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Antibacterial Effect of Human Mesenchymal Stem Cells Is Mediated in Part from Secretion of the Antimicrobial Peptide LL-37

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Abstract

Recent in vivo studies indicate that mesenchymal stem cells (MSCs) may have beneficial effects in the treatment of sepsis induced by bacterial infection. Administration of MSCs in these studies improved survival and enhanced bacterial clearance. The primary objective of this study was to test the hypothesis that human MSCs possessed intrinsic antimicrobial properties. We studied the effect of human MSCs derived from bone marrow on the bacterial growth of Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) and Gram-positive (*Staphylococcus aureus*) bacteria. MSCs as well as their conditioned medium (CM) demonstrated marked inhibition of bacterial growth in comparison with control medium or normal human lung fibroblasts (NHLF). Analysis of expression of major antimicrobial peptides indicated that one of the factors responsible for the antimicrobial activity of MSC CM against Gram-negative bacteria was the human cathelicidin antimicrobial peptide, hCAP-18/LL-37. Both m-RNA and protein expression data showed that the expression of LL-37 in MSCs increased after bacterial challenge. Using an in vivo mouse model of *E. coli* pneumonia, intratracheal administration of MSCs reduced bacterial growth (in colony-forming unit) in the lung homogenates and in the bronchoalveolar lavage (BAL) fluid, and administration of MSCs simultaneously with a neutralizing antibody to LL-37 resulted in a decrease in bacterial clearance. In addition, the BAL itself from MSC-treated mice had a greater antimicrobial activity in comparison with the BAL of phosphate buffered saline

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The authors indicate no potential conflicts of interest.

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(PBS)-treated mice. Human bone marrow-derived MSCs possess direct antimicrobial activity, which is mediated in part by the secretion of human cathelicidin hCAP-18/LL-37.

Keywords

Acute lung injury/acute respiratory distress syndrome; Antimicrobial peptides/proteins; *E. coli* pneumonia; LL-37; Mesenchymal stem cells

Introduction

The innate immune system provides the first line of defense against microbial infections. Among the key effector molecules responsible for bacterial killing are antimicrobial proteins and polypeptides, which comprise a diverse group, including lysozyme, lactoferrin, secretory leucoprotease inhibitor, and defensins, all of which are able to kill microorganisms [1]. The cathelicidin family is one of the main antimicrobial peptide families in mammals [2]. Peptides belonging to the cathelicidin family are either constitutively produced or induced on stimulation [3, 4]. Cathelicidins as well as the majority of known antimicrobial peptides exert their microbicidal activity through the disruption of the integrity of bacterial membranes [5]. In humans, the cathelicidin family of antimicrobial peptides is represented by a 4 kDa peptide, hCAP-18/LL-37, that is mainly produced by phagocytic leukocytes and epithelial cells, but it is also expressed in the bone marrow [6] and by mesenchymal stem cells (MSCs) [7]. LL-37 has a wide range of biological activities including direct killing of microorganisms, chemotaxis and chemokine induction, and regulation of inflammatory responses, as well as angiogenic, antiapoptotic, aids in horizontal DNA intracellular transfer, and wound healing effects [8, 9].

MSCs are multipotent adult stem cells found in the bone marrow and other anatomic niches, which have the capacity to differentiate into multiple cell types such as osteoblasts, adipocytes, and chondroblasts under in vitro conditions [10, 11]. Bone marrow (BM)-derived MSCs reside near the sinusoids and function as support cells for hematopoietic stem cells, perhaps providing some protection against microbial invasion. Although it is well established that MSCs have toll-like receptor (TLR) receptors [12–15] and are involved in inflammatory responses [16], little is known about their role in the innate immune system.

Acute lung injury (ALI) is a major cause of acute respiratory failure in critically ill patients. Bacterial pneumonia is the most common cause of ALI [17]. Recent studies have demonstrated that BM-derived MSCs reduce lung injury in experimental models of lipopolysaccharide (LPS)-induced ALI in mouse [18, 19] and in an ex vivo-perfused human lung [20]. In addition, other in vitro and in vivo studies have provided evidence for the beneficial effects of MSCs in the treatment of LPS- or bacteria-induced sepsis. In two mouse models of sepsis following cecal ligation and puncture (CLP), i.v. MSCs reduced total bacterial counts in the blood and peritoneal fluid [21, 22]. These survival benefits were explained in part by the immunomodulatory properties of MSCs, but the actual mechanism of enhanced bacterial clearance was not clearly identified. Although a recent publication by Mei et al. [23] showed that the improvement in bacterial clearance in MSC-treated septic mice following CLP could be in part explained by enhanced phagocytic activity of host immune cells, it is not known yet whether human BM-derived MSCs possess direct antimicrobial activity.

Thus, the primary hypothesis for these studies was that human MSCs might express direct antimicrobial activity through the secretion of antimicrobial peptides. We examined the effect of human MSCs on bacterial growth in vitro. Expression of different antimicrobial

peptides was investigated using reverse transcription polymerase chain reaction (RT-PCR), enzyme-linked immunosorbent assay (ELISA), and immunohistochemistry. Following stimulation with live *Escherichia. coli*, human MSCs produced one candidate antimicrobial peptide, LL-37, which was subsequently found to be responsible for antimicrobial activity in vitro. To determine if the secretion of LL-37 by MSCs would alter bacterial clearance in vivo, we tested BM-derived human MSCs in an *E. coli* pneumonia model in mice. Treatment with human MSCs, given 4 hours later, resulted in a significant reduction of *E. coli* colony-forming unit (CFU) in the lung homogenates (LHs) and the bronchoalveolar lavage (BAL) fluids. The effect was blocked with a neutralizing antibody to LL-37 demonstrating that human MSCs possessed antimicrobial activity, which is explained in part by the secretion of LL-37.

Materials and Methods

Chemicals and Reagents

LPS (*E. coli* O55:B5) was purchased from Sigma-Aldrich (St. Louis, MO). Mouse monoclonal antibody to human LL37/CAP18 clone 3D11 and mouse isotype IgG1 antibody were purchased from Hycult Biotechnology (Netherlands), goat Alexa-Fluor 488-labeled anti-mouse-IgG from Invitrogen, synthetic human LL-37 from AnaSpec (CA) and fetal bovine serum (FBS) from HyClone Laboratories Inc. (Utah).

Animals

C57BL/6 male mice (8- to 10-weeks old; Jackson Laboratory) were used in all experiments. Animals were maintained in the animal facility at the University of California, San Francisco (UCSF). All experimental protocols were approved by the Institutional Animal Care and Use Committee at UCSF.

