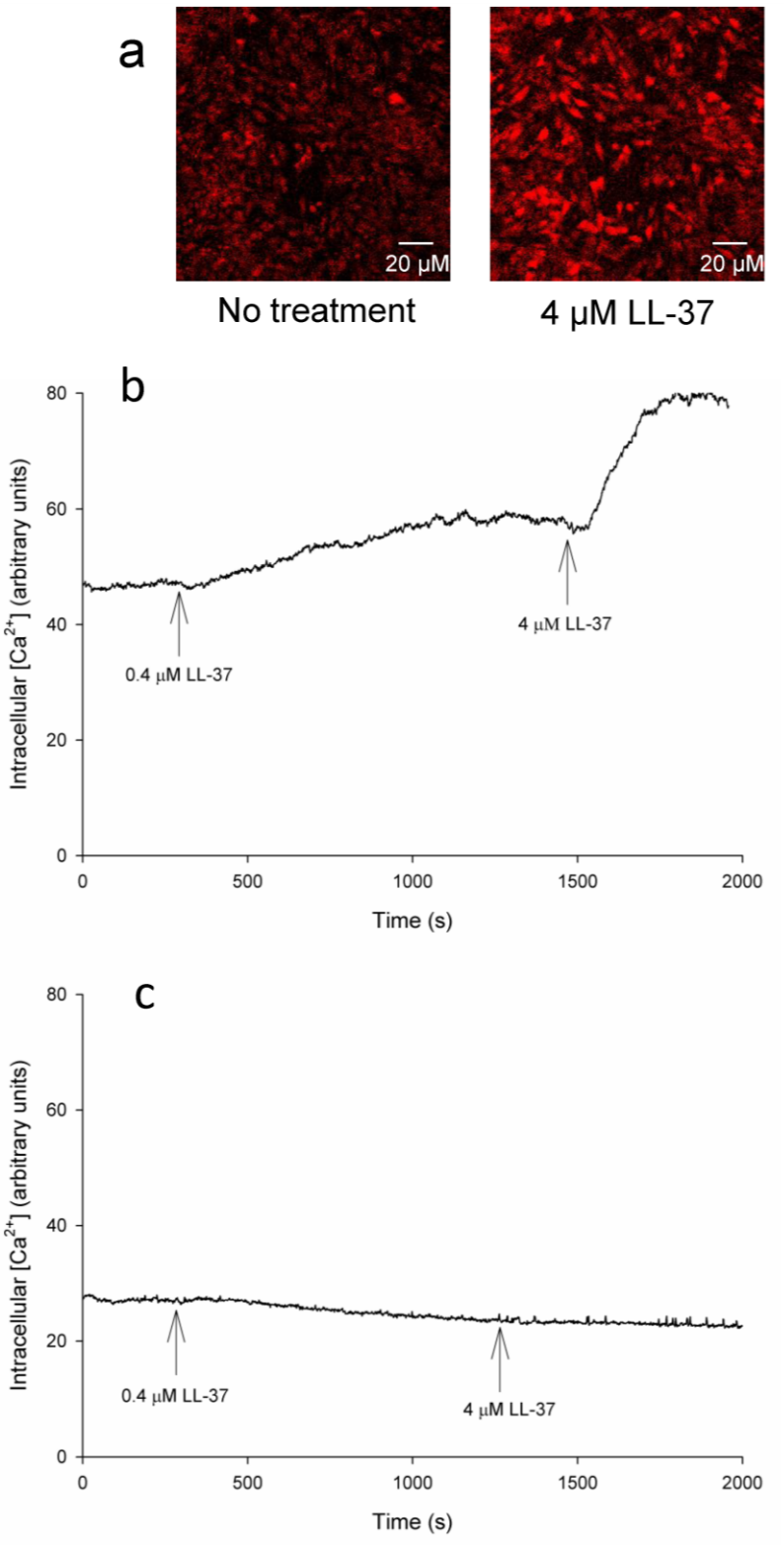

a
20 μM
20 μM
No treatment
4 μM LL-37
b
Intracellular [Ca2+] (arbitrary units)
0.4 μM LL-37
4 μM LL-37
Time (s)
c
Intracellular [Ca2+] (arbitrary units)
0.4 μM LL-37
4 μM LL-37
Time (s)

