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Human Mesenchymal Stem (Stromal) Cells Promote the Resolution of Acute Lung Injury in Part through Lipoxin A_4

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Previous studies demonstrated that bone marrow–derived mesenchymal stem (stromal) cells (MSCs) reduce the severity of acute lung injury in animal models and in an ex vivo perfused human lung model. However, the mechanisms by which MSCs reduce lung injury are not well understood. In the present study, we tested the hypothesis that human MSCs promote the resolution of acute lung injury in part through the effects of a specialized proresolving mediator lipoxin A_4 (LXA_4). Human alveolar epithelial type II cells and MSCs expressed biosynthetic enzymes and receptors for LXA_4. Coculture of human MSCs with alveolar epithelial type II cells in the presence of cytomix significantly increased the production of LXA_4 by 117%. The adoptive transfer of MSCs after the onset of LPS-induced acute lung injury (ALI) in mice led to improved survival (48 h), and blocking the LXA_4 receptor with WRW4, a LXA_4 receptor antagonist, significantly reversed the protective effect of MSCs on both survival and the accumulation of pulmonary edema. LXA_4 alone improved survival in mice, and it also significantly decreased the production of TNF-α and MIP-2 in bronchoalveolar lavage fluid. In summary, these experiments demonstrated two novel findings: human MSCs promote the resolution of lung injury in mice in part through the proresolving lipid mediator LXA_4, and LXA_4 itself should be considered as a therapeutic for acute respiratory distress syndrome. ***The Journal of Immunology*****, 2015, 195: 000–000.**

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are major causes of acute respiratory failure in critically ill patients. Despite improvements in supportive care, mortality from ALI remains high (1). Pathophysiologic mechanisms of ALI and ARDS include inflammation and increased endothelial and epithelial permeability to protein, resulting in extravascular accumulation of protein-rich edema fluid and alveolar epithelial injury (1). Several preclinical studies have demonstrated that bone marrow–derived mesenchymal stem (stromal) cells (MSCs) reduce the severity of ALI induced by endotoxin (2, 3), live *Escherichia coli* bacteria (4, 5), or following sepsis (6–8). Much of the therapeutic benefit of MSCs appears to derive from the release of paracrine soluble factors, which stabilize the injured alveolar epithelium and lung endothelium, reduce inflammation, increase the absorption of pulmonary edema fluid, and have antimicrobial activity (9). Some studies found that the therapeutic effect of MSCs may be related in part to release of microvesicles, which are involved in cell–cell communication, mitochondria transfer, and keratinocyte growth factor in the injured alveolus (10-13); however, there is not yet a complete understanding of the mechanisms that mediate the beneficial effect of MSCs in ALI.

Resolution of ALI and repair of alveolar structures depend on a balance of inflammatory interactions and molecular signaling. Recent findings indicate that resolution is not merely a passive termination of inflammation, but rather an active biochemical and metabolic process (14). Several mediators, pathways, and molecular systems contribute to altered alveolar endothelial and epithelial permeability (15). Among these, lipoxins were the first lipid mediators recognized to have dual anti-inflammatory and proresolution activities (14, 16). Lipoxins are biosynthesized by the sequential actions of lipoxygenases and other enzymes to produce bioactive trihydroxytetraenes, structures that are found in all eicosanoids of this class. In humans, initial oxidation of arachidonic acid via 15-lipoxygenase and then by 5-lipoxygenase is one route of lipoxin biosynthesis that has been observed in mucosal tissues such as the respiratory tract, gastrointestinal tract, and oral cavity that results from the interactions between epithelial cells and leukocytes (15). A role for lipoxin A_4 (LXA_4) in alveolar epithelial cells and ALI is still not fully understood, although previous studies suggested that aspirin-triggered 15-epi-LXA_4 induces apoptosis in neutrophils in situ and facilitates resolution of myeloperoxidase-sustained neutrophil-dependent pulmonary inflammation (17). In this study, we tested the hypothesis that MSCs promote resolution of ALI through LXA_4. We first investigated the role of MSCs and LXA_4 with an in vitro coculture system of human MSCs and alveolar epithelial type II (AT II) cells. We then tested the role of MSCs and LXA_4 in an in vivo mice model of ALI induced by *E. coli* endotoxin.

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X.F. conceived and designed the study, collected, assembled, analyzed, and interpreted the data, and wrote the manuscript; J.A., L.C., and J.K.C. collected and assembled data; J.W.L. conceived and designed data analysis and interpretation; B.D.L. conceived and designed assembly of data, data analysis, and interpretation and wrote the manuscript; and M.A.M. conceived and designed the study, collected, assembled, analyzed, and interpreted the data, wrote the manuscript, and provided final approval of the manuscript.