Cell Culture

Allogeneic BM-derived human MSCs were cultured as previously described [20]. Briefly, MSCs were obtained from the Texas A&M Health Science Center College of Medicine, Institute for Regenerative Medicine (Temple, Texas), a NIH repository. The cells met all the criteria for the classification as MSCs as defined by the International Society of Cellular Therapy [24]. In addition, we tested the cells by immunofluorescence and found them to be negative for CD45 and CD19. On arrival, cells were thawed and expanded in tissue culture flasks (BD Falcon, Belgium) at a density of 500,000 cells/150 cm^2. Cells were passaged every 3–4 days by trypsinization when they reached 70%–80% confluency and were used for the experiments between passages 5–10 as in our prior studies [20]. Between each passage, viability was measured with trypan blue exclusion. MSCs were cultured in α-Minimum Essential Medium without ribonucleosides or deoxyribonucleosides containing 2 mM L-glutamine and 16.5% FBS, without antibiotics. Cells were cultured in a humidified incubator at 5% CO_2 and 37°C under sterile conditions. Before each experiment, cells were trypsinized, counted, washed with phosphate buffered saline (PBS), and resuspended in appropriate medium (RPMI-1640 medium [RPMI] + 5% FBS in case of *E. coli* stimulation, Dulbecco's modified Eagle's medium (DMEM)-H21 and F-12 Ham's [1:1] in case of coculture with primary cultures of human alveolar epithelial type II [ATII] cells, and PBS when administered to the mice). Each experiment was done in triplicates, using cells from at least three different donors from the NIH repository. Normal adult human lung fibroblasts (NHLF; Lonza Inc., Switzerland) were used as controls and grown in the same conditions as MSCs.

ATII epithelial cells were isolated from human donor lungs (preserved at 4°C for 4–8 hours) as previously described [25]. Primary cultures of human ATII cells were plated on collagen

I-coated 24-well Transwell plates (0.4-μ;m pore size, polytetrafluoroethylene (PTFE) Membrane, CoStar, Corning Inc., NY) at 5% CO_2, at 37°C at a concentration of 1×10^6 cells/well. The cells were exposed to medium, DMEM-H21 and F-12 Ham's (1:1), 10% FBS with antibiotics for 72 hours and without FBS and antibiotics for 48 hours. Following 120 hours from the isolation, the type II cells were exposed to LPS at 100 ng/ml for 24 hours. For experiments with MSCs, allogeneic MSCs (250,000 cells/well) were added onto or to the bottom chamber of the Transwell plate (with or without cell contact) simultaneously with the LPS.

Bacterial Culture and Antimicrobial Assays

E. coli strain K1, *Pseudomonas aeruginosa* strain PA103, and *Staphylococcus aureus* Newman strain were used for these experiments. The methods used to passage, store, amplify, and quantify the bacteria have been described previously [26–28]. For each experiment, *E. coli* or *S. aureus* colonies were seeded from frozen stocks, *P. aeruginosa* colonies were seeded from selective agar-plate kept at −4°C and grown overnight at 37°C in liquid Luria-Bertani (LB) medium (Difco BD, MD) with slight agitation. Before each experiment, the bacterial cells were washed once and resuspended in PBS, and optical density (OD at λ = 600 nm) of the suspension was measured. Number of CFU was calculated as according to the following equation: OD_{600} = 1.0 corresponds to 4×10^8 CFU/ml for *E. coli* [26], OD_{600} = 0.5 corresponds to 5×10^8 for *P. aeruginosa* [28], and OD_{600} = 1.8 corresponds to 2×10^9 CFU/ml.

Assessment of direct inhibition of bacterial growth by MSCs or its conditioned medium (CM) was done by counting CFU. In brief, MSCs in 24-well plates (2×10^5 cells per well) in RPMI supplemented with 5% FBS were infected with 300 CFU *E. coli* or *S. aureus* and incubated for 6 hours in humidified CO_2 incubator, then aliquots of culture medium were taken from each well, serially diluted with sterile PBS, and plated on LB-agar plates (TEKnova, Hollister, CA). Colonies were counted after overnight incubation at 37°C.

Antimicrobial activity of MSC CM (or synthetic LL-37) was tested by a microdilution susceptibility test according to Andrä et al. [29] with slight modifications. Briefly, MSC CM was collected from the wells, centrifuged at 15,000 rpm for 10 minutes and frozen at −20°C (to eliminate any residual bacterial organisms). Prior to the experiments, samples were thawed on ice, and aliquots (90 μl) were transferred to a 96-well plate, inoculated with 100 CFU *E. coli*, *P. aeruginosa*, or *S. aureus* (in 10 μl of PBS) and incubated for 16 hours at 37°C. Then CFUs were counted as described earlier. In the case of *P. aeruginosa*, selective *Pseudomonas* isolation-agar plates (Difco, BD, MD) were used instead of LB-agar plates. In additional experiments, samples were preincubated with an anti-LL-37 antibody (1 μg/ml) or mouse isotype antibody control for 2 hours at reverse transcription (RT) on a plate-shaker (200 rpm). Antimicrobial activity of mouse BAL was tested according to Bergsson et al. [30] by adding 1×10^4 *E. coli* CFU to 90 μl of BAL sample and incubating at 37°C. After 2 hours, CFUs were counted.

RNA Isolation and RT-PCR

RNA was isolated from MSCs and NHLFs, using the Qiagen RNAeasy kit (Qiagen Inc, CA). After isolation, RNA samples were treated with DNase I for 60 minutes at RT to remove contaminating DNA. The quality of the RNA was assessed with the NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE) according to the manufacturer's instructions. 260/280 and 260/230 nm absorbance ratios of 1.8–2.0 indicated a pure RNA sample. Primers for LL-37 and GAPDH were custom made (Eurofins MWG Operon, Alabama). The sequences were as follows: LL-37 (forward 5′-TAACCTCTACCGCCTCCTGGACCTGGACC-3′; reverse 5′-

GGACTCTGTCCTGGGTACAAGATTCCGC-3′), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward 5′-GTCAGTGGTGGACCTGACCT-3′, reverse 5′-AGGGGTCTACATGGCAACTG-3′). The specificity of PCR product was confirmed by DNA sequencing. RT-PCR was done using the SuperScript III OneStep RT-PCR System with Platinum Taq DNA Polymerase protocol from Invitrogen according to manufacturer's instructions in the reaction volume of 25 μl. For LL-37 cDNA amplification, an initial reverse transcription step (52°C for 30 minutes) was followed by denaturing step (94°C for 2 minutes) and then by 40 cycles of denaturing (94°C for 20 seconds), annealing (60°C for 30 seconds), and extending (68°C for 30 seconds), followed by 5 minutes at 72°C for elongation. To normalize loading of the PCR products, *GAPDH* gene was amplified as an internal control (RT 58°C for 30 minutes, denaturation 94°C for 2 minutes, 18 cycles of amplification 94°C for 20 seconds, 64°C for 30 seconds, 68°C for 30 seconds, elongation 68°C for 5 minutes). The resulting amplified DNA product was run on a 1.4% agarose gel, and bands were visualized with the use of ethidium bromide.