Fig. 6

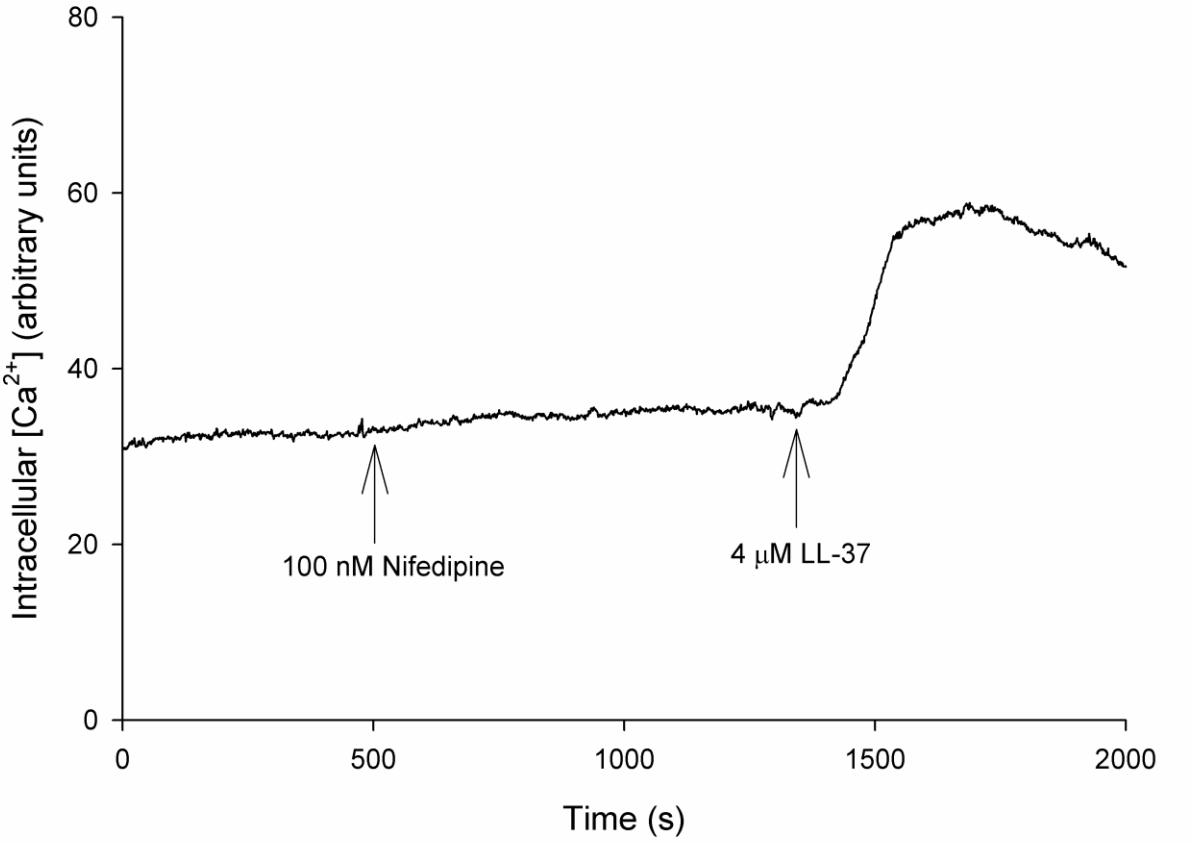

80
60
40
20
0
Intracellular [Ca2+] (arbitrary units)
100 nM Nifedipine
4 μM LL-37
0
500
1000
1500
2000
Time (s)