The data presented in this article have been submitted to the National Center for Biotechnology Information's Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE68610) under accession number GSE68610.

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Abbreviations used in this article: ALI, acute lung injury; ALOX15, arachidonate 15-lipoxygenase; AT II, alveolar epithelial type II; i.t., intratracheally; LXA_4, lipoxin A_4; MSC, mesenchymal stem cell.

Materials and Methods

Primary cultures of human AT II cells

All experiments using cadaver human lung tissues were approved by the Biosafety Committee of the University of California, San Francisco. Human AT II cells were isolated from donated adult human lungs, which were not used for lung transplantation. Lungs were obtained from five adult males without lung disease who died after head trauma or cerebrovascular accident. Type II cells were isolated by our published methods (18, 19). Freshly isolated human AT II cells were plated in the upper compartment of Transwells (0.4-μm pore size and collagen I coated; Costar, Corning) in DMEM-H21 and F-12 Ham's (1:1) plus 10% FBS. Cells reached confluence after 48 h and formed monolayers at 96–120 h that were ready for experiments.

Human MSC culture

Allogeneic human MSCs were obtained from Dr. D.J. Prockop at the Institute for Regenerative Medicine in Texas A&M Health Science Center. Upon arrival, cells were thawed and expanded in culture flasks (BD Falcon) at a density of 500,000 cells/150 cm^2. Cells were passaged every 3–4 d by trypsinization when they reached 70–80% confluence and were used for the experimental protocols between passages 5 and 10 (5). MSCs were cultured in α-MEM without ribonucleosides or deoxyribonucleosides containing 2 mm L-glutamine, 10% FBS, penicillin, and streptomycin. Cells were cultured in a humidified incubator at 5% CO_2 and 37°C under sterile conditions. Cells were trypsinized, collected, and washed with PBS before intratracheal instillation in the mice. Some cells were stained with an annexin V/dead cell apoptosis kit (Invitrogen) and analyzed with BD FACSDiva 8.0 software to measure the percentage of apoptotic cells.

Coculture of human AT II cells and human MSCs

To determine whether MSCs could generate anti-inflammatory lipid mediators, we cocultured MSCs and AT II cells in a Transwell system as before (19). When human AT II cells were grown in a tight monolayer at 96–120 h, the cells were washed and serum starved with plain medium for 24 h. MSCs were then plated in the bottom compartment of the Transwell at a density of 250,000 cells/well with no direct contact with AT II cells. To injure the cells, we exposed the AT II cells and MSCs to a mixture of three different cytokines referred to as cytomix (IL-1β, TNF-α, and IFN-γ, 50 ng/ml each; R&D Systems). We previously reported that these were the major proinflammatory cytokines in pulmonary edema fluid from patients with ARDS (20). Experiments were carried out in eight different groups: 1) AT II cells alone, 2) MSCs alone, 3) AT II cells plus MSCs, 4) AT II cells plus cytomix, 5) MSCs plus cytomix, 6) ATII cells plus culture medium of MSCs stimulated with cytomix, 7) MSCs and culture medium of AT II cells stimulated with cytomix, and 8) AT II cells plus cytomix plus MSCs. AT II cells, MSCs, and cytomix were added simultaneously, and conditions were maintained for 24 h.

RNA isolation and DNA microarray analysis

Total RNA was extracted from cocultured human AT II cells and human MSCs using RNA STAT (Tel-Test, Friendswood, TX) per the manufacturer's instructions. All RNA samples had high integrity and purity as assessed by an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA). For microarray analysis, RNA was converted to biotin-labeled cRNA using Affymetrix reagents and protocol (http://www.affymetrix.com). The cRNA was hybridized to U133A Affymetrix microarray chips that contained 16–20 unique 25-mer oligonucleotide probes for ~14,500 human genes plus corresponding 12–16 probes with a single nucleotide change (mismatch control). Hybridizations were performed with cRNA prepared from cocultured human AT II and human MSCs experiments (n = 3–5 different preparations were tested per group).

