Mesenchymal stem cells enhance survival and bacterial clearance in murine *Escherichia coli* pneumonia

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**ABSTRACT**

**Rationale** Bacterial pneumonia is the most common infectious cause of death worldwide and treatment is increasingly hampered by antibiotic resistance. Mesenchymal stem cells (MSCs) have been demonstrated to provide protection against acute inflammatory lung injury; however, their potential therapeutic role in the setting of bacterial pneumonia has not been well studied.

**Objective** This study focused on testing the therapeutic and mechanistic effects of MSCs in a mouse model of Gram-negative pneumonia.

**Methods and results** Syngeneic MSCs from wild-type mice were isolated and administered via the intratracheal route to mice 4 h after the mice were infected with *Escherichia coli*. 3T3 fibroblasts and phosphate-buffered saline (PBS) were used as controls for all in vivo experiments. Survival, lung injury, bacterial counts and indices of inflammation were measured in each treatment group. Treatment with wild-type MSCs improved 48 h survival (MSC, 55%; 3T3, 8%; PBS, 0%; p<0.05 for MSC vs 3T3 and PBS groups) and lung injury compared with control mice. In addition, wild-type MSCs enhanced bacterial clearance from the alveolar space as early as 4 h after administration, an effect that was not observed with the other treatment groups.

The antibacterial effect with MSCs was due, in part, to their upregulation of the antibacterial protein lipocalin 2.

**Conclusions** Treatment with MSCs enhanced survival and bacterial clearance in a mouse model of Gram-negative pneumonia. The bacterial clearance effect was due, in part, to the upregulation of lipocalin 2 production by MSCs.

**INTRODUCTION**

Bacterial pneumonia is the third most common cause of death worldwide, the most common cause of death in developing countries, and the most common infectious cause of death worldwide. The incidence of antibiotic resistance among bacterial isolates has been increasing at a rapid rate, thereby greatly limiting the armamentarium of medications available to treat patients with bacterial pneumonia. Therefore, given the large public health impact of bacterial pneumonia and the decreasing supply of effective antimicrobials, novel therapies are needed.

The innate immune response against bacterial pathogens in the lung classically consists of resident alveolar macrophages, recruited neutrophils, and endogenous antimicrobial factors present in the respiratory secretions. There is increasing evidence that lipocalin 2, which inhibits bacterial growth by blocking iron uptake, is one of the critical proteins involved in the innate immune response to bacterial infection in the lung. It has been recently demonstrated that lipocalin 2 is needed for effective host defence against pulmonary infection by *Escherichia coli*, *Mycobacterium tuberculosis* and *Klebsiella pneumoniae*. The lack of lipocalin 2 expression resulted in increased morbidity and mortality of infected mice, particularly in models of Gram-negative pneumonia. Although lipocalin 2 has been shown to be produced by multiple cell types, including neutrophils, macrophages and epithelial cells, there has also been recent literature reporting that a stromal cell line from bone marrow (ST2) may be a source of lipocalin 2.

Mesenchymal stem cells (MSCs) are multipotent, adult stem cells that are classically isolated from the bone marrow. Recent literature has demonstrated that these cells have potent immunomodulatory properties, including the suppression of pro-inflammatory processes in the lung in response to endotoxin and bleomycin. Given the predominantly immunosuppressive phenotype...
of MSCs, there has been concern over their effect on host defence to a live bacterial infection. Since bacterial pneumonia and sepsis are the two most common causes of respiratory failure from acute lung injury and acute respiratory distress syndrome, understanding the effect of MSCs in these settings is important. A recent study by Nemeth et al. demonstrated that MSCs improved survival in a cecal ligation and puncture model of sepsis and led to lower bacterial counts in the blood. However, the mechanism by which MSCs enhanced bacterial clearance was not presented. Also, a recent publication by Mei et al. demonstrated that MSCs can enhance the phagocytic capacity of host immune cells when administered in a mouse model of sepsis. Another recent study by our group provided evidence that human MSCs directly release the antibacterial peptide LL-37 in response to bacterial infection. We conducted this study to investigate the therapeutic and mechanistic effects of murine MSCs in a well characterised model of bacterial pneumonia in mice and to specifically determine the role of lipocalin 2 in the antimicrobial effect of MSCs in this model.

METHODS
Please see online supplement for more details.

