Protective Role of Adipose-Derived Stem Cells in Staphylococcus aureus-Induced Lung Injury is Mediated by RegIIIγ Secretion

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Key Words. Adipose-derived stem cells • Staphylococcus aureus • Acute lung injury • Regenerating islet-derived III • Signaling pathways

ABSTRACT

Effective and specific therapeutic approaches are still needed for treating acute lung injury caused by severe pneumonia. Adipose-derived stem cells (ADSCs) are well-characterized adult stem cells that have antibacterial and anti-inflammatory effects. In this study, we evaluated the therapeutic effect of ADSCs on Staphylococcus aureus-induced acute lung injury in mice. Our results showed that intratracheal injection of ADSCs could attenuate the severity of lung inflammation, and reduce the bacterial load as well as mortality among infected mice. Our experiments also revealed that the secretion of regenerating islet-derived IIIγ (RegIIIγ) is responsible for the protective effect of ADSCs. Moreover, the expression of RegIIIγ requires TLR2, MyD88, and JAK2/STAT3 activation. In conclusion, ADSCs exhibit a direct antimicrobial activity that is mediated primarily by the TLR2-MyD88-JAK2/STAT3-dependent secretion of RegIIIγ. STEM CELLS 2016;34:1947–1956

SIGNIFICANCE STATEMENT

Intratracheal administration of adipose-derived stem cells (ADSCs) attenuated S. aureus-induced lung injury and improved survival of infected mice by means of reducing bacterial load and alleviating inflammatory responses. An antimicrobial peptide RegIIIγ was found to be responsible for the protective effect of ADSCs. Moreover, upon S. aureus infection, RegIIIγ expression in ADSCs was induced by activation of the TLR2-MyD88-JAK2/STAT3 intracellular signaling cascades and amplified by interaction with macrophages.

INTRODUCTION

Severe pneumonia is one of the most common causes of acute lung injury (ALI)/acute respiratory distress syndrome (ARDS), a severe acute pathological condition characterized clinically by respiratory distress, refractory hypoxemia, and noncardiogenic pulmonary edema [1]. Severe bacterial infection, such as Staphylococcus aureus infection, whether community-acquired or nosocomial, is recognized as the primary cause of ALI development [2, 3].

Although efforts focus on two approaches to treat ALI induced by severe bacterial infection, that is, reducing bacterial load, and protecting against organ injury resulting from excessive inflammation [1], effective and specific therapeutic approaches are still needed. Mesenchymal stem cells (MSCs), also referred to as mesenchymal stromal cells) have been proposed as a promising cell therapy for ALI, potentially based on paracrine and/or autocrine secretion of a number of factors [4–7]. Of the various types of MSCs, adipose-derived stem cells (ADSCs) are frequently applied adult stem cells that have shown promising therapeutic potential in various regenerative medicine therapies in fields such as plastic surgery, orthopedic surgery, oral and maxillofacial surgery, and cardiac surgery [8] as well as in ALI [9].

Regenerating islet-derived IIIγ (RegIIIγ), also named heptocarcinoma-intestine-pancreas (HIP) or pancreatic associated protein (PAP), was firstly identified as a secreted intestinal antimicrobial protein. It belongs to the C-type lectin superfamily and functions in the control of bacterial growth [10–12]. Accumulating evidence indicates that RegIIIγ has roles in antibacterial and anti-inflammatory processes as well as in prompting cell proliferation and differentiation [13]. In addition, Reg proteins were recently shown to be involved in T helper (Th) 17 cell-mediated skin inflammation in relation to both antimicrobial activity and wound repair [14].
In this study, we evaluated the protective effect of ADSCs on *S. aureus*-induced lung injury. We showed that intratracheal injection of ADSCs markedly attenuated the severity of *S. aureus*-induced ALI and decreased mortality in infected mice. We also revealed that *S. aureus* can induce the expression of RegIIIγ, which is responsible for the bacterial killing capacity of ADSCs. Our work further demonstrated that the *S. aureus*-triggered expression of RegIIIγ in ADSCs was induced by activation of the TLR-like receptor (TLR) 2-Myeloid differentiation primary response gene (MyD) 88-Janus kinase (JAK) 2/signal transducer and activator of transcription (STAT) 3 intracellular signaling cascades and amplified by a paracrine secretion pathway engaged by macrophages.