Affymetrix Microarray Suite 5.0 was used to quantify and analyze mRNA content for expressed genes. The Microarray Suite software uses Wilcoxon signed rank tests to test whether a transcript is detectable on the array and the probability of a significant change between arrays, assigning p values and a change call (increase, decrease, or no change) for each probe. When more than one probe set was present for the same gene, data were combined to provide a mean value. Fold stimulation results are expressed as mean ± SD. All analyses were performed using the R/Bioconductor software (http://www.bioconductor.org). Array qualities were analyzed using the affyPLM package. The data were normalized by a robust multichip averaging method (21). Low-quality probesets identified as those with intensity value below the global 25th percentile and control probesets were filtered out. Limma package (22) was used to fit probeset level linear models to the data. The p values were adjusted by controlling the false discovery rate (23). A change in gene expression was identified as significant when the false discovery rate was <0.05. GOstat (24) was used to search for significant gene ontology terms for the significant genes.

Western blotting and immunostaining

Total cellular protein was extracted from the AT II cells and human MSCs exposed to different experimental conditions using 0.1 ml lysis buffer per well containing 1% Triton X-100, 20 mM Tris-Base (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM vanadate, 2 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 mM Pefabloc and homogenized. Protein levels were measured by the bicinchoninic acid method (Pierce). Each sample was first reduced and denatured with sample buffer and run on a 4–12% gradient Bis-Tris gel (Invitrogen), 10–20 μg proteins per lane, using a MOPS-SDS buffer (Invitrogen) at 100 V for roughly 2 h. The proteins were then transferred onto a nitrocellulose membrane and blocked with 5% milk in TBS with Tween 20 for 1 h. The nitrocellulose membrane was then exposed to the primary Ab overnight at 4°C. Primary Abs used were rabbit anti-human FPR2 (1:4000 dilution, Lifespan Biosciences). The protein bands were then visualized with a chemiluminescence agent, ECL+ (Amersham Biosciences), and quantitated with Carestream molecular imaging software.

For immunostaining of FPR2 in AT II cells, after completion of the experimental conditions, the AT II cell monolayer was washed twice with cold PBS and fixed in 4% paraformaldehyde for 30 min. Then the monolayer of cells was processed and embedded in paraffin section. Thin sections of the monolayer were cut and processed through deparaffinization/rehydration, Ag unmasking with citrate, blocking and incubation with primary Ab FPR2 (1:400 dilution) overnight, then followed by incubation with secondary goat anti-rabbit Ab (Molecular Probes). Nuclei were counterstained with DAPI. Images were obtained by Zeiss LSM 510 laser scanning confocal microscopy.

E. coli *endotoxin-induced ALI in mice*

All experiments using mice were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco. C57BL/6 male mice (8–10 wk old, ~24 g, The Jackson Laboratory) were first anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) i.p. ALI was then induced by the instillation of LPS from *E. coli* O55:B5 (Sigma-Aldrich) at 5 mg/kg intratracheally (i.t.). Mice for sham control were instilled with 30 μl PBS. Mice were then allowed to recover until they awakened from anesthesia. Four hours after the induction of injury, mice were anesthetized again with isoflurane and were divided into five groups: 1) PBS, 2) human bone marrow MSCs (500,000 cells in 30 μl PBS), 3) recombinant LXA_4 (10 μg/kg, i.t, Santa Cruz Biotechnology), 4) WRW4 (1 mg/kg), and 5) MSCs plus WRW4. Mice were followed for 72 h. Survival in each group was noted as well as body temperature and activity. At the end of either 24 or 48 h, samples were collected from each mouse for assessment of lung injury, biochemical analysis, and histology.

Measurement of LXA_4, arachidonate 15-lipoxygenase B protein content, MIP-2, TNF-α in coculture supernatant, and bronchoalveolar lavage fluid

The level of LXA_4 in bronchoalveolar lavage (BAL) fluid of mice and coculture supernatant of human AT II and MSCs were measured with the LXA_4 ELISA kit (Neogen, Lexington KY). Fresh samples were collected in amber vials mixed with methanol and then diluted and acidified with 1 N HCl. The samples were then passed through the C18 Sep-Pak light column (Waters) and eluted with methyl formate. A stream of N_2 was used to evaporate methyl formate and the residue was reconstituted with diluted extraction buffer and assay for LXA_4 content as the manufacturer instructed. Arachidonate 15-lipoxygenase (ALOX15)B protein level in lysates of human AT II cells, human MSCs, and macrophages were measured with an ALOX15B ELISA kit (MyBioSource, San Diego, CA). Total cell count in BAL fluid of mice was determined with Z1 Coulter particle counter (Beckman Coulter). Mouse MIP-2 and TNF-α were measured in the BAL fluid with ELISA kits (R&D Systems).

