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^{o} 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^{o}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 $1:10^4$.

Phagocytosis studies. To determine if MSCs can phagocytose *E. coli*, 10^5 MSCs were incubated with 10^6 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 (10^5 per cell type) ± MSCs (10^5 cells) were incubated with 10^6 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 ΔΔCt 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 Ultracel-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 3×10^3 pg/ml which increased significantly at a concentration of 3×10^4 pg/ml and higher. Therefore, the range of 3×10^3 to 3×10^4 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

1. **Gupta N**, Su X, Popov B, et al. Intrapulmonary delivery of bone marrow-derived mesenchymal stem cells improves survival and attenuates endotoxin-induced acute lung injury in mice. *J Immunol* 2007;**179**:1855-1863.
2. **Su X**, Looney M, Robriquet L, et al. Direct visual instillation as a method for efficient delivery of fluid into the distal airspaces of anesthetized mice. *Exp Lung Res* 2004;**30**:479-493.
3. **Modelska K**, Pittet JF, Folkesson HG, et al. Acid-induced lung injury : protective effect of anti-interleukin-8 pretreatment on alveolar epithelial barrier function in rabbits. *Am J Respir Crit Care Med* 1999;**160**:1450-1456.
4. **Ma T**, Fukuda N, Song Y, et al. Lung fluid transport in aquaporin-5 knockout mice**.** *J Clin Invest* 2000;**105**:93-100.
5. **Su X**, Johansen M, Looney MR, et al. CD47 deficiency protects mice from lipopolysaccharide-induced acute lung injury and Escherichia coli pneumonia. *J Immunol* 2008;**180**:6947-53.
6. **Looney MR**, Su X, Van Ziffle JA, et al. Neutrophils and their Fc gamma receptors are essential in a mouse model of transfusion-related acute lung injury. *J Clin Invest* 2006;**116**:1615-23.

7. **Looney MR**, Nguyen JX, Hu Y, et al. Platelet depletion and aspirin treatment protect mice in a two-event model of transfusion-related acute lung injury. *J Clin Invest* 2009;**119:**3450-61.
8. **Zhang X**, Goncalves R, Mosser DM. The isolation and characterisation of murine macrophages. *Curr Protoc Immunol* 2008;**83**:14.1.1-14.1.14.

FIGURE LEGENDS

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 3×10^3 pg/ml compared to bacterial growth in RPMI media alone. A lipocalin 2 blocking ab completey 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, √ $p < 0.01$ vs RPMI). Data are mean ± SD.