**MATERIALS AND METHODS**

**Reagents**

Recombinant RegIIIγ (rRegIIγγ) peptide was prepared by transient expression of a his-tagged RegIIIγ expression vector in *E. coli* followed by Ni-NTA agarose purification (Qiagen, Shanghai, China, http://www.qiagen.com) and renaturalization. The plasmid and purification protocols were kindly provided to us by Dr. Lai [14]. Limulus test was performed to exclude the trace expressions for endotoxin. Anti-RegIIIγ neutralization antibody and isotype control IgG were kindly provided to us by Dr. Lai [14]. Antibodies against phospho-STAT3, phospho-AKT, phospho-p38 mitogen-activated protein kinase (MAPK), phospho-JNK MAPK, phospho-ERK42/44 MAPK, AKT, and MAPKs of p38, JNK, and ERK42/44 were from Cell Signaling Technology (Beverly, MA, http://www.cellsignal.com); antibody against β-actin was from Sigma-Aldrich (St. Louis, MO); AG490, the specific pharmaceutical inhibitor for JAK2/STAT3, was purchased from Beyotime Institute of Biotechnology (Shanghai, China, http://www.beyotime.com/); horseradish peroxidase conjugated IgGs were purchased from Protein A (Shanghai, China, http://www.beyotime.com/); and protein A + G sepharose beads used for co-immunoprecipitation (co-IP) were from Qiagen.

**Animals**

Age- and sex-matched mice with a C57BL/6 genetic background were used in all experiments. Wild-type mice were purchased from the Animal Center of Slaccas (Shanghai, China, http://www.slaccas.com/). Wild-type (wt), TLR2 and TLR4 knockout mice, both on a C57BL/6J background, were maintained in our laboratories. All animal experiments were in accordance with the guide for the care and use of laboratory animals and were approved by the Animal Care and Use Committee at both Zhejiang University and Hangzhou Normal University.

**Bacterial Culture**

The *S. aureus* strain was a clinical isolate from the Second Affiliated Hospital, Zhejiang University School of Medicine, and its identification was confirmed by both biochemical assays and 16S ribosomal RNA gene sequencing [15, 16]. Bacteria were quantified according to the OD600-based bacterial growth curve and colony forming units (CFU) assay.

**Antimicrobial Assay**

To test the effect of ADSCs on bacterial growth, $1 \times 10^5$ of ADSCs grown in 24-well plates was infected with 10 μl of *S. aureus* at a multiplicity of infection (moi) of 1 or 10, respectively, and continued cultured for 6 hours. Afterward, cell culture medium was collected. Samples were serially diluted with sterile PBS, and plated on TSA agar plates. Colonies were counted after overnight incubation at 37°C. To assess the direct antibacterial effect for either conditional medium (CM) derived from ADSCs or rRegIIγ, bacterial CFU counting was performed as described above. Cell culture medium was passed through a 0.22 μm filter for preparation of CM. Aliquots (90 μL) were transferred to a 96-well plate, inoculated with 100 CFU of *S. aureus* (in 10 μl of PBS) and incubated for 16 hours at 37°C before CFUs were counted [18].

**S. aureus-Induced ALI Mice Model**

Pathogen free, 8-week old female C57BL/6 mice were used to establish *S. aureus*-induced ALI model [15, 16]. In brief, 40 μl of *S. aureus* (1 $\times 10^8$ CFU) was inoculated directly intratracheally (i.t.) into lightly anesthetized mice. To perform noninvasive i.t. inoculation, a mouse endotracheal intubation kit was applied (BiosciEquip Supply International Co., Limited, Hongkong, China, http://www.beyotime.com/). Cells were passaged every 3 days by trypsinization when they reached to 80% confluence. The cells were used between generation three and six depending on cell growth situation [17]. Raw 264.7 mouse macrophage cells (ATCC TIB-71) were maintained in RPMI-1640 medium containing 10% FBS, 2 mM glutamine, 50 μg/ml penicillin, 50 μg/ml streptomycin and 0.05 mM β-mercaptoethanol. An indirect coculture with Raw 264.7 macrophages and ADSCs was performed using a Transwell coculture system (0.4 μm pore size, Corning Costar Corp., Cambridge, MA, http://www.corning.com) in the 6-well tissue culture system (0.4 μm filter for preparation of CM). Polyvinylidene difluoride membrane with 0.4 μm pores of the Transwell system allows the exchange of the medium but limits the cell–cell interactions between the ADSCs and macrophages. Eight hours before bacterial infection, the culture medium for ADSCs was replaced by serum- and antibiotic-free medium DMEM-hg.
Mice were observed for viability and behavior every 8 hours up to 120 hours or alternatively, at indicated time points, sacrificed for harvesting whole lung homogenate (LH) and bronchoalveolar lavage (BAL) fluid. The application of ADSCs or RegIIIγ was performed by i.t. administration of $5 \times 10^5$ ADSCs or 20 μg rRegIIIγ (both in 40 μL PBS) 2 hours after infection. In another separate experiment, 40 μg of anti-RegIIIγ neutralization antibody or control IgG was applied together with bacteria instillation.