Extravascular lung water and lung histology

Gravimetric lung water determination was done by standard methods as we have reported (2, 25). Lungs from endotoxin injured with or without treatment with LXA_4 and MSCs were excised at 48 h. The lungs were inflated with 0.5 ml air and the trachea was ligated. The lungs were then fixed in 4% paraformaldehyde. After fixation, lungs were embedded in paraffin, cut into 5-μm sections, and stained with H&E.

Statistical analysis

For in vivo animal experiments and in vitro coculture assays, comparisons between two groups were made using an unpaired t test. For comparisons

Table I. Gene expression of synthetic enzymes and receptors for anti-inflammatory lipid mediators

Gene Title	Gene Symbol	MSC Cells			AT II Cells				
		MSC	MSC + Cyto	*p* Value (MSC + Cyto versus MSC)	AT II Alone	AT II + Cyto	AT II + Cyto + MSC	*p* Value (AT II + Cyto versus ATII)	*p* Value (ATII + Cyto + MSC versus ATII + Cyto)
		Log_2 Intensity			Log_2 Intensity				
Phospholipase A_2	PLA2G2A	4.18	4.46	0.16	4.26	4.28	4.64	0.93	0.04
	PLA2G2D	4.11	4.42	0.40	4.07	4.17	4.53	0.47	0.002
	PLA2G4A	6.89	9.03	0.0002	6.95	7.41	8.38	0.26	0.005
	PLA2G5	4.29	4.41	0.66	4.02	4.08	4.7	0.86	0.003
	PLA2G6	5.74	5.79	0.89	5.43	5.53	5.92	0.55	0.003
Arachidonate 5-lipoxygenase	ALOX5	3.75	3.93	0.53	4.62	4.5	4.81	0.78	0.33
Arachidonate 12-lipoxygenase	ALOX12	3.7	3.77	0.87	3.64	4.36	4.25	0.05	0.7
Arachidonate 15-lipoxygenase	ALOX15B	4.67	5.47	0.03	6.81	6.96	7.99	0.9	0.13
Lipoxin A_4 receptor	ALX/FPR2	3.85	3.99	0.65	3.13	3.27	3.78	0.48	0.01

Gene expression of PLA2, ALOX5, ALOX12, ALOX15B, and ALX/FPR2 in cocultured human mesenchymal stem cells and human alveolar epithelial type II cells with or without cytomix stimulation. Transcript levels were measured with Affymetrix microarrays. Data were normalized using robust multichip averaging method and are expressed as average log_2 intensity. The *p* values were adjusted by controlling the false discovery rate. Cyto, cytomix.

among multiple groups, ANOVA with Bonferroni correction was used. A p value < 0.05 was considered statistically significant. Analyses were done using STATA software. Data are shown as mean ± SD.

Results

Gene expression of biosynthetic enzymes and receptors for LXA_4 in human MSCs and human AT II cells

The data discussed in this study have been deposited in National Center for Biotechnology Information's Gene Expression Omnibus and are accessible through accession number GSE68610 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE68610). The results of Affymetric microarray analysis indicated that both human MSCs and AT II cells expressed several isoforms of phospholipase A_2 (Table I), an enzyme that catalytically hydrolyzes the S_N2 acyl bond of membrane phospholipids to release arachidonic acid for subsequent conversion to bioactive eicosanoids. In coculture, MSCs upregulated expression of most isoforms of phospholipase A_2 in AT II cells after cytomix stimulation (Table I). Human MSCs and human AT II cells also expressed arachidonate 5-lipoxygenase, arachidonate 12-lipoxygenase, and ALOX15 (Table I, no data for ALOX15A isoform because of no available probe at the time for the experiment). These enzymes are involved in the oxidation of arachidonic acid and biosynthesis of LXA_4. ALOX15B expression was significantly increased in MSCs stimulated with cytomix. ALX/FPR2, a receptor for LXA_4, was expressed in both human MSCs and human AT II cells. ALX/FPR2 expression in AT II cells was significantly increased by MSCs with cytomix stimulation.