Fluorescent Immunohistochemistry

For detection of the LL-37 in cell cultures, MSCs or NHLFs were seeded on Lab-Tek II chamber slides (Nalge Nunc International; Thermo Fisher Scientific, PA) at a density of 5×10^4 cells/cm^2. Cells were grown for 24 hours and then stimulated with *E. coli* (300 CFUs) for 6 hours. After completion of the experimental conditions, the cell monolayer was washed twice with cold PBS and fixed in 4% paraformaldehyde for 30 minutes. The cells were then washed three times with PBS for 10 minutes in a gently shaking chamber at room temperature. The cells were then permeabilized by 0.2% Triton X-100 for 2 minutes, washed three times with PBS for 10 minutes, and blocked with 5% BSA for 2 hours. The slides were incubated with a primary antibody for the LL-37 at dilution 1:50 in 5% BSA overnight at 4°C. The slides were washed three times with PBS for 10 minutes and then exposed to the secondary antibody, goat Alexa-Fluor 488-labeled anti-mouse-IgG. After washing, slides were incubated with 4,6-diamidino-2-phenylindole (DAPI) (300 μM, Sigma Aldrich) for 5 minutes to counterstain the nuclei. The slides were mounted with Vectashield mounting medium. Images were obtained by Leica DM 1,000 microscope.

Protein Assays

Levels of SP-D in MSC CM were determined by ELISA kit (Yamasa Corporation, Japan). Levels of β-defensin-2 and β-defensin-3 were measured by ELISA (Phoenix Pharmaceuticals Inc., CA). hCAP-18/LL-37 concentration in MSC CM was measured by ELISA (Hycult Biotechnology, Netherlands).

E. coli Pneumonia Mouse Model

Mice were anesthetized with Avertin and *E. coli* 1×10^6 CFU in the volume of 30 μl were instilled into the lung. The details of direct visualized intratracheal (IT) instillation method were described in our previous publications [18, 27]. After 4 hours, the mice were anesthetized with isoflurane and given MSC (1×10^6 cells in 30 μl of PBS) or PBS intratracheally. Eighteen hours after *E. coli* instillation, mice were sacrificed to carry out BALs.

E. coli Quantification in the LH and BAL

Mice were euthanized at 18 hours after instillation of *E. coli*. BAL was done after euthanizing the mice by placing a 20-gauge catheter into the trachea through which 1 ml of cold PBS was flushed back and forth three times. Aliquots of BAL were serially diluted and cultured on LB agar plate overnight at 37°C. CFUs were then counted. For a different set of lungs, 1 ml PBS was added and the lungs were homogenized under sterile conditions. The

homogenate was serially diluted and cultured on a LB plates overnight at 37°C. Bacteria CFUs were then counted as previously described.

Measurement of Leukocytes and Neutrophils in BAL

BAL leukocytes and neutrophils were measured by a Coulter counter (Z1 series; Beckman Coulter). A cell smear was also made using a cytospin centrifuge (Thermo Shandon), and cells were visualized with Wright-Giemsa staining (Fisher Scientific). A differential of the white blood cell count was estimated microscopically by counting 100 cells per representative portion of the slide.

BAL Cytokine and Protein Measurements

BAL samples were centrifuged at 1,000 rpm for 10 minutes, and the supernatants were collected and stored at −80°C. Macrophage inflammatory protein (MIP)-2, a mouse neutrophil chemokine, in the BAL supernatants was measured by ELISA (R&D Systems). BAL protein concentration as a marker of lung endothelial and epithelial permeability was measured by Bio-Rad protein assay kit.

Statistical Analysis

All experimental groups were carried out at least three times in triplicates. Results are expressed as mean ± SD if the data were normally distributed. Comparisons between two groups were made by an unpaired *t* test. Comparisons between more than two groups were made by repeated measures of analysis of variance using the Bonferroni correction for multiple-comparison testing with Statview (SAS Institute Inc.). A value of $p < .05$ was considered statistically significant.

Results

Human MSCs Inhibit *E. coli* Bacterial Growth

To test the effect of human MSCs on bacterial growth, we infected MSCs with 300 CFU of *E. coli* for 6 hours. BM-derived MSCs significantly inhibited bacterial growth compared with control medium (RPMI) and compared with NHLFs (Fig. 1A).

To determine if the observed antibacterial effect was associated with soluble secreted factors, we assessed the ability of the CM to inhibit bacterial growth by incubation with *E. coli* or with another Gram-negative bacteria, *P. aeruginosa*. There was no significant effect of unstimulated CM of MSCs on bacterial growth in comparison with control medium or CM of normal human lung fibroblasts. However, CM of MSC previously stimulated with *E. coli* displayed marked antimicrobial activity against *E. coli* (Fig. 1B) and against *P. aeruginosa* (Fig. 1C). These results suggested that the mechanism of MSC antimicrobial activity against Gram-negative bacteria was associated with a secreted product, which was induced with previous bacterial challenge.

Similar inhibitory effect was detected in coculture experiments of MSCs with Gram-positive bacteria, *S. aureus*. MSC CM after *S. aureus* stimulation also retained antimicrobial activity against *S. aureus* (Supporting Information Fig. S1A, S1AB). Because the primary focus of this investigation was to test the role of direct antimicrobial properties of MSCs in Gram-negative infections, we concentrated on the activity against Gram-negative bacteria.