Animal care
C57BL/6 male mice (8–10 weeks old; Jackson Laboratory) were used in all experiments. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco and the University of Pittsburgh.

Experimental design
The experimental design was similar to that used by our group previously, with the exception that lung injury was induced with the intratracheal instillation of live Escherichia coli K1 bacteria at a concentration of 106 cfu/25 μl of phosphate-buffered saline (PBS).

Cell cultures
Wild-type mouse MSCs were isolated and expanded from the bone marrow of green fluorescent protein positive (GFP+)-C57BL/6 mice using the same protocol described previously with the exception that cells were used at passage numbers 5–10 not at higher passages as used in the previous study. Lower passage numbers were used in this study because recent literature has demonstrated that long-term cultures of MSCs are predisposed to malignant transformation and loss of biological activity. Cells fulfilled the criteria for MSCs as established by the International Society of Cellular Therapy.

Protein assays
Cytokines and lipocalin 2 levels were measured using standard ELISA kits from R&D (Minneapolis, MN, USA). Myeloperoxidase was measured using standard methods as a way to quantify neutrophil degranulation.

In vivo lipocalin 2 blocking studies
In vivo lipocalin 2 blocking studies were performed to determine the importance of the upregulation of lipocalin 2 observed with MSC treatment, and its relationship to the antibacterial effect.

Lipocalin 2 production by lipopolysaccharide-stimulated MSCs
Conditioned media from unstimulated and lipopolysaccharide (LPS)-stimulated MSCs were obtained, and lipocalin 2 was measured using an ELISA kit (R&D).

Alveolar macrophage isolation and stimulation
Alveolar macrophages were isolated from normal C57BL/6 mice by bronchoalveolar lavage (BAL) using calcium- and magnesium-free PBS supplemented with 0.6 mM ethylenediaminetetraacetic acid (EDTA). The cells were then plated and stimulated with either LPS (±MSCs in a transwell) or with conditioned media obtained from LPS-stimulated MSCs. Supernatants were collected and analysed by ELISA for lipocalcin 2 and tumour necrosis factor α (TNFα) levels.

Stimulation of MSCs with alveolar macrophage conditioned media and inflammatory cytokines
MSCs were stimulated with either conditioned media from LPS-stimulated alveolar macrophages or with inflammatory cytokines in the presence of LPS. The supernatant from these studies was used to measure lipocalcin 2 concentrations.

Statistical analysis
Comparisons between the two groups were made using an unpaired t test. The log-rank test was used for comparing survival data. A value of p<0.05 was considered statistically significant. Analyses were done using SPSS software and GraphPad Prism software. Data are shown as mean±SD.

RESULTS
MSCs improve survival and phenotype in the E coli pneumonia model
MSC-treated mice had a significantly higher rate of survival over 48 h compared with fibroblast (3T3)-treated and PBS-treated mice (figure 1). MSC treatment also resulted in an improvement in the activity and phenotype of the mice at 48 h compared with fibroblast-treated and PBS-treated mice.

MSCs reduce the severity of lung injury in the E coli pneumonia model
MSC-treated mice had a significant reduction in the severity of lung injury as measured by excess lung water (figure 2A). The quantity of excess lung water was measured for mice in each group at the time of death or at the endpoint of the experiment (48 h). Histological analysis also demonstrated qualitatively less...
lung injury in the MSC-treated mice compared with the other groups (figure 2B). Histology was obtained from a mouse from each group that survived the longest in order to accurately compare the severity of lung injury between groups. Immunohistochemistry for GFP expression demonstrated no detectable GFP+ MSCs in the lung at 48 h after infection with *E. coli*.

**MSC treatment enhanced bacterial clearance from the lung as early as 4 h after instillation**

Treatment with MSCs enhanced bacterial clearance from the alveolar space as early as 4 h after instillation. This was determined by culturing the BAL fluid 4 h after treatment with MSCs or control (figure 3A).

This reduction in bacterial burden persisted at 24 h when the number of *E. coli* in the whole lung homogenate was measured in MSC-treated and control mice (figure 3B).

**MSCs reduced the early pro-inflammatory response to *E. coli* pneumonia**

Treatment with MSCs reduced the early pro-inflammatory response to *E. coli* instillation as measured by 8 h BAL levels of macrophage inflammatory protein 2 (MIP-2) and TNFα (figure 4A,B). MSC-treated mice also had evidence of less neutrophil degranulation in the alveolar space as assessed by BAL levels of myeloperoxidase (figure 4C). MSC treatment had no effect on the level of interleukin 10 (IL-10) in the BAL fluid compared with control mice (data not shown).