ALOX15B and ALX/FPR2 protein levels in human MSCs and human AT II cells

To further validate the results from gene expression, we then measured the protein concentration of ALOX15B and ALX/FPR2 in cell lysates of human MSCs and AT II cells. ALOX15B protein concentration was higher in AT II cells than in MSCs under baseline conditions (Fig. 1A). Coculture of MSCs with AT II further increased the protein concentration of ALOX15B in AT II cells under cytomix stimulation (Fig. 1A). Western blotting showed that the LXA_4 receptor ALX/FPR2 was also expressed in human AT II cells and MSCs (Fig. 1B, *upper panel*). MSCs upregulated the ALX/FPR2 protein expression in human AT II cells stimulated with cytomix. Immunofluorescent staining of cultured

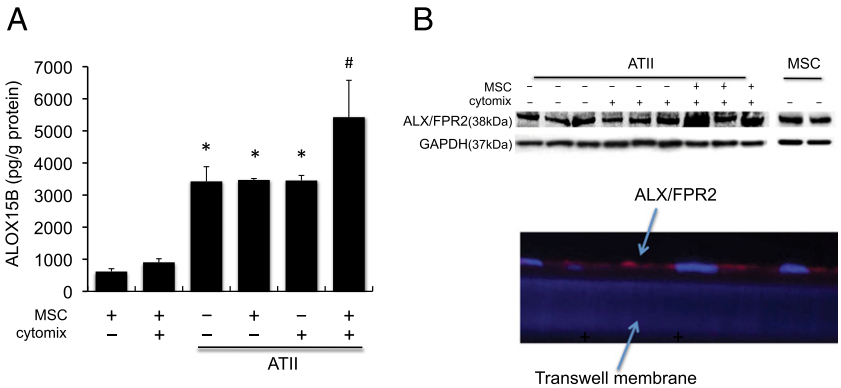

FIGURE 1. (**A**) Measurements of ALOX15B protein levels in lysates of human MSCs and human AT II cells. The data are expressed as ALOX15B concentration divided by the grams of protein. Data are presented as mean ± SD for each group ($n = 6$ for each groups). $^*p < 0.05$ versus MSCs alone or with cytomix, $^\#p < 0.05$ versus AT II, AT II plus MSC, or ATII plux cytomix. (**B**) ALX/FPR2 protein expression. *Upper panel*, ALX/FPR2 protein levels were assessed by immunoblot. Total cellular protein was extracted from cell lysates of human AT II cells exposed to cytomix and MSCs. Control blots were derived from the same samples. Images were analyzed with Carestream molecular imaging software. *Lower panel*, Immunofluorescent staining of ALX/FPR2 on human AT II cell monolayer (red). Nuclei were counterstained with DAPI (blue).

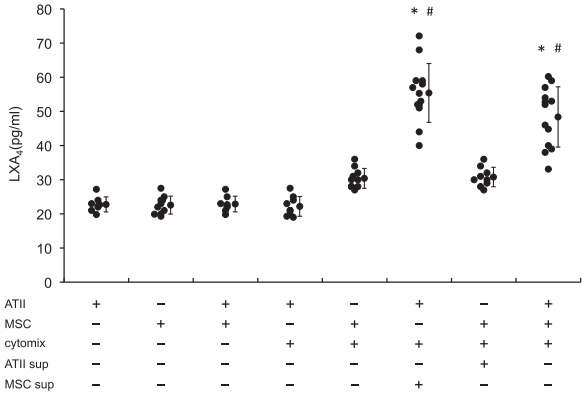

FIGURE 2. LXA_4 concentrations in coculture supernatant of human AT II cells and human MSCs. Human AT II cells were plated and cultured in the upper compartment of the Transwell for 96–120 h, after which 250,000 MSCs were added to the lower compartment with or without cytomix. Data are presented as mean ± SD. $^*p < 0.05$ versus AT II alone or with cytomix, $^{\#}p < 0.05$ versus MSCs alone or with cytomix. ATII sup, culture supernatant of AT II cells stimulated with cytomix; MSC sup, culture supernatant of MSCs stimulated with cytomix.

human AT II monolayer demonstrated staining of ALX/FPR2 on the apical side of human AT II cells (Fig. 1B, *lower panel*).

LXA_4 level in the coculture supernatant of human MSCs and human AT II cells

To identify the source of LXA_4, we carried out in vitro coculture experiments with MSCs and AT II cells. Freshly isolated human AT II cells were plated on collagen I–coated Transwells for 96 h in culture medium containing 10% FBS and an additional 24 h in serum-free medium (19, 20). Then, 250,000 MSCs were added to the lower compartment. These cells were then cocultured for 24 h more with cytomix or vehicle control. Human AT II cells or MSCs alone had low levels of LXA_4 in the culture supernatant under basal and cytomix-stimulated conditions. The LXA_4 levels were significantly increased when AT II cells were cocultured with MSCs or when AT II cells were exposed to the supernatant of MSCs stimulated with cytomix (Fig. 2). There is no significant effect of AT II supernatant (cytomix stimulated) on LXA_4 production by MSCs (Fig. 2).