**Histopathological Changes in the ALI Mice**

The whole lung of the mouse was fixed with 10% paraformaldehyde and embedded with paraffin. Four micrometer sections were sliced for haematoxylin and eosin staining. Morphometric analysis was conducted under an automatic photo-microscope (Leica, Wetzlar, Germany http://www.leica.com/). The severity for ALI was histopathologically evaluated, by means of inflammatory cell infiltration and alveolar wall thickening.

**S. aureus Quantification in the LH and BAL Fluid**

After harvesting, whole lungs were homogenized in 1 mL of PBS supplemented with protease inhibitor cocktail (Roche Applied Science, Basel, Switzerland, http://www.roche-applied-science.com/) before homogenates were serially diluted 1:10 in PBS and plated on TSA agar to determine lung CFUs. The BAL fluid supplemented with protease inhibitor cocktail (Roche Applied Science, Basel, Switzerland, http://www.roche-applied-science.com/) before homogenates were serially diluted 1:10 in PBS and plated on TSA agar to determine lung CFUs. The BAL fluid was collected by lavaging the lungs with 1 mL of sterile PBS for five times. The CFUs in the first 1 mL of BAL fluid were counted.

**Cell Counting and Measurement of Cytokines in the BAL Fluid**

Erythrocytes were lysed using lysis buffer (eBioscience San Diego, CA http://www.ebioscience.com/) before the number of total cells was counted in BAL fluid. 2 × 10^5 cells were loaded onto a slide by cytospin (Statspin, Westwood, MA http://www.statspin.com/) and stained with Giemsa (BASO, Zhuhai, China, http://www.baso.com.cn/) for neutrophil counting. Cell-free BAL fluid was applied for Enzyme-linked immunosorbent assays (ELISA) to measure the concentration of keratinocyte-derived chemokine (KC), macrophage inflammatory protein (MIP)-2, and interleukin (IL)-1β (all kits from R&D systems Inc. Minneapolis, MN, https://www.rndsystems.com/) according to the manufacturer’s protocol.

**Quantitative Reverse Transcription-PCR (qRT-PCR)**

The whole lung of the mouse was conducted for RNA extraction with Trizol reagent (Invitrogen). For cultured cells, total RNA was extracted using RNA simple Total RNA Kit (Tiangen, Beijing, China). cDNA was synthesized using reverse transcriptase cDNA synthesis system (Ferments Burlington, Ontario, Canada http://www.fermentas.com). Two microgram of total RNA and random primers were included in the reaction. The real-time semiquantitative PCR was performed in triplicates using the SYBR Green PCR assay and an ABI IQTM apparatus (both from Applied Biosystems, Foster City, CA http://www.appliedbiosystems.com/). Primers were designed using Primer 5. The sequences for the primers were listed as follows. β-actin: forward primer: 5’-AGAGGGAAAATCTGGCCTGAGC-3’, reverse primer: 5’-CCATAGTGATGACCTGCCGT3’; KC: forward primer: 5’-ACCCCAAACCGAAGTCA-3’, reverse primer: 5’-GTTGCCCCATGACAGGCT-3’; MIP-2: forward primer: 5’-CCAAGACAGTGACTGAA-3’, reverse primer: 5’-TCCCTTCCAGGTCA GTTA-3’; IL-1β: forward primer: 5’- CCTTGCTCTGATGG-3’, reverse primer: 5’-AGTGTCCTCATAATCTCCCC-3’; RegIIIγ: forward primer: 5’-TTCTCTGTCCTCCAGATGACAAA-3’, reverse primer: 5’-CATCCACCTCTGTGGGT TCA-3’. β-actin was amplified as an endogenous reference gene. Expression of target genes was measured after normalization RNA with the reference gene, as fold increased expression above control calculated by corrected ΔΔ with a Sequence Detection Software (Applied Biosystems).

**Western Blotting and Co-IP**

For western blotting, 1 × 10^6 cultured cells were lysed in 1 × RIPA buffer (CST, Danvers, MA) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail. Protein concentrations were determined by the Bradford protein assay. Total proteins (40 μg) were separated by SDS-PAGE and blotted onto to PVDF membranes. The membranes were probed with antibodies against individual phosphorylated or total proteins for p38, JNK, AKT, ERK42/44, and STAT3, as well as β-actin (all from CST). Horseradish-peroxidase-conjugated goat anti-rabbit antibody was used as the secondary antibody (Lianke, Hangzhou, China, http://www.liankebio.com/). ECL reagent was used to develop the membrane and signal was detected on a digital image system (FluorChem E; Proteinsimple, Santa Clara, CA, http://www.proteinsimple.com/). For co-IP, we established an in-direct coculture model with Raw 264.7 macrophages and ADSCs using a Transwell coculture system in 6-well tissue culture plates. S. aureus (moi: 1) was added to the lower chamber. After 6 hours, the upper chamber with the Raw 264.7 cells was removed and the cell culture supernatants were collected and incubated for 2 hours at 4°C with 1 μg/μL anti-RegIIIγ antibody and protein A + G sepharose. After vigorous washes, the precipitates were boiled in sample buffer, separated on SDS-PAGE, and transferred to PVDF membranes for anti-RegIIIγ western blotting.