MSCs Secrete Human Cathelicidin Antimicrobial Peptide, LL-37

To investigate potential candidates responsible for the observed antimicrobial effect, we analyzed the cell culture supernatants of both unstimulated and *E. coli*-stimulated MSCs for

the presence of proteins and peptides with known antimicrobial activity. Protein levels by ELISA for human β-defensins hBD-2 and hBD-3, Lipocalin-2, and SP-D were negative or showed very low levels, which were insufficient to elicit an antimicrobial effect (data not shown). However, by RT-PCR, immunofluorescence, and ELISA, MSCs expressed and secreted the human cathelicidin antimicrobial peptide hCAP-18/LL-37. By RT-PCR, baseline levels of LL-37 mRNA was low but increased significantly with stimulation with *E. coli*. Normal human lung fibroblasts also expressed LL-37 but at lower levels (Fig. 2A). By immunofluorescence (Fig. 2B) and ELISA (Fig. 2C), secreted levels of LL-37 were significantly higher in MSCs compared with the control NHLFs. MSCs stimulated by incubation with *E. coli* secreted a significantly higher level of LL-37 as compared with NHLFs (Fig. 2C).

Antimicrobial Activity of Synthetic LL-37

Synthetic LL-37 alone demonstrated a significant antibacterial effect against *E. coli* and against *P. aeruginosa,* when tested in the same medium (RPMI 5% FBS) and in the same conditions as MSC CM. The concentration necessary for the effect in comparison with control medium became significant starting at 1 ng/ml and increased in a dose-dependent manner (Fig. 3A, 3B). Prior experiments demonstrated that MSCs, previously stimulated with *E. coli*, secreted LL-37 in the range of 10 ng/ml (Fig. 2C).

Antimicrobial Activity of MSC CM Is Mediated by LL-37 Secretion

To test the effect of LL-37 secretion on antimicrobial effects of MSC CM, we preincubated the CM (previously stimulated with *E. coli*) with a blocking antibody, anti-LL-37 at a concentration of 1 μg/ml, or a mouse isotype control antibody for 2 hours and then cultured the CM with 100 CFU of *E. coli* or *P. aeruginosa* for additional 16 hours. Preincubation with the anti-LL-37 antibody removed all of the antibacterial effect of the CM for MSCs against both bacterial strains. In contrast, preincubation with the mouse isotype control IgG antibody had no significant effect on antibacterial activity, suggesting the importance of LL-37 secretion (Fig. 3C, 3D).

In Vivo Antimicrobial Effect of MSCs in a Model of *E. coli* Pneumonia

To determine if the antimicrobial effect of MSCs was applicable in vivo, we infected C57BL/6 mice with IT instillation of *E. coli* 1×10^6 CFU. Four hours after *E. coli* instillation, mice were treated with either 1×10^6 BM MSC or PBS intratracheally. Eighteen hours after infection, LH and BAL samples of mice treated with BM MSC showed a sharp reduction in total bacterial counts (Fig. 4A, 4B). The total bacterial counts in the BAL were in order of magnitude less then bacterial counts in the LHs. In contrast, mice that received the NHLF did not demonstrate any difference in bacterial counts in LHs (3.3 ± 1.1 for NHLF and $3.9 \pm 1.7 \times 104$ CFU/ml for PBS, mean CFU ± SD) or BAL (9.6 ± 14.9 for NHLF and $8.26 \pm 10.6 \times 10^3$ CFU/ml for PBS, mean CFU ± SD) compared with the PBS-treated group.

Evaluation of In Vivo MSC Treatment at 18 Hours on BAL Cell Counts and Protein Influx

Total BAL cell counts and absolute neutrophil counts were also significantly lower in the BM MSC-treated group compared with PBS-treated mice (Fig. 5A, 5B), suggesting that bacterial clearance in the BAL of MSC-treated mice did not primarily depend on the recruitment of immune cells. The BAL level of the neutrophil chemokine, MIP-2, was also significantly lower in mice treated with MSCs, compared with the PBS-treated group (Fig. 5C). BAL protein level, a marker of lung endothelial and epithelial permeability, was significantly lower in the mice given MSCs compared with PBS (Fig. 5D). In separate

experiments, NHLF-treated mice did not show any difference from PBS-treated group in any of these parameters (data not shown).

Neutralization of LL-37 Activity Abolishes the Antimicrobial Effect of MSCs In Vivo

Administration of MSCs together with neutralizing anti-LL-37 Ab (10 μg), but not the mouse IgG control (10 μg), resulted in a nearly 10-fold increase in bacterial number both in the LHs and BAL (Fig. 6A, 6B), compared with MSC-treated group, indicating that LL-37 activity is required for the antimicrobial effect of MSCs observed in vivo.

In separate experiments, administration of neutralizing anti-LL-37 antibodies alone (10 μg) did not change the level of bacterial growth in mouse BAL (Supporting Information Fig. S2A) compared with PBS-treated mice, indicating that anti-LL-37 antibodies did not interfere with antimicrobial activity of endogenous mouse antimicrobial proteins, including potentially mouse cathelin related antimicrobial peptide (CRAMP).

To confirm that antimicrobial effect in vivo was linked to the secretion of soluble products by MSCs, we performed an additional investigation of the antimicrobial activity of mouse BAL. The supernatant of the BAL of mice treated with MSCs, when incubated with 1×10^5 CFU of *E. coli* for 2 hours, inhibited bacterial growth by 49%, in comparison to the BAL supernatant of PBS-treated mice, suggesting the presence of an antimicrobial factor (Fig. 6C). The antimicrobial effect of BAL supernatant was significantly reduced in mice treated with MSCs together with anti-LL-37 antibody (Fig. 6C), whereas the isotype IgG did not have any effect (data not shown).

Also, the antimicrobial activity of mouse BAL following the administration of anti-LL37 Ab in vivo alone showed no difference in comparison to BAL from PBS-treated mice (Supporting Information Fig. S2B), providing additional evidence that antibodies used in our experiments did not neutralize the activity of endogenous mouse cathelicidin.

Coculture of MSCs with Human ATII Cells

Because total BAL cell numbers were significantly lower in the MSC-treated group, another possible source of antimicrobial peptides and proteins in the BAL could be alveolar epithelial cells. Therefore, we tested whether or not MSCs may have any influence on the production of antimicrobial peptides by primary cultures of human ATII cells. We cocultured MSCs together with human ATII cells in a Transwell system (Fig. 7A) with or without contact in the presence of LPS (100 ng/ml for 24 hours). ELISA of the CM for LL-37 showed that epithelial cells alone produced very low amounts of LL-37, and there was no difference between levels of LL-37 produced by MSCs alone or in coculture with ATII (with or without cell contact; Fig. 7B), indicating that MSCs were the main source of LL-37. In addition, the antimicrobial activity of the CM demonstrated 60% reduction in *E. coli* CFU only in the case of coculture of ATII together with MSCs (without contact) compared with ATII cells alone or stimulated with LPS in comparison with control medium (Fig. 7C).