**MSCs upregulate the concentration of lipocalin 2 in the alveolar space**

Levels of lipocalin 2 were quantified by ELISA, which demonstrated a 56% increase in the quantity of lipocalin 2 in the BAL fluid of MSC-treated mice compared with PBS-treated mice (figure 5A). Other antimicrobial factors were also screened for, including defensins and collectins such as surfactant protein-D, but the results were no different for MSC-treated and control mice.

**Blocking lipocalin 2 inhibits the in vivo antibacterial activity of MSCs**

To determine if lipocalin 2 was an important mediator of the bacterial clearance effect seen with MSC treatment, blocking studies were done using the in vivo model of *E. coli* pneumonia. When the lipocalin 2 blocking antibody was premixed with...
MSCs at the time of cell delivery, the antibacterial effect of MSC treatment was eliminated compared with control mice. When MSCs were premixed with the isotype control antibody, the cells had preserved antibacterial activity compared with PBS-treated mice (figure 5B).

MSCs upregulate mRNA and protein production of lipocalin 2 in response to LPS stimulation
To determine whether MSCs, themselves, were an important source of lipocalin 2 or other known antimicrobial proteins, quantitative reverse transcriptase PCR was carried out for lipocalin 2 gene expression in unstimulated and LPS-stimulated MSCs. As seen in figure 6A, MSCs increased their transcription of the lipocalin 2 gene by approximately twofold upon LPS stimulation.

To determine if MSCs produced lipocalin 2 protein in a measurable quantity, cells were stimulated with LPS for 24 h to allow for adequate time for protein synthesis and secretion and then the conditioned media was collected to measure lipocalin 2 levels by ELISA. As shown in figure 6B, MSCs significantly upregulated production of lipocalin 2 protein with LPS stimulation, which is consistent with the enhanced mRNA expression observed. However, the absolute quantity of lipocalin 2 in the MSC-conditioned media was still relatively low compared with that measured in the BAL fluid from in vivo experiments (figure 5A).

Co-culture of LPS-stimulated MSCs and alveolar macrophages enhances production of lipocalin 2
Given that the amount of lipocalin 2 produced by MSCs was much less than that measured in the BAL fluid obtained from in vivo experiments, we hypothesised that MSCs upregulated lipocalin 2 production by innate immune cells in the lung based on previous literature demonstrating that neutrophils and...
Macrophages can contribute to intrinsic lipocalin 2 production in the lung. The principal immune cell present in the lung during the initial inflammatory response to bacterial infection is the alveolar macrophage. Because MSCs were delivered by the intratracheal route in this study, we hypothesised that the interaction between MSCs and alveolar macrophages may contribute to enhanced lipocalin 2 production.

To test this hypothesis, MSCs were co-cultured with alveolar macrophages in a transwell system and stimulated with LPS for up to 24 h. At this time, the media was collected and lipocalin 2 measured by ELISA. As shown in figure 7A, culturing MSCs and alveolar macrophages together in the presence of LPS resulted in a synergistic effect on lipocalin 2 production compared with levels measured by either cell type alone. The presence of the transwell to physically separate the MSCs and macrophages suggested that the upregulation of lipocalin 2 production occurred by a paracrine mechanism. Furthermore, the amount of lipocalin 2 detected under these conditions was comparable to that measured in the in vivo BAL samples, suggesting that the interaction between MSCs and alveolar macrophages was relevant to the in vivo findings.

**Conditioned media from LPS-stimulated alveolar macrophages enhances lipocalin 2 production by MSCs, an effect that is recapitulated by TNFα.**

To test our original hypothesis that MSCs enhanced alveolar macrophage production of lipocalin 2, we incubated alveolar macrophages with conditioned media obtained from LPS-stimulated MSCs and measured the amount of lipocalin 2 that was produced. However, contrary to our hypothesis, the addition of LPS-stimulated MSC conditioned media did not result in an increase in lipocalin 2 levels compared with stimulation of alveolar macrophages with LPS alone (figure 7B). Unexpectedly, incubation of MSCs with LPS-stimulated alveolar macrophage

![Figure 7](http://thorax.bmj.com/)