Fig. 7

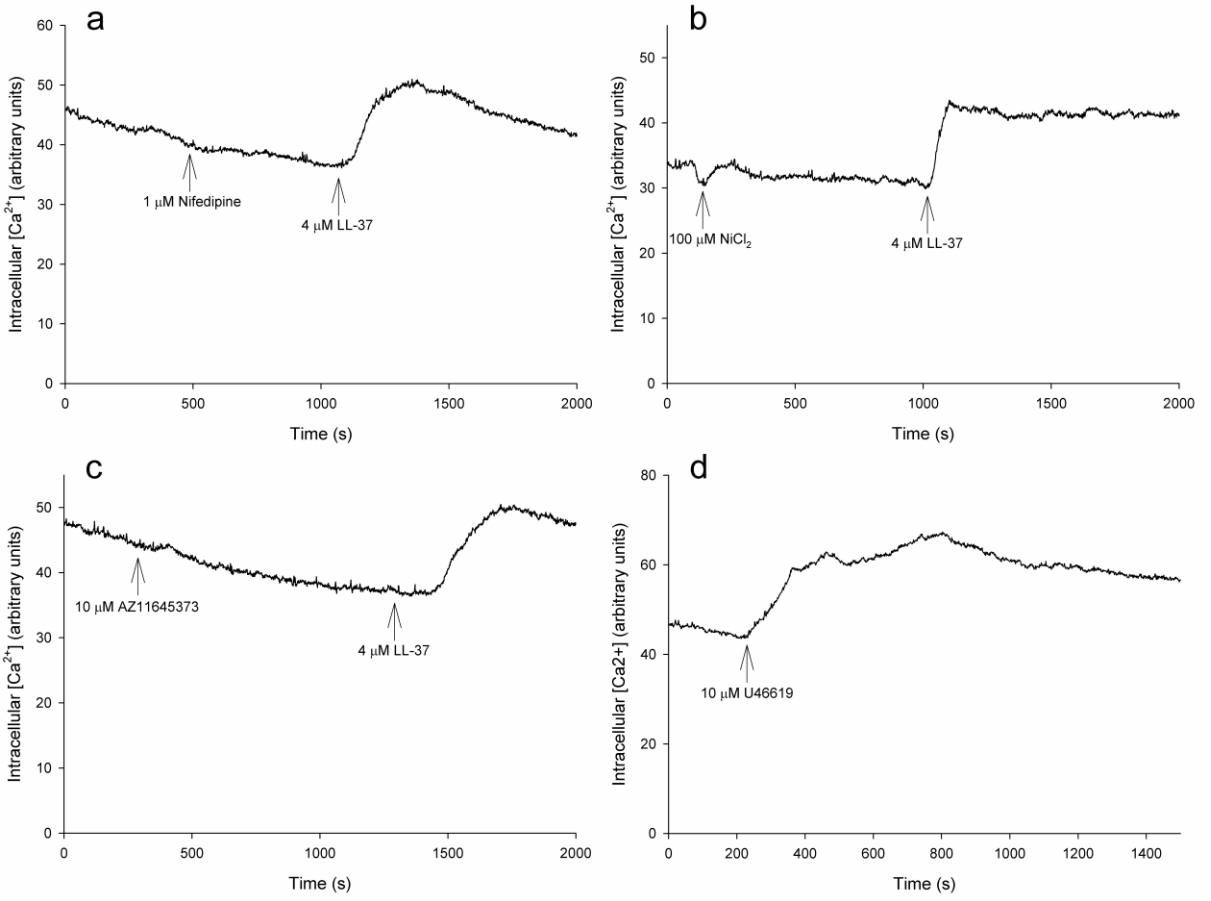

a
Intracellular [Ca2+] (arbitrary units)
1 μM Nifedipine
4 μM LL-37
Time (s)
b
Intracellular [Ca2+] (arbitrary units)
100 μM NiCl2
4 μM LL-37
Time (s)
c
Intracellular [Ca2+] (arbitrary units)
10 μM AZ11645373
4 μM LL-37
Time (s)
d
Intracellular [Ca2+] (arbitrary units)
10 μM U46619
Time (s)