Discussion

The main findings of this study are that (a) human BM-derived MSCs are able to inhibit bacterial growth directly, and the activity is conserved in its CM, indicating the presence of a secreted soluble factor; (b) MSCs produce and secrete substantial quantities of the antimicrobial peptide, human cathelicidin hCAP-18/LL-37, which inhibits bacterial growth of *E. coli* and *P. aeruginosa* in vitro; (c) the expression of LL-37 by MSCs is inducible with *E. coli* exposure both on mRNA and protein levels; (d) and, in a mouse model of *E. coli* pneumonia, IT MSC administration led to a significant reduction in bacterial counts in the

BAL and LHs 18 hours after infection, which was dependent on LL-37 activity. These results demonstrate that MSCs can participate in host defense through the secretion of an antimicrobial peptide.

Antimicrobial peptides, such as LL-37, or host defense peptides are endogenous peptides with antimicrobial properties, which are essential parts of the innate immune system. Although there is evidence that synthetic or native LL-37 exhibits potent antimicrobial activity in vitro against a wide range of pathogens including viruses [31], fungus [32], and both Gram-positive and Gram-negative bacteria [5, 31, 33], very few studies have established the importance of biological levels of LL-37 to its antimicrobial activity in vivo. Brandenburg et al. [34] reported on the antimicrobial activity of cerebrospinal fluid of patients with infectious meningitis, which was mediated primarily by LL-37. This finding was further supported by the expression of rCRAMP (a rat analog of LL-37) in rat glial cells after stimulation with bacterial supernatants and its antimicrobial activity. In their subsequent study [35], these investigators reported antimicrobial activity of rat meningeal cell culture supernatants against a wide spectrum of Gram-positive and Gram-negative bacteria. Using siRNA, the authors proved that rCRAMP was the main factor responsible for the antimicrobial effect. Bergsson et al. [30] demonstrated the potential relevance of LL-37 antimicrobial activity in the BAL of patients with cystic fibrosis (CF). Despite high levels of LL-37, it was inactive against microbes in the CF lung because of complex formation with glycosaminoglycans (GAGs). GAG lyases and hypertonic saline can break these complexes, liberating LL-37 and restoring antimicrobial effects.

Although LL-37 secretion was previously detected in the bone marrow [6] and by BM MSC [7], the potential role of LL-37 for antimicrobial activity by MSCs has not yet been investigated. Previous studies emphasized the role of LL-37 as a chemoattractant for stem cells. Coffelt et al. [7] showed that LL-37 increased the migration of MSCs through its formyl peptide receptor, FPLR1. In another study, Tomchuck et al. [14] reported that LL-37 could be a ligand for the TLR4 receptor on MSCs and stimulate their migration and even change their immunomodulatory properties. From these studies, two important properties of MSCs were described in relation to their antimicrobial potential: (a) MSCs have both TLR and FPLR receptors, which are necessary to recognize pathogen-associated molecular patterns, suggesting the possibility that MSCs may be involved in innate immune reactions; (b) LL-37, through a concentration gradient, can activate MSCs and induce their migration toward the source of infection.

In our experiments, we found that LL-37 expression in BM-derived MSC was upregulated by *E. coli* stimulation. Following exposure with live *E. coli* for 6 hours, the upregulation of LL-37 by MSCs were detected at both the mRNA (Fig. 2A) and protein levels, which was confirmed by immunofluorescence (Fig. 2B) and ELISA (Fig. 2C). The level of LL-37 in the unstimulated and *E. coli*-stimulated MSC CM was in the range of 2.7 ± 1.2 and 7 ± 1 ng/ml (mean ± SD), respectively. Coffelt et al. [7] previously reported 140–150 pg/ml levels of LL-37 in CM of unstimulated bone-marrow derived MSC from three different donors. We detected a higher concentration of LL-37 in CM of unstimulated MSCs, which could be explained by difference in cell culture conditions. Regardless, LL-37 secretion by MSCs is a general property and does not appear to depend on the condition of the donor.

More significantly, these results were associated with an increase in antimicrobial activity of MSC CM against *E. coli* and *P. aeruginosa* (Fig. 1B, 1C). Synthetic LL-37, starting at a dose of 1 ng/ml and dissolved in the same medium (RPMI, 5% FBS), showed a dose-dependent antimicrobial activity against both Gram-negative bacteria as well (Fig. 3A, 3B). Preincubation of MSC CM with a neutralizing anti-LL37 Ab markedly reduced antibacterial activity of the samples (Fig. 3B), providing a direct link between LL-37 secretion and the

antimicrobial effect of MSC CM. We also demonstrated that MSCs are capable of inhibiting the growth of Gram-positive bacteria, *S. aureus*, and that this effect is mediated by a secreted soluble product (Supporting Information Fig. S1A, S1B). These data are in agreement with the recent study by Cruse et al. [36], in which the investigators found that ex vivo human lung mast cells exhibit direct antimicrobial activity against *S. pneumonia*, and this activity was contact-independent and mediated in part by the release of LL-37.

To extend our in vitro studies, we infected C57BL/6 male mice with IT *E. coli* (1×10^6 CFU/mice). IT administration of MSCs 4 hours after infection was associated with a reduction in both total cell counts, protein and MIP-2 levels in the BAL as compared with the *E. coli*-infected mouse (Fig. 5A–5D), reflecting the immunomodulatory properties of MSCs [18–22]. MSC administration also led to increased bacterial clearance as reflected in lower bacterial CFU number in the LHs and BAL (Fig. 4A, 4B). To test the role of LL-37 secretion, we administered MSCs together with a neutralizing antibody to LL-37 following *E. coli* infection. The application of the blocking antibody abrogated the improvement in bacterial clearance (Fig. 6A, 6B) and resulted in increased BAL neutrophil counts, similar to the PBS-treated animals. In separate experiments, when anti-LL37 Abs were given in the absence of MSCs, bacterial growth was unchanged as compared with PBS-treated mice (Supporting Information Fig. S2A), suggesting that the antibody used in these experiments was specific for the human peptide and did not neutralize the functional activity of mouse endogenous cathelicidin (CRAMP), which is a normal component of mouse respiratory secretions. The benefit of MSC administration on bacterial growth was not replicated with the IT administration of normal human lung fibroblasts. These data indicate that the activity of LL-37 of MSC may be important following infection and may directly or in concert with other antimicrobial factors in the lung, such as the lung collectins SP-A and SP-D, inhibit bacterial growth. The decrease in MIP-2 in the BAL as well as the decrease in number of neutrophils (Fig. 5A–5C) may reflect a decrease in bacterial burden or the immunomodulatory properties of the MSCs as well. Similar results for LL-37 antimicrobial properties were reported in the study by Cirioni et al. [37]. In their experiments, the authors described the beneficial effects of LL-37 treatment in models of LPS, *E. coli*, and CLP-induced sepsis in rats. They attributed the protective effect of LL-37 to its bactericidal activity and to the ability to decrease the plasma levels of endotoxin and cytokines in septic animals. In a follow-up study [38], the same group showed that LL-37 alone given systemically, provided protection against lethal *P. aeruginosa* infection in cyclophosphamide-treated neutropenic mice. The results in our experiments demonstrate the benefit of both the antimicrobial and the immunosuppressive properties of MSCs in *E. coli* pneumonia.