Mesenchymal stem cells (MSCs) enhance their secretion of lipocalin 2 in response to a combination of inflammatory signals including tumour necrosis factor α (TNFα), produced by activated macrophages, and lipopolysaccharide (LPS). (A) Co-culture of MSCs and alveolar macrophages (AMs) led to a significant increase in the quantity of lipocalin 2 produced (n=8–12 per group, *p<0.0000001, #p<0.0001). (B) The conditioned media of LPS-stimulated AMs augmented MSC secretion of lipocalin 2, whereas the conditioned media of LPS-stimulated MSCs had no effect on AM production of lipocalin 2 (n=4 per group, √p<0.00001 vs AM + LPS group, *p<0.0000001 vs MSC + LPS group). (C) Incubation of MSCs with cytomix resulted in an increase in lipocalin 2 production commensurate with that seen with LPS-stimulated AM-conditioned media. This effect was driven primarily by TNFα (n=3–4 per group, √p<0.00001 vs MSC + LPS group, *p<0.001 vs MSC + LPS + TNFα group). (D) Incubation of LPS-stimulated MSCs with TNFα resulted in a significant increase in lipocalin 2 production starting at 200 pg/ml of TNFα; in the absence of LPS, TNFα had no effect on lipocalin 2 production by MSCs (n=4 per group, *p<10−11 vs MSC + LPS group, √p=0.0003 vs MSC + LPS + 200 pg/ml TNFα group, #p<0.05 vs MSC + 1000 pg/ml TNFα). Data are mean±SD.
conditioned media led to a pronounced increase in lipocalin 2 production, similar to that observed when both cells were incubated together in the presence of LPS (figure 7B).

Given that alveolar macrophages are well known to produce significant quantities of inflammatory mediators in response to endotoxin or bacterial stimulation, we hypothesised that the principal mediators involved in upregulating MSC production of lipocalin 2 could be the pro-inflammatory cytokines TNFα and IL-1β. This hypothesis was also derived from recent studies that reported that IL-1β and TNFα have the capacity to upregulate cellular production of lipocalin 2 at the transcriptional level. Therefore, in the presence of LPS, MSCs were incubated with TNFα, IL-1β, and interferon γ (INFγ) simultaneously (cytomix) and individually to assess their effect on MSC production of lipocalin 2. As shown in figure 7C, cytomix led to an upregulation of lipocalin 2 production by MSCs in a quantity similar to that seen with LPS-stimulated alveolar macrophage conditioned media. The strongest effect on lipocalin 2 production by MSCs was seen with TNFα alone, while there was no effect with INFγ.

Therefore, the amount of TNFα was measured in LPS-stimulated alveolar macrophages (8080±642 pg/ml), and in the presence of LPS, MSCs were then incubated with a range of TNFα concentrations to reflect the quantity produced by alveolar macrophages in vitro and what was measured in vivo (figure 4B). As shown in figure 7D, TNFα stimulated MSC production of lipocalin 2 at a concentration as low as 200 pg/ml and resulted in maximal production at 500 pg/ml, suggesting that physiological levels of TNFα are sufficient to induce upregulation of lipocalin 2 in MSCs. However, in the absence of LPS, TNFα had no effect on lipocalin 2 production by MSCs, indicating that LPS is a required co-factor in the stimulatory effect.

**DISCUSSION**

There are three main findings of the current study: (1) MSCs reduce lung injury and improve survival in a model of Gram-negative bacterial pneumonia; (2) MSCs enhance bacterial clearance in the alveolar space as early as 4 h after administration, while still retaining their classic immunosuppressive properties; (3) MSCs significantly upregulate their production of lipocalin 2 in response to LPS and inflammatory mediators generated by activated macrophages, and this response contributes to the antibacterial effect observed with MSC treatment.

The finding that MSC treatment can lead to enhanced bacterial clearance from the lung is of particular importance because MSCs have been thought to have a primarily immunosuppressive phenotype and therefore a potentially deleterious effect on host defence to bacterial infection. To explain the antibacterial effect observed with MSC treatment, we hypothesised that lipocalin 2, a protein that binds bacterial siderophores and restricts iron utilisation by bacteria, may be an important antimicrobial factor. Our data demonstrated that treatment with MSCs led to a significant upregulation of lipocalin 2 levels in the alveolar space, and that blocking lipocalin 2 in vivo eliminated the bacterial clearance effect observed with MSCs. These results suggest that lipocalin 2 is an important mediator of the antibacterial effect seen with MSC treatment in the model of pneumonia used in this study. It is important to note that the general role of lipocalin 2 in host defence to pulmonary infection is unclear as there are reports of the ability of clinical isolates of the Gram-negative bacteria *K pneumoniae* to cause respiratory infection through evasion of the inhibitory effects of lipocalin 2.