**Statistical Analysis**

All experiments were performed for at least three times. Survival curves were compared using the log-rank test. For other data, 2-tailed unpaired t test was applied for the comparison between 2 groups and 1-way ANOVA was used for the comparison within three and more groups. A p value of .05 or less was considered as statistically significant. All calculations were performed using the Prism software program for Windows (GraphPad Software, Inc.). All bars across CFU data are presented as medians of triplicates. Error bars in all graphs indicate SEM and represent triplicates.

**Results**

Administration of ADSCs Reduced Bacterial Burden and Attenuated S. aureus-Induced ALI in Mice

To evaluate the effect of ADSCs on ALI, a mouse model of S. aureus-induced lung inflammation and ALI was applied. C57BL/6 mice were infected via i.t. instillation of $1 \times 10^8$ CFU S. aureus. Two hours after S. aureus instillation, mice were treated with either $5 \times 10^5$ ADSCs or PBS intratracheally. Mice were either kept alive for observation of their viability and behavior every 8 hours up to 120 hours or sacrificed 12 hours after infection for evaluation of lung inflammation and...
Figure 1. ADSCs reduced bacterial burden and attenuated *S. aureus*-induced ALI in mice. C57BL/6 mice were infected with i.t. instillation of *S. aureus* (1 × 10^8 CFU). Two hours after *S. aureus* stimulation, mice were i.t. treated with either 5 × 10^5 ADSCs or PBS. Mice were kept alive for observation of their viability and behavior every 8 hours for 120 hours (A, n = 15 per each group, *p < .05) or sacrificed 12 hours after infection for the evaluation of lung inflammation as well as bacterial loads (B–F, n = 5–8 per group, *p < .05). (A) Survival curves for mice; (B) representative H&E staining of lung tissues, scale bar = 20 μm; (C) CFU counts in LH and BAL samples; (D) total cell counts and percentage of neutrophils in BAL fluid; (E) concentrations of inflammatory cytokines of IL-1β, KC, and MIP-2 in BAL fluid; and (F) mRNA expression of IL-1β, KC, and MIP-2 in LH. Abbreviations: ADSCs, Adipose-derived stem cells; CFU, colony forming units; PBS, phosphate buffer saline; LH, lung homogenize; BAL, bronchoalveolar lavage; KC, keratinocyte-derived chemokine.
bacterial loads. The application of ADSCs improved the survival rates in mice infected with *S. aureus* (*p* < .05, Fig. 1A). At 12 hours after infection, ADSCs-treated mice showed greater preservation of lung tissue with remarkably reduced inflammatory cell infiltration and alveolar wall thickening (Fig. 1B). Lung homogenate and BAL samples from mice treated with ADSCs showed lower bacterial counts, compared with those in control mice (Fig. 1C). Total BAL cell counts and the percentage of neutrophils were also significantly lower in the ADSC-treated group than in PBS-treated mice (Fig. 1D). Moreover, the BAL protein levels (Fig. 1E) and the mRNA levels (Fig. 1F) of the inflammatory cytokines of IL-1β, KC, and MIP-2 in LH samples were significantly lower in mice treated with ADSCs as compared with levels in the PBS-treated group. These results suggest that bacterial clearance in the lung of ADSC-treated mice was an important mechanism of ADSCs against *S. aureus*-induced ALI.

**ADSCs Secreted Antibacterial Factors upon *S. aureus* Challenge**

To test the effect of ADSCs on bacterial growth, ADSCs were infected with *S. aureus* at a moi of 1 or 10 for 6 hours before the cell culture medium was collected for a bacterial titer test or passed through a 0.22 μm filter for preparation of CM. ADSCs significantly inhibited bacterial growth compared with control medium (Fig. 2A), as did the CM collected from infected ADSCs (Fig. 2B), indicating that the observed antimicrobial activity against *S. aureus* was associated with the secretion of antibacterial factors upon bacterial challenge.

**Antimicrobial Activity of ADSCs in a *S. aureus*-Induced ALI Mouse Model is Mediated in part by RegIIIγ**

Next, we investigated whether *S. aureus* stimulates the production of RegIIIγ in ADSCs. Cells were infected with *S. aureus* as described for the previous experiment. After 6 hours, cells were collected for RNA extraction and protein preparation. RTPCR and western blot analyses revealed that the levels of RegIIIγ mRNA and protein in ADSCs increased significantly upon stimulation with an abundance of *S. aureus* (moi: 10) (Fig. 3A, 3B). To evaluate the bioactivity of RegIIIγ in vivo, mice models of *S. aureus* infection and application of ADSCs were performed. In addition, 40 μg of anti-RegIIIγ neutralization antibody or control IgG was applied together with *S. aureus* instillation. Notably, treatment with ADSCs reduced the bacterial burden in LH, and the application of anti-RegIIIγ neutralization antibody inhibited the protective effect of ADSCs (Fig. 3C).