There are some limitations to the current study. As the blocking antibody to LL-37 only partially reduced the antimicrobial activity of MSCs in the BAL (Fig. 6C), it is likely that there are other antimicrobial factors that may be induced by MSCs. Although we tested for antimicrobial effects and release of LL-37 from ATII cells (Fig. 7), we did not study the contribution of LL-37 to acute inflammatory responses by ATII cells. Interestingly, a study by Tjabringa et al. [39] showed that LL-37, in addition to its antimicrobial activities, might contribute to innate immunity by activation of airway epithelial cells through ERK1/2 activation and increased IL-8 release, suggesting that LL-37 can play a regulatory function in both the antimicrobial and the inflammatory response of airway epithelial cells. Also, in the in vivo model, we did not explore whether or not MSCs altered the antimicrobial activity of other cells, such as neutrophils or macrophages.

Conclusion

In conclusion, human MSCs participate in the innate immune response against Gram-negative bacteria through the secretion of the antimicrobial peptide, LL-37. The secretion of this peptide is inducible with prior bacterial stimulation and has antimicrobial effect both in vitro and in vivo. Thus, human allogeneic human MSCs may be beneficial in bacterial infections because of their antimicrobial properties as well as their immunomodulatory effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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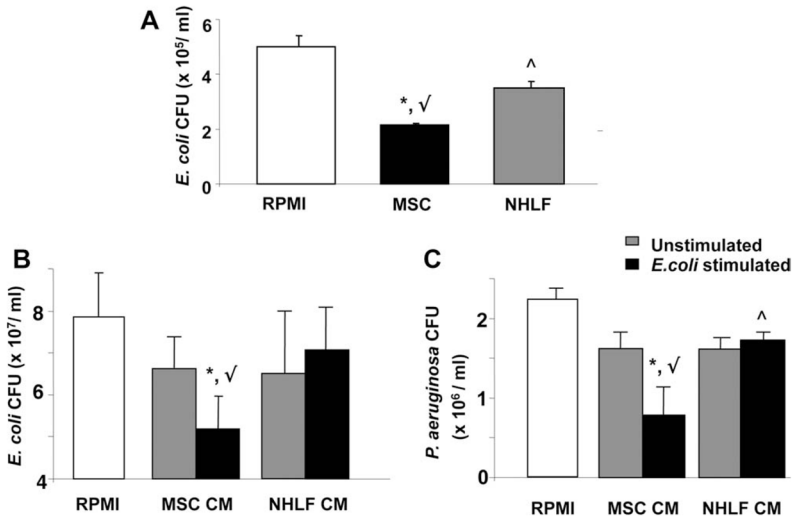

Figure 1.

MSC or its conditioned medium has antimicrobial activity against Gram-negative bacteria. **(A):** MSC directly inhibits *E. coli* growth after 6-hour coincubation. MSC or NHLF were incubated with live *E. coli* K1 for 6 hours. Bacterial growth was assessed by CFU counts. *, $p < .0001$ versus RPMI; √, $p < .007$ versus NHLF; ^, $p < .02$ vs. RPMI by analysis of variance (ANOVA; Bonferroni), $n = 8$. **(B):** The CM of MSC or NHLF with or without prior stimulation with live *E. coli* was examined for antimicrobial activity against *E. coli*. MSC CM after stimulation with *E. coli* inhibited bacterial growth by 40%, whereas CM from unstimulated cells or from NHLF did not have a significant effect. Data are mean ± SD; *, $p < .0001$ versus RPMI; √, $p < .001$ versus NHLF CM (*E. coli* stimulated), by ANOVA (Bonferroni), $n = 8–11$. **(C):** The CM of MSC or NHLF with or without prior stimulation with live *E. coli* was tested for antimicrobial activity against *P. aeruginosa*. MSC CM after stimulation with *E. coli* inhibited bacterial growth by 70%. Data are mean ± SD; *, $p < .0001$ versus RPMI; √, $p < .003$ versus NHLF CM (*E. coli* stimulated), ^, $p < .007$ versus RPMI by ANOVA (Bonferroni), $n = 6$. Abbreviations: CFU, colony-forming units; CM, conditioned medium; MSC, mesenchymal stem cell; NHLF, normal human lung fibroblasts; RPMI, RPMI-1640 medium.

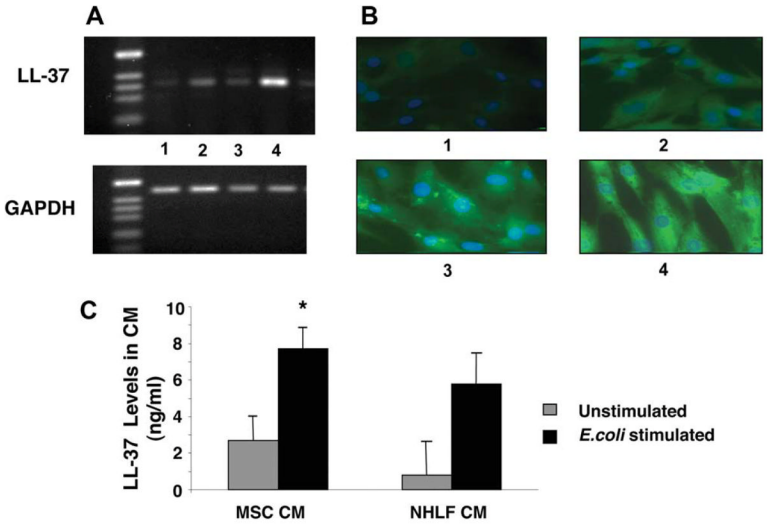

Figure 2.