Following the finding that lipocalin 2 is an important antibacterial protein upregulated by MSC administration, we focused on determining the cellular sources of lipocalin 2 in this model. We carried out a series of in vitro experiments that demonstrated that alveolar macrophages stimulated MSCs to enhance their secretion of lipocalin 2 to levels comparable to that measured in vivo. This effect was recapitulated by TNFα stimulation in the presence of LPS. It is important to note that the ability of TNFα to upregulate lipocalin 2 production by MSCs was observed in the presence of concurrent LPS stimulation, which was added to the conditions to model the effect of LPS-stimulated alveolar macrophage conditioned media. When MSCs were stimulated with TNFα alone there was no increase in lipocalin 2 production. These data are consistent with previous studies that have shown that TNFα alone does not lead to upregulation of lipocalin 2. Therefore, in the presence of LPS, MSCs were incubated with TNFα, IL-1β, and interferon γ (INFγ) simultaneously (cytomix) and individually to assess their effect on MSC production of lipocalin 2. As shown in figure 7C, cytomix led to an upregulation of lipocalin 2 production by MSCs in a quantity similar to that seen with LPS-stimulated alveolar macrophage conditioned media. The strongest effect on lipocalin 2 production by MSCs was seen with TNFα alone, while there was no effect with INFγ.

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There are still some limitations and unanswered questions from this study. Despite the finding that MSCs can produce significant quantities of lipocalin 2 in response to stimulation with LPS and TNFα, we do not know the contributions to lipocalin 2 production by other cell types in the lung and other innate immune cells. It is well known that neutrophils and lung epithelial cells make lipocalin 2, so understanding more completely the relative contributions from other cell types will be important. This can likely only be assessed through a combination of strategies using selective deletion of cell populations and genetically modified mice deficient for lipocalin 2.

Also, additional work will be needed to determine if camp, the mouse homologue of the antibacterial peptide LL-37, interacts with the production of lipocalin 2. Our group recently published a study demonstrating that human MSCs secrete LL-37 in quantities sufficient to kill bacteria in a similar Gram-negative pneumonia model. However, it is unclear what the relevance of this finding will be in mouse MSCs, which is the focus of the current study. Therefore, it will be necessary to determine if mouse MSCs secrete camp, and if this results in direct bacterial killing or participates in the regulation of the secretion of other antimicrobial proteins such as lipocalin 2. It is also possible that MSCs secrete other antimicrobial factors in addition to lipocalin 2 and LL-37, and these factors may also participate in the regulation of the antimicrobial effects of MSCs.

In summary, this study demonstrates that MSC-based therapy improves survival, reduces lung injury and enhances bacterial clearance when used as a treatment for bacterial pneumonia in mice. Part of the bacterial clearance effect observed with MSC treatment is mediated by their ability to upregulate lipocalin 2 production in response to inflammatory stimuli such as LPS and TNFα. These findings demonstrate that MSCs can directly enhance the innate immune response to bacterial infection in the lung.

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SUPPLEMENTARY FILE

METHODS

Experimental design. To briefly summarise, mice were anaesthetised with ketamine (90 mg/kg) and xylazine (10 mg/kg) i.p. E. coli K1, at a concentration of $10^6$ cfu/25 µl, was then delivered intratracheally (IT) to each mouse. The concentration of E. coli was determined with the use of a (Beckman) spectrophotometer. Mice were then recovered in a chamber with supplemental oxygen while they awakened from anesthesia. 4 h after the exposure to E. coli, mice were reanaesthetised with ketamine and xylazine, at half the original dose, and then given treatment IT with either wild type MSCs, 3T3 mouse lung fibroblasts, or PBS. 3T3 lung fibroblasts were chosen as a cell control in this study since they are a somatic cell line of mesenchymal lineage and have been used by our group as a cell control in earlier studies (7). The cells were delivered at a concentration of 750,000 cells/30 µl and PBS was given at a volume of 30 µl. This dose was based upon the work that our group previously published (7). Mice were then recovered again as before and the time course of the experiments ranged from 8-48 h, at which time survival was noted and samples were collected for microbiological, biochemical, and physiological analyses.