**Antimicrobial Activity of rRegIIIγ In Vitro and in a *S. aureus*-Induced ALI Mouse Model**

We further explored the antimicrobial activity of rRegIIIγ against *S. aureus* both in vitro and in the *S. aureus*-induced ALI mouse model in vivo. For the in vitro experiment, serial diluted rRegIIIγ was added to *S. aureus* (1×10^6^ CFU) cultures for 3 hours. For the in vivo experiment, 20 μg rRegIIIγ peptide was administered to mice together with *S. aureus* instillation. The bacterial burden was detected by CFU, and dose-dependent inhibition of *S. aureus* growth by RegIIIγ was observed (Fig. 4A). In the mouse model of *S. aureus*-induced ALI, similar to the application of ADSCs, i.t. instillation of 20 μg rRegIIIγ markedly reduced the bacterial load in both BAL (Fig. 4B) and LH (Fig. 4C) samples, and this effect was accompanied by decreases in the total BAL cell counts and the percentage of neutrophils (Fig. 4D). In addition, protein expression of the inflammatory cytokines IL-1β, KC, and MIP-2 was reduced in BAL samples of rRegIIIγ-treated mice (Fig. 4E), compared to levels in samples from the mock group.

**Coculture of ADSCs with Macrophages Enhanced the Production and Secretion of RegIIIγ upon *S. aureus* Infection**

Previous research indicates that the secretion of antibacterial peptides by MSCs may be amplified via autocrine and/or paracrine pathways in the presence of neighboring cells [6]. To investigate whether this is true for ADSCs, we established an in-direct coculture model with Raw 264.7 macrophages and ADSCs using a Transwell coculture system (Fig. 5A). The presence of Raw 264.7 macrophages significantly enhanced the expression of RegIIIγ mRNA in *S. aureus*-stimulated ADSCs as compared with that in ADSCs not cultured with macrophages (Fig. 5B). Western blot analysis also showed that RegIIIγ protein expression was initially low in both ADSCs and Raw 264.7 macrophages. Coculture with Raw 264.7 macrophages resulted in abundant RegIIIγ expression in ADSCs upon *S. aureus* infection (Fig. 5C). To further confirm that RegIIIγ was secreted by ADSCs, samples of cell culture supernatant were collected for co-IP with 1 μg/μl anti-RegIIIγ antibody and protein A+G sepharose beads, followed by Western blotting with anti-RegIIIγ antibody. The combination of *S. aureus* infection and...
RegIIIγ-mediated protective role of adipose-derived stem cells

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Antimicrobial activity of ADSCs in S. aureus-induced ALI is mediated by RegIIIγ production. (A) Levels of RegIIIγ mRNA as detected by RT-qPCR in ADSCs infected with S. aureus at a moi of 1 or 10 for 6 hours. (B) Levels of RegIIIγ protein as detected by western blotting in ADSCs infected with S. aureus at a moi of 1 or 10 for 6 hours. (C) CFU counts in LH samples from mice infected with S. aureus and treated with ADSCs (as described for the experiment shown in Fig. 1) and with application of 40 μg anti-RegIIIγ–neutralizing antibody or control IgG together with S. aureus instillation. n = 5 per group. *p < .05. Abbreviations: ADSCs, Adipose-derived stem cells; CFU, colony forming units; LH, lung homogenize.

Figure 3.

DISCUSSION

The main findings of our study are that (a) i.t. administration of ADSCs attenuated S. aureus-induced lung injury and improved survival rates of infected mice; (b) ADSCs reduced the bacterial load as well as the inflammatory response in infected mice; (c) RegIIIγ exhibited antimicrobial activity against S. aureus in vitro and in vivo and mediated the protective effect of ADSCs on S. aureus-induced lung injury; (d) RegIIIγ secretion in response to S. aureus was enhanced in ADSCs cocultured with macrophages; and (e) S. aureus infection induced RegIIIγ expression in ADSCs via activation of the TLR2-MyD88-JAK2/STAT3 signal pathway.