MSC LL-37 expression is upregulated by *E. coli* stimulation. **(A):** Level of LL-37 mRNA in MSC is increased after *E. coli* stimulation as detected by semiquantitative RT-PCR. GAPDH was used as an internal control to normalize loading of the RNA samples. Lane (1) NHLF, (2) NHLF (*E. coli* stimulated), (3) MSC, and (4) MSC (*E. coli* stimulated). **(B):** Levels of LL-37 protein expression in MSC are increased after *E. coli* stimulation as indicated by immunofluorescence (green staining). (1) NHLF, (2) NHLF (*E. coli* stimulated), (3) MSC, and (4) MSC (*E. coli* stimulated). Nuclei were visualized with DAPI staining (blue). Images are representative for each condition run in triplicates. All representative examples are shown at a magnification of 1 × 1,000. **(C):** Using ELISA, it was found that both MSC and NHLF secreted higher levels of LL-37 after bacterial stimulation. Data are mean ± SD; *, $p < .001$ versus NHLF (*E. coli* stimulated), $n = 12$. Abbreviations: CM, conditioned medium; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; DAPI, 4,6-diamidino-2-phenylindole; ELISA, enzyme-linked immunosorbent assay; MSC, mesenchymal stem cell; NHLF, normal human lung fibroblasts; RT-PCR, reverse transcription polymerase chain reaction.

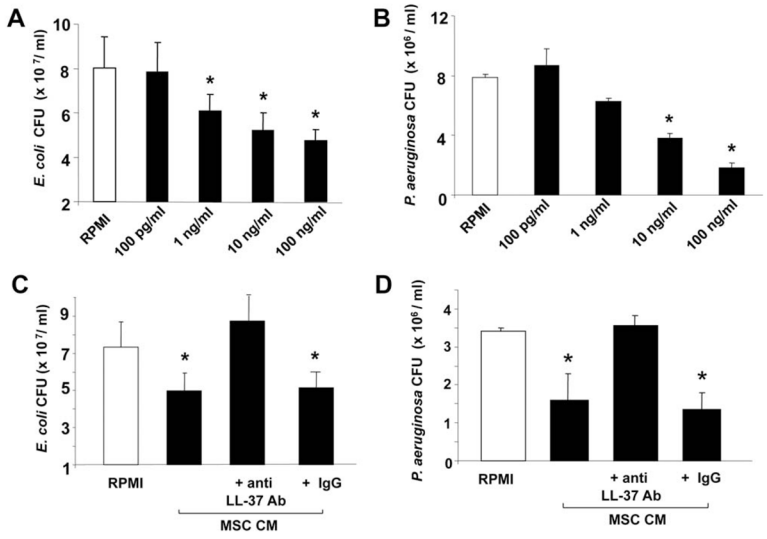

Figure 3.

Antimicrobial activity of MSC CM is mediated by LL-37 secretion. **(A):** Synthetic LL-37 showed significant antimicrobial activity against *E. coli* in a dose-dependent manner. Data are mean ± SD; *, $p < .001$ versus RPMI by analysis of variance (ANOVA; Bonferroni) $n = 3$. **(B):** Synthetic LL-37 displayed dose-dependent antimicrobial effect against *P. aeruginosa*. Data are mean ± SD; *, $p < .001$ versus RPMI by ANOVA (Bonferroni) $n = 3$. Preincubation of MSC CM with anti-LL-37 antibody (1 μ;g/ml), but not with mouse IgG (1 μg/ml), significantly reduced the antimicrobial effect of MSC CM against *E. coli* **(C)** and *P. aeruginosa* **(D)**. Data are mean ± SD; *, $p < .0002$ versus MSC CM + anti-LL-37 by ANOVA (Bonferroni), $n = 5–7$. Abbreviations: CFU, colony-forming unit; MSC CM, mesenchymal stem cell conditioned medium; RPMI, RPMI-1640 medium.

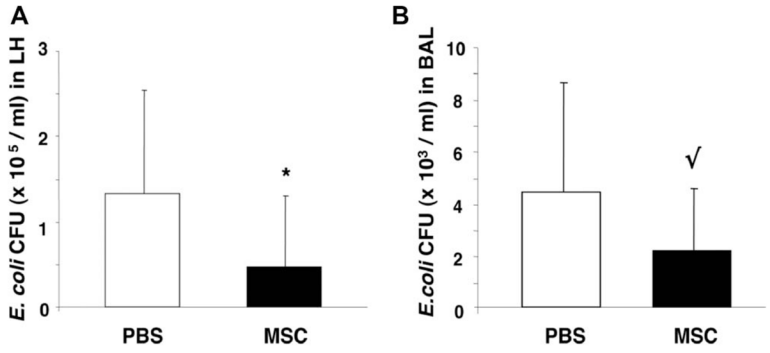

Figure 4.

MSCs reduce bacterial growth in an *E. coli* pneumonia mice model. Intratracheal administration of MSCs 4 hours following *E. coli* instillation significantly reduced *E. coli* CFU growth in mouse lung homogenates **(A)** and BAL fluid **(B)** 18 hours after infection. Data are mean ± SD; *, $p < .03$ versus PBS-treated control mice for lung homogenates; √, $p < .04$ versus PBS-treated control mice for BAL; $n = 12–14$. Abbreviations: BAL, bronchoalveolar lavage; CFU, colony-forming unit; LH, lung homogenates; MSC, mesenchymal stem cell; PBS, phosphate buffered saline.

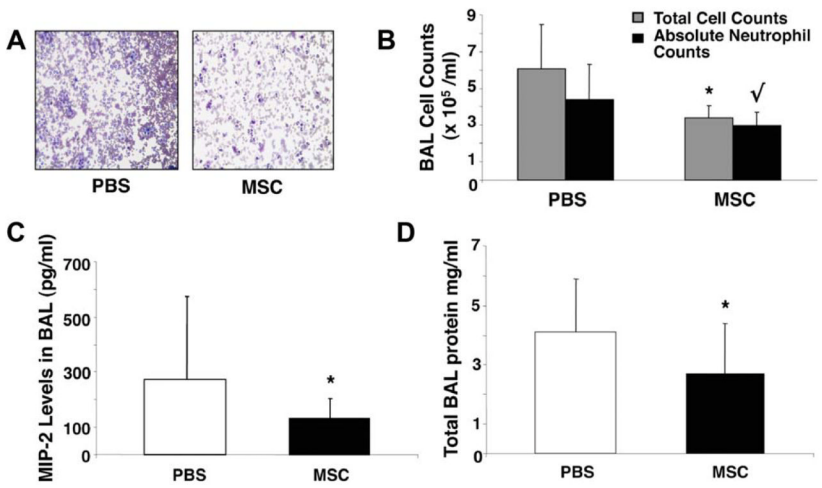

Figure 5.