Cell culture. MSCs were cultured in alpha-MEM + 15% FBS + 1% Penicillin/Streptomycin culture medium. MSC differentiation potential was verified using the Mouse Mesenchymal Stem Cell Functional Identification Kit (R&D Systems, MN, USA, See Supplementary Figure 1). 3T3 fibroblasts were obtained from ATCC, and were maintained in cell culture using DMEM + 10% FBS + 1% Penicillin/Streptomycin.
culture medium. In all experiments, fibroblasts were used at passage numbers 5-10, similar to the MSCs.

**Intratracheal instillations.** The details of this method have been previously published by our group (1,2). Briefly, mice were anesthetised as described above and then fixed at a 60° angle on an intubating board. A fiber optic light source was placed immediately over the neck, and the oropharynx was opened with forceps. The instillate was then injected directly into the trachea using a PE-10 catheter attached to a 0.5-ml syringe.

**Excess lung water measurements.** These calculations were made using standard techniques as previously published by our group (3,4).

**Histology and immunohistochemistry.** Lungs from all treatment groups were excised, fixed with 100% ethanol IT and immersed in 100% ethanol. After fixation, lungs were embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin and eosin (H&E). For immunohistochemistry, paraffin sections were passed through xylene, graded alcohol, and rinsed in phosphate buffered saline (PBS). Endogenous peroxide was inactivated using 3% hydrogen peroxide (Sigma). Slides were heated in citrate buffer for 15 min and blocked in blue blocker (Shandon Lipshaw). Sections were incubated overnight at 4°C in a humidour or for 1 to 2 h at room temperature with primary antibody (rabbit polyclonal anti-GFP, Abcam) diluted 1:100 with PBS containing 1 mg/mL bovine serum albumin. Three 5-min rinses with PBS were performed after each successive step. Sections were incubated in PBS containing the secondary antibody (Chemicon) for 1 h at
room temperature. After the washes, the signal was detected using the ABC Elite kit (Vector Laboratories) for GFP according to the manufacturer’s instructions. Colour development was monitored under a microscope. Sections were counterstained with modified Harris hematoxylin solution (Sigma). After dehydration by passage through graded alcohol concentrations and xylene, sections were mounted using DPX (Fluka Laboratories) before observation. For negative control, all steps were performed as described above with the exception of the application of primary antibody.

**Bronchoalveolar lavage (BAL).** As described in previous publications (1,5), BAL was done after euthanizing the mice, placing a 20 gauge catheter into the trachea, and then flushing 1ml of cold PBS into the trachea back and forth three times. The BAL sample was then processed to obtain cell counts and the supernatant was frozen for future protein analyses.

**ELISA measurements.** Levels of TNF-α, MIP-2, IL-10, and lipocalin 2 were measured in BAL fluid and cell conditioned medium using R&D ELISA or DuoSet kits (R&D Systems, MN, USA, mean minimum detectable dose ranged from 1.5 – 4.0 pg/ml).

**Quantification of bacterial colonies.** The number of *E. coli* cfu in the BAL was determined by diluting the freshly obtained samples 1:100 in a total volume of 1ml of PBS and then plating 100 μl of this diluted sample on LB Agar plates. Plates were placed in a 37°C incubator overnight and then the number of colonies were counted the
following day. A similar protocol was followed for determining the number of *E. coli* cfu in the whole lung homogenate except that the dilution factor was increased to 1:10⁴.