Effects of human MSCs, recombinant LXA_4, and WRW4 on 48-h survival in mice with LPS-induced lung injury

To further test the role of MSCs and LXA_4 in acute lung injury, we studied the effect of the ALX/FPR2 receptor agonist and antagonist using an in vivo experimental model of acute lung injury induced by LPS, a model we have used in a prior study (2). The percentage of apoptotic human MSCs at the time of i.t. instillation was 5.6%. Intratracheal administration of human MSCs (500,000 cells) 4 h after LPS injury significantly improved the 48 h survival in mice (Fig. 3A). LXA_4 also improved the 48 h survival in LPS-challenged mice (Fig. 3B). When WRW4, an ALX/FPR2 receptor antagonist, was administered 4 h after LPS injury along with the MSCs, the protective effect of MSCs was significantly reduced (Fig. 3B). WRW4 alone had no effect on control mice.

At 48 h, MSC-treated mice had significantly less histological evidence of lung injury compared with mice given PBS (Fig. 4A). Mice treated with LXA_4 also had a significant reduction in the degree of hemorrhage and edema as assessed by the lung injury score (Fig. 4B). WRW4, administered 4 h after LPS injury, abrogated the effect of MSCs on reducing influx of inflammatory cells, RBCs, and thickening of the interstitium (Fig. 4A). Mice given MSCs or LXA_4 showed a reduction in excess lung water at 48 h, and WRW4 partially reversed the effect of MSCs on reducing the quantity of excess lung water (Fig. 4C).

Effect of human MSCs on LXA_4, TNF-α, and MIP-2 production in BAL fluid of mice with LPS-induced lung injury

To further understand the mechanisms for human MSCs and LXA_4 on improving the survival of LPS-challenged mice, we measured LXA_4, TNF-α, and MIP-2 levels in BAL fluid from mice with the in vivo experiments described above. LXA_4 level was increased by LPS as compared with control, although the difference was not significant. MSC treatment significantly increased the LXA_4 level in BAL fluid of mice with lung injury (Fig. 5A). Administration of MSCs 4 h after LPS decreased the production of TNF-α and MIP-2 by 34 and 47%, respectively. In the absence of MSCs, LXA_4 markedly decreased the production of TNF-α and MIP-2. Exposure to WRW4 attenuated the inhibitory effect of both MSCs and LXA_4 on proinflammatory mediator levels (Fig. 5B, 5C). WRW4 alone had no effect on TNFα and MIP-2 production. Also, WRW4 did not have an additive effect on cytokine production when combined with LPS (Fig. 5B, 5C).

Discussion

The main findings of this study can be summarized as follows: 1) both human MSCs and human AT II cells demonstrated gene and protein expression of biosynthetic enzymes and receptors for LXA_4; 2) coculture of human MSCs and human AT II cells under proinflammatory stimulations significantly increased the production of LXA_4; 3) human MSCs improved 48 h survival in mice with LPS-induced lung injury; blocking ALX/FPR2 receptors with WRW4 during adoptive transfer of MSCs negated these beneficial effects; and 4) human MSCs inhibited TNF-α and MIP-2 production in BAL fluid of mice, an effect that was partially reversed by WRW4.

LXA_4 was the first identified anti-inflammatory and pro-resolving lipid mediator (14, 16). Biosynthesis of LXA_4 has been established at sites of lung inflammation in the upper and lower

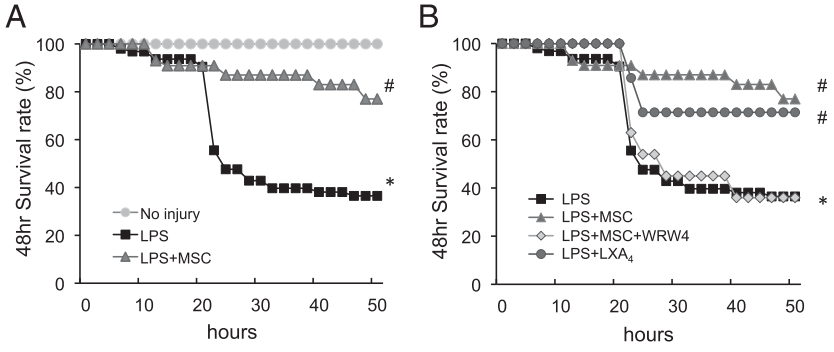

FIGURE 3. The effects of MSCs, ALX/FPR2 agonists (LXA_4), and antagonist (WRW4) on 48 h survival of LPS-injured mice. (**A** and **B**) Four hours after LPS injury (5 mg/kg, i.t.), mice received MSCs (500,000 cells), LXA_4 (10 μg/kg), WRW4 (1 mg/kg), or vehicle i.t. Statistical analysis was performed using log-rank test. Results are expressed as percentage survival (n = 25–35 per group). $^*p < 0.05$ versus no injury, $^{\#}p < 0.05$ versus LPS group.