Effect of MSCs on cellular responses and protein influx in the airspaces of *E. coli* pneumonia in mice. **(A):** MSC-treated mice had reduced levels of cells, blood, and edema in the BAL as compared with PBS treated following *E. coli* pneumonia in mice. Images are representative for each condition performed at least in triplicates. **(B):** Mice treated with MSCs had reduced total BAL cell and neutrophil counts. *, $p < .04$ versus PBS-treated mice for total cell counts; √, $p < .05$ versus PBS-treated mice for absolute neutrophil counts; n = 12–14. **(C):** Levels of the neutrophil chemokine, MIP-2, were significantly lower in the BAL of MSC-treated group as compared with control. *, $p < .05$ versus PBS-treated mice; n = 15–16. **(D):** MSC significantly improved lung endothelial/epithelial permeability to protein as represented by total BAL protein at 18 hours. Data as mean ± SD; *, $p < .05$ versus PBS-treated mice; n = 12–14. Abbreviations: BAL, bronchoalveolar lavage; MIP-2, macrophage inflammatory protein; MSC, mesenchymal stem cells; PBS, phosphate buffered saline.

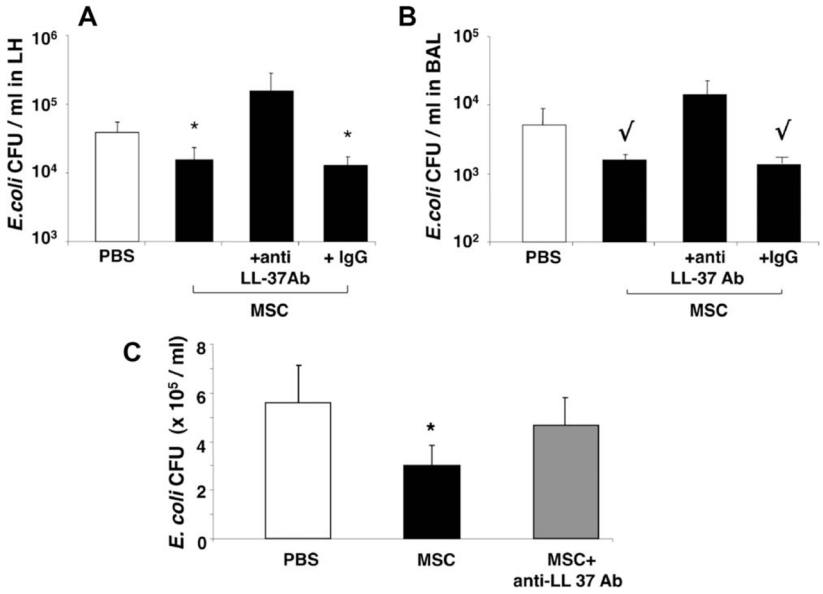

Figure 6.

Intratracheal administration of anti-LL 37 antibody reduced the therapeutic effect of MSC in *E. coli* pneumonia. Coadministration of MSC together with an anti-LL 37 neutralizing antibody (10 μg), but not with mouse IgG isotype antibody, inhibited the therapeutic effect of MSC in bacterial clearance in lung homogenates **(A)** and BAL fluid **(B)**. Values are mean CFU ± SD; *, $p < .002$ versus MSC + anti-Ll-37 antibody treated mice; √, $p < .005$ versus MSC + anti-LL-37 antibody-treated mice by analysis of variance (ANOVA; Bonferroni), $n = 5$. **(C):** MSC administration enhanced antimicrobial activity of mouse BAL. BAL samples were incubated with *E. coli* (10^5 CFU/ml) for 2 hours, and CFU growth was counted. Data are mean ± SD; *, $p < .002$ versus BAL of PBS-treated mice by ANOVA (Bonferroni), n = 8–9. Abbreviations: BAL, bronchoalveolar lavage; CFU, colony-forming unit; LH, lung homogenates; MSC, mesenchymal stem cell; PBS, phosphate buffered saline.

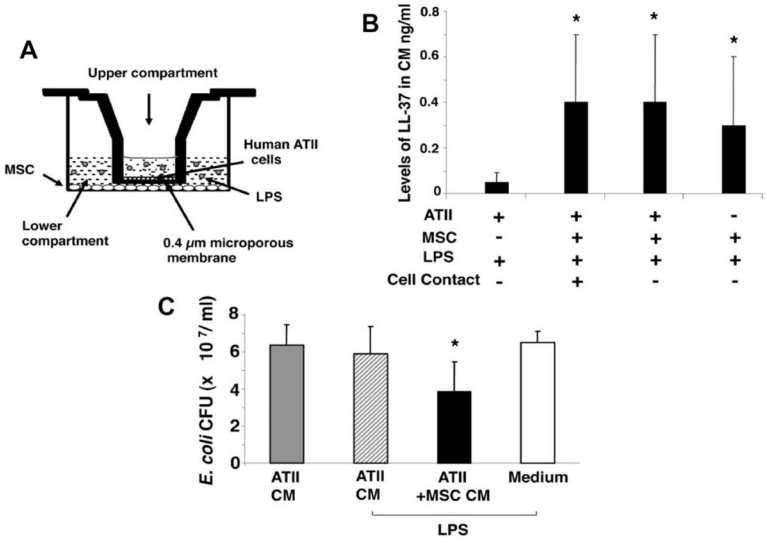

Figure 7.

MSCs are the primary source of LL-37 secretion in cocultures of MSCs and human ATII cells. **(A):** Human MSCs and ATII cells were cocultured, with or without contact, in the presence of LPS. **(B):** LL-37 was measured following each condition. *, $p < .02$ versus ATII alone by analysis of variance (ANOVA; Bonferroni), $n = 8–9$. **(C):** Conditioned medium from cocultures of ATII cells and MSC in the presence of LPS (100 ng/ml) significantly inhibited bacterial growth. Data are mean ± SD; *, $p < .002$ by ANOVA (Bonferroni) versus ATII + LPS, $n = 8–9$. Abbreviations: ATII, alveolar epithelial type II cells; CFU, colony-forming unit; CM, conditioned medium; LPS, lipopolysaccharide; MSC, mesenchymal stem cell.