**Phagocytosis studies.** To determine if MSCs can phagocytose *E. coli*, 10⁵ MSCs were incubated with 10⁶ cfu of bacteria for 60-90 minutes. This was done in a 5 ml polypropylene tube that was placed on a rotator in a 37°C incubator. After the incubation time period was done, the MSCs were collected by centrifugation and then lysed with 0.2% Triton-X. 100 μl of the lysate was then plated on an LB Agar plate and incubated overnight at 37°C; the colonies were counted the following day. As a negative control, 3T3 mouse lung fibroblasts were subjected to the same conditions to assess for phagocytosis, while freshly isolated peritoneal macrophages were used as a positive cell control. In addition to determining if MSCs themselves can phagocytose bacteria, MSCs were co-cultured with freshly isolated peritoneal macrophages or bone marrow neutrophils to see if MSCs could enhance phagocytosis of bacteria by other innate immune cells. Peritoneal macrophages and bone marrow neutrophils were isolated using standard protocols (6-8). In these studies, equal numbers of macrophages or neutrophils (10⁵ per cell type) ± MSCs (10⁵ cells) were incubated with 10⁶ cfu of *E. coli* for 30-60 minutes to allow for phagocytosis. Then the cells were isolated, lysed and plated as described above. In the co-culture conditions with MSCs, all recovered bacteria was presumed to be due to phagocytosis from the macrophages or neutrophils since it was determined from the prior experiments that MSCs did not exhibit significant phagocytic capacity.
**In vitro dose response effect of lipocalin 2.** A dose response for the antibacterial killing activity of lipocalin 2 was done to confirm that the levels that were measured in the BAL fluid represented a quantity of lipocalin 2 that could account for the bacterial clearance effect observed with MSC treatment. For these studies, $10^4$ cfu of *E. coli* was incubated with different concentrations of recombinant lipocalin 2 (R & D) in 100 µl of RPMI + 10% FBS for 5 hours and then the medium from each well was collected, diluted and plated to count colonies. In addition, conditions were done in which an anti-mouse lipocalin 2 blocking antibody (R & D) and isotype control antibody (R & D) were added to determine the effect of neutralizing lipocalin 2 on bacterial growth. Antibodies were used at a concentration of 2 µg/well (20 µg/ml) for these studies.

**In vivo lipocalin 2 blocking studies.** The experimental design of these studies was similar to that outlined above. First mice were infected with *E. coli* at a concentration of $10^6$ cfu/25 µl, and then 4 hours later mice received one of four treatments: (1) MSCs alone at the same concentration as in the initial studies (2) PBS alone, (3) MSCs + lipocalin 2 blocking ab, (4) MSCs + isotype control antibody. Antibodies were the same as the ones used for the *in vitro* dose response studies and were used at a concentration of 40 µg/mouse. Four hours after the treatment was delivered, mice were sacrificed and a BAL was done to measure the bacterial counts in each of the 4 groups. Bacterial counts were determined as before by diluting and plating the BAL fluid onto LB Agar plates.

**Quantitative RT-PCR of lipocalin 2 in MSCs.** RNA from 5 LPS-treated and 5 untreated MSC samples were checked with TaqMan RNA-to Ct 1-Step PCR protocol
using Taqman Gene Expression Assays for Lcn2 and Gusb as an endogenous control (Mm01324470_m1 and Mm00446954_g1, respectively, Applied Biosystems). RT-PCR experiments were performed in triplicates including the appropriate negative controls on an Applied Biosystems 7900HT instrument. Fold-change between the LPS-treated and the control samples was calculated using the relative quantitation $\Delta\Delta$Ct method followed by an unpaired Student’s t test for statistical significance verification.

**Lipocalin 2 production by LPS stimulated MSCs.** Conditioned media from cultures of MSC stimulated with LPS were obtained to determine if they produced lipocalin 2. MSCs were plated at a density 100,000 cells per well in a 24 well plate in RPMI-1640 supplemented with 10% FBS + 1% penicillin/streptomycin. Then the cells were either left unstimulated or were stimulated with LPS (1 µg/ml) for 24 hr. Supernatants were collected, centrifuged and concentrated 20, 30, or 40 times with Amicon Ultra-0.5 Centrifugal Filter Unit with Ultrace-3 membrane (3kDA) according to manufacturer instructions (Millipore). Lipocalin-2 protein levels in the cell culture supernatants were measured by mouse Lipocalin-2/NGAL ELISA or Duoset kit (R&D Systems, MN, USA).

**Alveolar macrophage isolation and stimulation.** A total of 10 ml was used in each mouse in 0.5-ml increments with a 30 second dwell time. The lavage fluids were pooled and centrifuged at 600 x g for 10 min, and the cells were collected for the co-culture assay. An aliquot of the harvested cell fraction was analyzed by cytospin and H & E staining to determine the percentage of alveolar macrophages. The cell suspension were
> 95% alveolar macrophages with no evidence of neutrophils. The cells were resuspended in RPMI-1640 containing 10% FBS and 1% penicillin/streptomycin at a concentration of 100,000 cells per well in 24 well plates. Cells were incubated for 24 hr hours, washed, and then stimulated with LPS (1 μg/ml) (E.coli 0111:B4; Sigma-Aldrich) with or without MSC (100,000 cells/insert) in a transwell (0.4-mkm pore size; Costar; Corning) for 24 hr. In separate experiments, alveolar macrophages were incubated with conditioned media obtained from MSCs stimulated with LPS for 24 hours.