MSCs possess intrinsic antimicrobial properties, including the ability to secrete antimicrobial factors upon infection. To date, only a few peptides with antibacterial activities have been identified. For example, Krasnodembskaya et al. revealed that bacterial challenge stimulates bone marrow-derived MSCs to produce a human cathelicidin antimicrobial peptide, hCAP-18/LL-37. LL-37 exhibits a broad antibacterial activity against both Gram-positive and Gram-negative bacteria and contributes, in part, to the therapeutic effect of MSCs on mice with E. coli pneumonia [18]. Gupta et al. used a mouse infection model to show that an antibacterial protein lipocalin 2 contributes to improvements in the injured lung and the survival of mice with bacterial pneumonia, as well as enhanced bacterial clearance from the alveolar space following i.t. treatment with MSCs [19]. In a recent study, using a Gram-negative bacteria infection model, Sung et al. identified beta-defensin-2 as an antibacterial factor secreted by MSCs [20]. They also noticed that the spectrum of antimicrobial factors may be diverse due to different sources of MSCs and different stimuli. In our study, we identified a new antimicrobial

Raw 264.7 macrophage coculture resulted in the greatest concentration of RegIIIγ in the cell culture supernatant (Fig. 5D).

TLR2-MyD88-JAK2/STAT3 Signaling Mediates RegIIIγ Expression in S. aureus-Infected ADSCs

Finally, we explored the mechanisms by which ADSCs produce RegIIIγ upon S. aureus infection. After 6 hours of in vitro stimulation, signal transduction was assessed by immunoblotting for the phosphorylation of relevant signaling molecules and with the application of pharmaceutical inhibitors. ADSCs derived from TLR2−/− and MyD88−/− mice were included to study RegIIIγ expression in response to S. aureus stimulation as compared to that in ADSCs from wt mice. S. aureus stimulation increased the phosphorylation of STAT3 in ADSCs but had no effects on the phosphorylation of p38, JNK, AKT, and ERK42/44 (Fig. 6A, 6B). RegIIIγ expression seemed to be dependent on the activation of STAT3, given that the application of AG490, a specific pharmaceutical inhibitor of the JAK2/STAT3 signaling, abolished RegIIIγ expression (Fig. 6C). Upon infection of ADSCs with S. aureus (moi: 10), expression of TLR2 was increased dramatically (Fig. 6D). Compared to ADSCs derived from wt mice, ADSCs from either TLR2−/− or MyD88−/− mice showed deficient expression of RegIIIγ (Fig. 6E).
Figure 4. Antimicrobial activity of recombinant RegIIIγ in vitro and in a mouse model of S. aureus-induced ALI. (A) Serial dilutions of rRegIIIγ (90 μl in PBS) were incubated with 1 × 10⁶ CFU S. aureus (10 μl in PBS) for 3 hours to observe bacterial growth. (B–E) In the mouse model of S. aureus-induced ALI, 20 μg of rRegIIIγ was given to mice via i.t instillation. (B) Bacterial load in BAL fluid; (C) bacterial load in LH; (D) total cell counts and percentage of neutrophils in BAL fluid; and (E) protein expression of IL-1β, KC, and MIP-2 in BAL fluid. n = 5 per group. *p < .05. Abbreviations: CFU, colony forming units; KC, keratinocyte-derived chemokine; BAL, bronchoalveolar lavage.

Figure 5. Coculture with macrophages enhanced the production and secretion of RegIIIγ in S. aureus-infected ADSCs. (A) An in-direct coculture model was established with Raw 264.7 macrophages and ADSCs using a Transwell coculture system. ADSCs were infected with S. aureus at a moi of 1 for 6 hours with or without the presence of macrophages. (B) Levels of RegIIIγ mRNA in ADSCs as detected by RT-qPCR. *p < .05. (C) Levels of RegIIIγ protein as detected by western blotting in ADSCs. (D) Levels of secreted RegIIIγ as detected by co-IP. Abbreviation: ADSCs, Adipose-derived stem cells.
protein RegIIIγ that contributes to the ability of ADSC to kill \textit{S. aureus} and protect against \textit{S. aureus}-induced ALI in a mouse model. As a distinct antimicrobial protein, RegIIIγ works by interacting with bacterial peptidoglycan carbohydrate [10–12], which is responsible for the bacterial killing capacity of ADSCs against \textit{S. aureus}.

Toll-like receptors are known to recognize pathogen-associated molecular patterns (PAMPs) from various pathogens. MSCs derived from human umbilical cord (UC-MSCs), adipose tissue (ADSCs) and bone marrow (BMSCs) express various TLRs including TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, and TLR9 [21, 22]. Some ligands for TLRs are known to have influence on the immune potentiality of MSCs. Poly(I:C) has been shown to improve immunosuppressive function and therapeutic effect of MSCs on sepsis via inhibiting microRNA-143 [23]. Interestingly, it seems that TLR signaling is not limited to an immune role but also affects the proliferation (CpG-ODN, TLR9), osteogenic differentiation (LPS/TLR4, PGN/TLR2) [21, 22], and angiogenesis (TLR 2/6) [24] of MSCs. In macrophages, TLR2 predominately recognizes peptidoglycan (PGN) and lipoteichoic acid (LTA), the two major cell wall constituents of \textit{S. aureus}, and activates MAPK-AKT-STAT3 signaling pathway to induce IL-33 production [14, 25]. In this study, we showed that stimulation of ADSCs with \textit{S. aureus} enhances RegIIIγ expression.