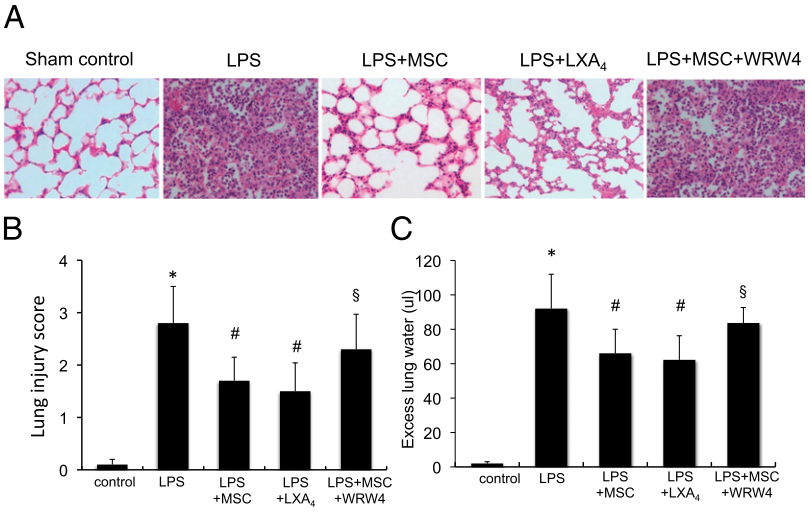

FIGURE 4. The effects of MSCs, ALX/FPR2 agonists (LXA_4), and antagonist (WRW4) on LPS-induced lung injury in mice. (**A**) Representative lung histology at 48 h after LPS induced lung injury. (**B**) Lung injury scores. The degree of lung injury (atelectasis, alveolar and interstitial inflammation, alveolar and interstitial hemorrhage, alveolar and interstitial edema, necrosis, and overdistension) in six sections from the lower lobes using the following criteria: 0, no injury; 1, injury to 25% of the field; 2, injury to 50% of the field; 3, injury to 75% of the field; 4, diffuse injury. (**C**) Measurement of excess lung water in mice at 48 h after LPS-induced lung injury (n = 12/group). $^{*}p < 0.05$ versus control group, $^{\#}p < 0.05$ versus LPS group, $^{\S}p < 0.05$ versus LPS plus MSC or LPS plus LXA_4.

airways and the vasculature (26, 27). In response to lung inflammation, airway epithelial cells 15-LOX–derived 15*S*-hydroxyeicosatetraenoic acid can be transformed by neutrophil 5-LOX to an unstable epoxytetraene intermediate that is converted by enzymatic hydrolysis to LXA_4 and LXB_4 (15). LXA_4 binds specifically and reversibly to ALX/FPR2, a G protein–coupled receptor, with a K_d of ~0.5 nM (15, 28, 29). In the present study, we found that both human MSCs and human AT II cells express biosynthetic enzymes and receptors for LXA_4 at the gene and protein level. During cell–cell interactions, human MSCs upregulated ALX/FPR2 expression in human AT II cells under inflammatory stimulation.

The results from in vitro coculture experiments of human MSCs and AT II cells indicated that AT II cells and MSCs alone generate modest amounts of LXA_4. Combination of two cells together resulted in significantly higher levels of LXA_4. The production of LXA_4 by AT II cells was upregulated by MSCs. The experiment provides further evidence that a single cell type could generate LXA_4, but multicellular host responses and cell–cell interactions produce LXA_4 in significantly higher amounts. To our knowledge, this study demonstrates for the first time that LXA_4 can be generated in human alveolar epithelial type II cells as well as by human MSCs.

To further understand the role of MSCs and LXA_4 in acute lung injury, we studied the effect of LXA_4 and the ALX/FPR2 antagonist in experimental ALI induced by LPS. Previous studies have shown that administration of apoptotic cells to an area of inflammation, including inflamed lungs, is anti-inflammatory (30, 31). Therefore, we measured the percentage of apoptotic MSCs before intratracheal instillation. A result of 5.6% apoptotic MSCs at the time of administration indicates that most of the beneficial effect of MSCs comes from live cells. The experiments demonstrated that treatment of MSCs and LXA_4 after the onset of LPS-induced lung injury led to improved 48 h survival in mice. Blocking the receptor for LXA_4 with WRW4 during adoptive transfer of MSCs negated these beneficial effects. MSC treatment increased LXA_4 but decreased TNF-α and MIP-2 levels in BAL fluid in mice. Taken together, these results indicated that the beneficial effect of MSCs promoting resolution of LPS-induced lung injury in mice is in part mediated through the proresolving lipid mediator LXA_4 signaling via ALX/FPR2 receptors.