**Stimulation of MSCs with alveolar macrophage conditioned media and inflammatory cytokines.** MSCs (100,000 cells/well) were stimulated for 24 hours with conditioned media from alveolar macrophages that had been stimulated with LPS for 24 hours, to determine if alveolar macrophages were producing a soluble factor to upregulate MSC production of lipocalin 2. Additionally, MSCs were stimulated with TNF-α, IL-1β, and IFN-γ (50 ng/ml, R & D systems) simultaneously and individually to test the effect of these cytokines on MSC production of lipocalin 2. A dose response analysis of MSC production of lipocalin 2 with a range of TNF-α concentrations was subsequently done after measuring the amount of TNF-α in the media of LPS stimulated macrophages (R & D ELISA).

**RESULTS**
MSCs do not phagocytose bacteria or enhance the phagocytosis of bacteria by other immune cells in vitro. To determine if the protective effect of MSCs was related to an effect on phagocytosis, in vitro studies were done in which MSCs +/- macrophages or neutrophils were incubated with *E. coli*. When MSCs alone were incubated with *E. coli* there was no evidence of phagocytosis beyond the level seen with the negative control, 3T3 fibroblasts. Furthermore, when MSCs were incubated with either neutrophils or macrophages, there was no enhancement of phagocytosis as compared to neutrophils or macrophages alone (Supplementary Figure 2).

**In vitro lipocalin 2 dose response study.** In order to determine if the levels of lipocalin 2 measured in the BAL fluid of the control and MSC treated mice were sufficient to result in bacterial killing, an in vitro dose response study was done using escalating quantities of lipocalin 2. As shown in Supplementary Figure 3, lipocalin 2 had measurable bacterial killing activity starting at a concentration of $3 \times 10^3$ pg/ml which increased significantly at a concentration of $3 \times 10^4$ pg/ml and higher. Therefore, the range of $3 \times 10^3$ to $3 \times 10^4$ pg/ml of lipocalin 2 represents a dynamic part of the dose response curve, and as a result, the differences measured in vivo could be expected to contribute to the differential bacterial killing activity observed with MSC treatment. Also, importantly, the dose response study demonstrated the ability of the lipocalin 2 blocking antibody to effectively neutralise the bacterial killing effect of lipocalin 2, while the isotype control had no significant effect.
REFERENCES


Figure 1S. MSCs demonstrated the ability to differentiate into bone, fat, and cartilage cells upon appropriate stimulation. (A) Adipogenic differentiation was determined by oil red staining (red: neutral triglycerides); (B) osteogenic differentiation was determined by immunofluorescent staining with anti-osteoponin Ab; (C) chondrogenic differentiation was detected by alcian blue staining (blue: mucopolysaccharides and glycosaminoglycans).

Figure 2S. MSCs neither directly phagocytose bacteria nor enhance the phagocytic capacity of immune cells, such as neutrophils and macrophages, using an in vitro model. MSCs exhibited similar phagocytic levels as the negative control, 3T3 fibroblasts. Neutrophils and macrophages both exhibited significantly higher levels of phagocytosis compared to MSCs and 3T3 cells. When MSCs were combined with either macrophages or neutrophils, the phagocytic capacity of these immune cells did not increase (n = 4-8 per group; * p < 0.01 vs MSC group, # p < 0.01 vs 3T3 group). Data as mean ± SD.

Figure 3S. Lipocalin 2 demonstrates significant antibacterial effects at concentrations similar to those measured in vivo. Lipocalin 2 exhibited significant
antimicrobial activity starting at a concentration of $3 \times 10^3$ pg/ml compared to bacterial growth in RPMI media alone. A lipocalin 2 blocking ab completely neutralised the antibacterial effect of lipocalin 2, while an IgG isotype ab had no effect (ab concentration was 20 μg/ml, $n = 3-8$ per group, $\sqrt{p} < 0.01$ vs RPMI). Data are mean ± SD.
Supplementary Figure 2

E. coli cfu (x10/ml)

MSC  3T3  Mac  Mac + MSC  PMN  PMN + MSC

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