**Figure 6.** The TLR2-MyD88-STAT3 signaling partly mediates RegIIIγ expression in \textit{S. aureus}-stimulated ADSCs. ADSCs derived from wt, TLR2−/−, or MyD88−/− mice were infected with \textit{S. aureus} at the indicated moi for 6 hours. Cells were then collected for immunoblotting for the phosphorylation of relevant signal molecules and the application of pharmaceutical inhibitors. (A) Phosphorylation of p38, JNK, AKT, and ERK44/42; (B) protein expression of RegIIIγ and phosphorylation of STAT3; (C) mRNA expression of RegIIIγ with or without the application of AG490, a specific pharmacological inhibitor of JAK2/STAT3; (D) mRNA expression of TLR2; and (E) mRNA expression of RegIIIγ in ADSCs derived from wt mice vs. those from TLR2−/− or MyD88−/− mice with or without \textit{S. aureus} infection. n = 4 per group. *p < .05.
dependent IgG responses, and the induction of antimicrobial proteins. Recently, Sung et al. demonstrated a link between TLR4 signaling and beta-defensin-2 secretion in MSCs challenged by *E. coli* [20].

Our study revealed that both TLR2 and MyD88 are involved in the regulation of RegIIIα production in *S. aureus*-infected ADSCs. This phenomenon seems to be unique to ADSCs, given that the MAPK and PI3k/AKT pathways have been shown to be required for TLR2-coupled macrophages activation upon *S. aureus* infection [16, 25]. Activated STAT3 may work as a transcription factor for Th17 cells differentiation, T cell-dependent IgG responses, and the induction of antimicrobial factors (e.g., β-defensins) [28]. Interestingly, it was recently reported that activated STAT3 may stimulate the production of RegIIIα by directly binding at its promoter region [29]. Inhibition of STAT3 resulted in reduced RegIIIα production coupled with impaired clearance of methicillin-resistant *S. aureus* in the pulmonary epithelium [30]. It is likely that in ADSCs, activated STAT3 also directly binds at the promoter region of RegIIIα and initiates the transcriptional and translational processes.

Based on our results, we proposed a model in which activation of TLR2 by *S. aureus* infection stimulates the expression of RegIIIα in ADSCs mainly through the TLR2-MyD88-JAK2/STAT3 signaling pathway (Fig. 7).

**CONCLUSION**

In conclusion, ADSCs ameliorate ALI in *S. aureus*-infected mice by secretion of the antimicrobial protein RegIIIα. In addition, ADSCs may coordinate with macrophages to enhance RegIIIα expression in order to augment their protective effect. Therefore, treatment with ADSCs may be beneficial in cases of bacterial infection due to their antimicrobial properties.

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**AUTHOR CONTRIBUTIONS**

J.Q.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, financial support; Y.H.: collection and/or assembly of data, data analysis and interpretation; L.F.Z.: conception and design, collection and/or assembly of data; J.Y.X.: collection and/or assembly of data; C.W.L.: collection and/or assembly of data; L.Y.S.: conception and design, collection and/or assembly of data, data analysis and interpretation; F.X.: conception and design, financial and administrative support, data analysis and interpretation, manuscript writing, and final approval of manuscript.

J.Q. and Y.H. contributed equally to this article.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicate no potential conflicts of interest.