Fig. 8

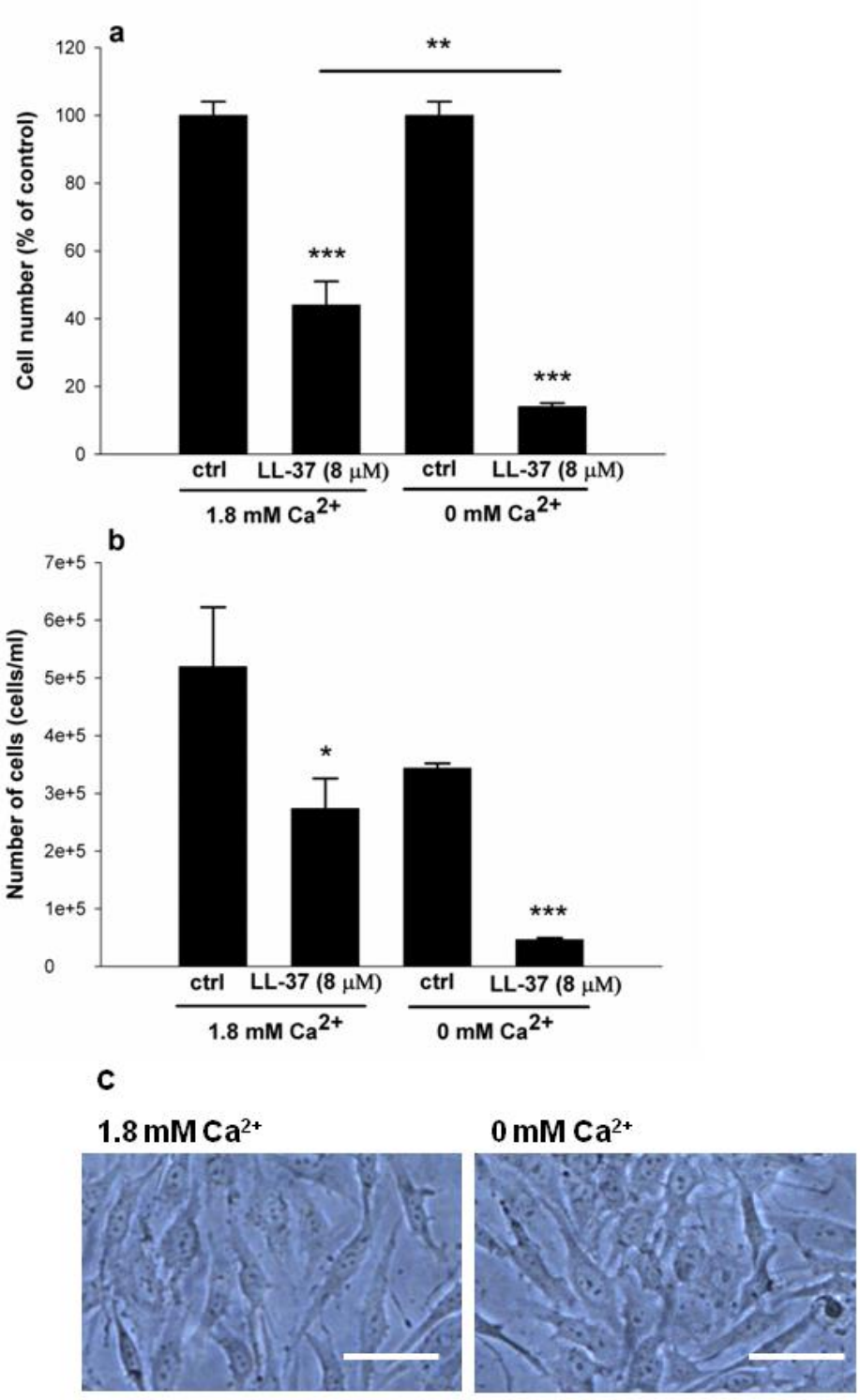
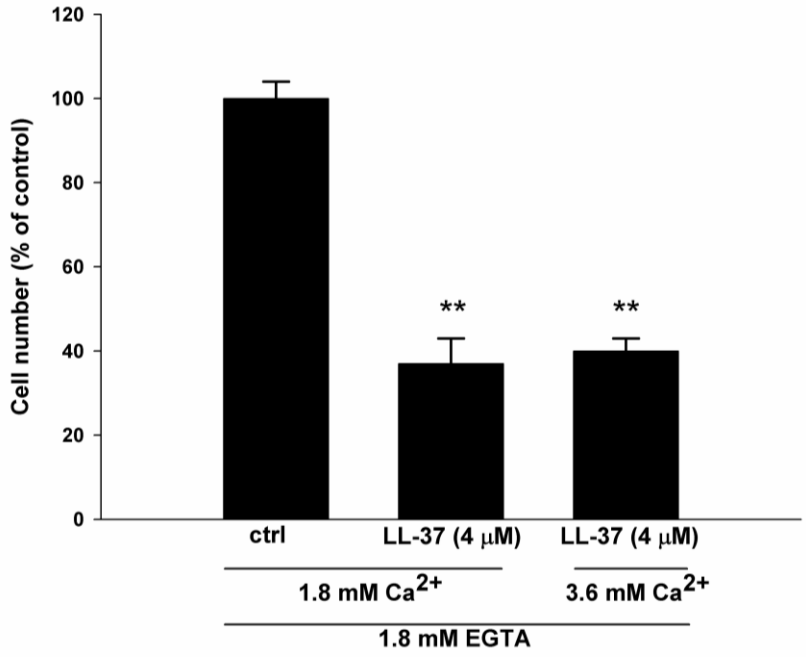

a
Cell number (% of control)
120
100
80
60
40
20
0
**
***
***
ctrl
LL-37 (8 μM)
ctrl
LL-37 (8 μM)
1.8 mM Ca2+
0 mM Ca2+
b
Number of cells (cells/ml)
7e+5
6e+5
5e+5
4e+5
3e+5
2e+5
1e+5
0
*
***
ctrl
LL-37 (8 μM)
ctrl
LL-37 (8 μM)
1.8 mM Ca2+
0 mM Ca2+
c
1.8 mM Ca2+
0 mM Ca2+

Fig. 9


120
100
80
60
40
20
0
Cell number (% of control)
**
**
ctrl
LL-37 (4 μM)
LL-37 (4 μM)
1.8 mM Ca2+
3.6 mM Ca2+
1.8 mM EGTA