We have previously reported that clinical-grade human MSCs restored alveolar fluid clearance to a normal level and decreased inflammation in ex vivo human lungs injured with live *E. coli* bacteria (5). The effects were associated with increased bacterial killing in part through enhanced phagocytosis activity of alveolar macrophages. LXA_4 is a potent stimulus for macrophage phagocytosis (32). Additionally, LXA_4 inhibits neutrophil transepithelial migration (33) and airway epithelial proinflammatory mediator expression in an ALX/FPR2-dependent manner (33, 34). LXA_4 also displays mucosal protection by stimulating ZO-1 expression and transepithelial electrical resistance in human airway epithelial cells (35). Moreover, LXA_4 activates the alveolar epithelial cell basolateral sodium channel, Na,K-ATPase, and increases alveolar fluid clearance in rats (36), and interestingly MSCs increase alveolar fluid clearance in endotoxin-injured

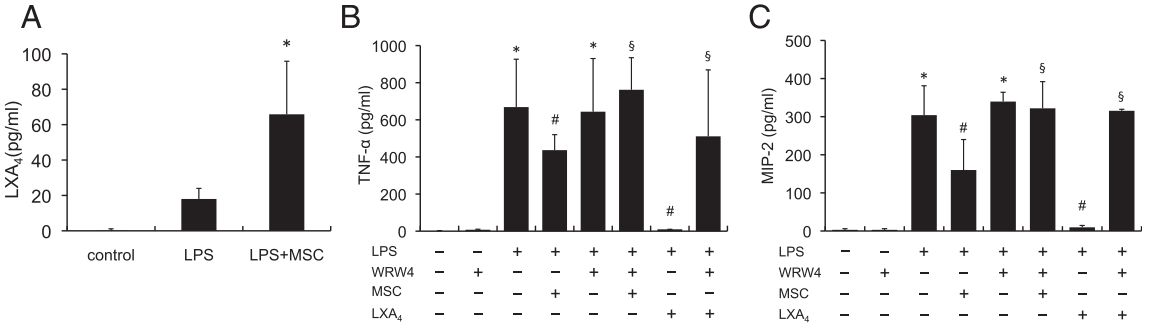

FIGURE 5. The effects of human MSCs, ALX/FPR2 agonists (LXA_4), and antagonists (WRW4) on LXA_4, TNF-α, and MIP-2 levels in BAL fluid of mice. MSCs, LXA_4, or WRW4 were administered i.t. 4 h after LPS injury. BAL fluid was collected at 48 h after injury. (**A**) LXA_4. (**B**) TNF-α. (**C**) MIP-2. Data are presented as mean ± SD for each group (n = 8–10 for each group). $^{*}p < 0.05$ versus control group, $^{\#}p < 0.05$ versus LPS group, $^{\S}p < 0.05$ versus LPS plus MSC or LPS plus LXA_4.

human lungs (37). In the present study, both the in vitro and in vivo experiments demonstrated that MSCs enhanced the generation of LXA_4 by cytokine-activated alveolar epithelial cells and decreased the production of TNF-α and MIP-2 in BAL fluid of mice with LPS-induced lung injury. In sterile models of ALI, administration of LXA_4 or its stable analogs attenuates lung inflammation and edema (38, 39). Taken together, these findings point toward a pivotal role for ALX/FPR2 receptors in mediating lung protection. Of interest, LXA_4 is not the only specialized proresolving mediator with agonist properties for ALX/FPR2. For example, selected D-series resolvins are also ALX/FPR2 ligands (40, 41). Moreover, because LXA_4 can also interact with other receptors, the findings in the present study for LXA_4 interactions with ALX/FPR2 may have broader implications for specialized proresolving mediators in MSC-initiated lung protection.

In summary, these experiments provide evidence that MSCs can promote the resolution of acute lung injury in mice in part through LXA_4 signaling via ALX/FPR2 receptors. In addition to mediating some of the potential therapeutic effects of MSCs themselves, the proresolving mediator LXA_4 has the potential to be a new therapeutic approach for patients with ARDS.

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Disclosures

The authors have no financial conflicts of interest.

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