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**REFERENCES**

1. Johnson ER, Matthay MA. Acute lung injury: Epidemiology, pathogenesis, and treatment. *Aerosol Med Pulm Drug Deliv* 2010;23:243–252.
2. Klevens RM, Morrison MA, Nadle J et al. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *JAMA* 2007;298:1763–1771.
3. Otto M. Basis of virulence in community-associated methicillin-resistant *Staphylococcus aureus*. *Annu Rev Microbiol* 2010;64:143–162.
4. Lee JW, Fang X, Krasnodembskaya A et al. Concise review: Mesenchymal stem cells for acute lung injury: Role of paracrine soluble factors. *STEM CELLS* 2011;29:913–919.
5. Matthay MA, Thompson BT, Read EJ et al. Therapeutic potential of mesenchymal stem cells for severe acute lung injury. *Chest* 2010;138:965–972.
6. Qin ZH, Xu JF, Qu JM et al. Intrapleural delivery of MSCs attenuates acute lung injury by paracrine/endocrine mechanism. *J Cell Mol Med* 2012;16:2745–2753.
7. Xu F, Hu Y, Zhou J et al. Mesenchymal stem cells in acute lung injury: Are they ready for translational medicine? *J Cell Mol Med* 2013;17:927–935.
8. Tobita M, Orbay H, Mizuno H. Adipose-derived stem cells: current findings and future perspectives. *Discov Med* 2011;11:160–170.
9 Zhang S, Danchuk SD, Imhof KM et al. Comparison of the therapeutic effects of human and mouse adipose-derived stem cells in a murine model of lipopolysaccharide-induced acute lung injury. Stem Cell Res Ther 2013;4:13
10 Cash HL, Whitham CV, Behrendt CL et al. Symbiotic bacteria direct expression of an intestinal bactericidal lectin. Science 2006;313:1126–1130.
11 Brandl K, Plitas G, Mihu CN et al. Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits. Nature 2008;455:804–807.
12 Vaishnava S, Yamamoto M, Severson KM et al. The antibacterial lectin RegIIIgamma promotes the spatial segregation of microbiota and host in the intestine. Science 2011;334:255–258.
13 Lehotzky RE, Partch CL, Mukherjee S et al. Molecular basis for peptidoglycan recognition by a bactericidal lectin. Proc Natl Acad Sci USA 2010;107:7722–7727.
14 Lai Y, Li D, Li C et al. The antimicrobial protein REG3A regulates keratinocyte proliferation and differentiation after skin injury. Immunity 2012;37:74–84.
15 Xu F, Diao R, Liu J et al. Curcumin attenuates staphylococcus aureus-induced acute lung injury. Clin Respir J 2015;9:87–97.
16 Xu F, Kang Y, Zhang H et al. Akt1-mediated regulation of macrophage polarization in a murine model of Staphylococcus aureus pulmonary infection. J Infect Dis 2013;208:528–538.
17 Taha MF, Hedayati V. Isolation, identification and multipotential differentiation of mouse adipose tissue-derived stem cells. Tissue Cell 2010;42:211–216.
18 Krasnodembskaya A, Song Y, Fang X et al. Antibacterial effect of human mesenchymal stem cells is mediated in part from secretion of the antimicrobial peptide LL-37. Stem Cells 2010; 28:2229–2238.
19 Gupta N, Krasnodembskaya A, Kapetanaki M et al. Mesenchymal stem cells enhance survival and bacterial clearance in murine Escherichia coli pneumonia. Thorax 2012;67:533–539.
20 Sung DK, Chang YS, Sung SI et al. Antibacterial effect of mesenchymal stem cells against Escherichia coli is mediated by secretion of beta-defensin-2 via toll-like receptor 4 signalling. Cell Microbiol 2016;18:424–436.
21 Chen D, Ma F, Xu S et al. Expression and role of Toll-like receptors on human umbilical cord mesenchymal stromal cells. Cytotherapy 2013;15:423–433.
22 Hwa Cho H, Bae YC, Jung JS. Role of toll-like receptors on human adipose-derived stromal cells. Stem Cells 2006;24:2744–2752.
23 Zhao X, Liu D, Gong W et al. The toll-like receptor 3 ligand, poly(I:C), improves immunosuppressive function and therapeutic effect of mesenchymal stem cells on sepsis via inhibiting MIR-143. Stem Cells 2014;32:521–533.
24 Grote K, Petri M, Liu C et al. Toll-like receptor 2/6-dependent stimulation of mesenchymal stem cells promotes angiogenesis by paracrine factors. Eur Cell Mater 2013;26:66–79.
25 Li C, Li H, Jiang Z et al. Interleukin-33 increases antibacterial defense by activation of inducible nitric oxide synthase in skin. PloS Pathog 2014;10:e1003918.
26 Frantz AL, Rogier EW, Weber CR et al. Targeted deletion of MyD88 in intestinal epithelial cells results in compromised antibacterial immunity associated with downregulation of polymeric immunoglobulin receptor, mucin-2, and antibacterial peptides. Mucosal Immunol 2012;5:501–512.
27 Wu YY, Hsu CM, Chen PH et al. Toll-like receptor stimulation induces nondefensin protein expression and reverses antibiotic-induced gut defense impairment. Infect Immun 2014;82:1994–2005.
28 Quinton LJ, Mizgerd JP. NF-kappaB and STAT3 signaling hubs for lung innate immunity. Cell Tissue Res 2011;343:153–165.
29 Choi SM, McAleer JP, Zheng M et al. Innate Stat3-mediated induction of the antimicrobial protein Reg3gamma is required for host defense against MRSA pneumonia. J Exp Med 2013;210:551–561.
30 Choi SM, McAleer JP, Trevejo-Nunez G et al. Acute alcohol intoxication impairs methicillin-resistant Staphylococcus aureus clearance in the lung by impeding epithelial production of Reg3gamma. Infect Immun 2014;82:1402–1407.