

Antibacterial effect of mesenchymal stem cells against *Escherichia coli* is mediated by secretion of beta-defensin-2 via toll-like receptor 4 signalling

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Summary

Recently, we demonstrated that intratracheal transplantation of human umbilical cord blood-derived mesenchymal stem cells (MSCs) attenuates *Escherichia (E) coli*-induced acute lung injury primarily by down-modulating inflammation and enhancing bacterial clearance in mice. This study was performed to elucidate the mechanism underlying the antibacterial effects of MSCs. The growth of *E. coli* *in vitro* was significantly inhibited only by MSCs or their conditioned medium with bacterial preconditioning, but not by fibroblasts or their conditioned medium. Microarray analysis identified significant up-regulation of toll-like receptors (TLR)-2 and TLR-4, and β -defensin 2 (BD2) in MSCs compared with fibroblasts after *E. coli* exposure. The increased BD2 level and the *in vitro* antibacterial effects of MSCs were abolished by specific antagonist or by siRNA-mediated knockdown of TLR-4, but not TLR-2, and restored by BD2 supplementation. The *in vivo* down-modulation of the inflammatory response and enhanced bacterial clearance, increased BD2 secretion and the resultant protection against *E. coli*-induced pneumonia observed only with MSCs, but not fibroblasts, transplantation in mice, were abolished by knockdown of TLR-4 with siRNA transfection. Our data indicate that BD2 secreted by the MSCs via the TLR-4 signalling pathway is one of the critical

paracrine factors mediating their microbicidal effects against *E. coli*, both *in vitro* and *in vivo*. Furthermore, TLR-4 from the transplanted MSCs plays a seminal role in attenuating *in vivo* *E. coli*-induced pneumonia and the ensuing acute lung injury through both its anti-inflammatory and antibacterial effects.

Introduction

Bacterial pneumonia and the ensuing acute lung injury (ALI) are important causes of morbidity and mortality in critically ill patients despite appropriate antimicrobial therapy (Ware and Matthay, 2000; Rubenfeld *et al.*, 2005). Moreover, the incidence of antibiotic resistance among bacterial isolates has been rapidly increasing. Therefore, there is an urgent need to develop novel therapies to improve the outcome of this intractable disease.

Both the microbial pathogen and the host inflammatory responses are known to be responsible for the pathophysiological mechanism of bacterial pneumonia and the resultant ALI. Recent studies have demonstrated that mesenchymal stem cells (MSCs) not only attenuate the inflammatory responses but also enhance bacterial clearance in bacterial pneumonia and/or sepsis (Kim *et al.*, 2011; Gupta *et al.*, 2012). These findings suggest that MSCs could be a promising novel therapeutic modality for bacterial pneumonia and the ensuing ALI. However, although paracrine mediators such as keratinocyte growth factor, antimicrobial polypeptide LL-37, or lipocalin 2 secreted by the transplanted MSCs are known to enhance bacterial clearance (Krasnodembskaya *et al.*, 2010; Gupta *et al.*, 2012; Zhu *et al.*, 2014), the precise mechanisms and specific factors responsible for the antimicrobial action of transplanted MSCs have not yet been elucidated.

Previously, we showed that intratracheal transplantation of human umbilical cord blood (UCB)-derived MSCs attenuates *Escherichia (E) coli*-induced ALI primarily by down-modulating the inflammatory responses and by enhancing bacterial clearance (Kim *et al.*, 2011). In the present study, we tried to identify the precise mechanism

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and specific factors mediating the antibacterial effects of MSCs transplantation. We initially conducted microarray analyses of MSCs to screen for the genes up-regulated after exposure to *E. coli* and were able to identify the up-regulation of β -defensin-2 (BD2) and toll-like receptor (TLR) 4 in MSCs but not in fibroblasts after *E. coli* exposure. Next, to test the hypothesis that BD2 secreted by MSCs via TLR-4 mediates the antibacterial effects, the antibacterial effects of MSCs with or without specific antagonist for TLR-4, or knockdown of TLR-4 with small interfering RNA (siRNA), and supplementation of BD2 was evaluated *in vitro* and *in vivo* in *E. coli*-induced ALI in mice.

Results

MSCs, but not fibroblasts, inhibit bacterial growth *in vitro*

After infection with 10^3 *E. coli* and incubation for 6 h, human UCB-derived MSCs, but not human fibroblasts (MRC-5), significantly inhibited bacterial growth *in vitro* (Fig. 1A). These findings indicate that the antibacterial effects observed *in vitro* are specific to MSCs.

Conditioned media obtained from MSCs only with bacterial preconditioning, that is, media obtained from MSCs after infection with 10^3 *E. coli* and incubation for 6 h, significantly inhibited bacterial growth *in vitro* (Fig. 1B). However, neither conditioned media obtained from MSCs without bacterial preconditioning nor conditioned media obtained from fibroblasts with or without bacterial preconditioning significantly inhibited bacterial growth *in vitro*.

These findings suggest that some paracrine molecules secreted by MSCs upon stimulation by bacterial preconditioning mediate the antibacterial effects of MSCs.

Changes in gene expression profiles of MSCs after bacterial exposure

In microarray analyses performed to identify gene expression changes in MSCs responsible for their antibacterial action after infection with 10^3 *E. coli* and incubation for 6 h, 400 genes were significantly up-regulated (Fig. 2A) in the MSCs compared with the human fibroblasts (MRC-5).

The 400 significantly up-regulated genes in MSCs compared with fibroblasts were related to 139 functional biological processes including regulation of cell proliferation, intracellular signalling cascades, immune responses and defence responses (Fig. 2B), and 6 KEGG signalling pathways including cytokine–cytokine receptor interaction, Jak-STAT signalling pathway, and TLR signalling pathway (Fig. 2C).

Among the up-regulated genes in biological processes of the intracellular signalling cascade, defence response, and immune response, both the TLR-2 and TLR-4 genes were commonly involved in gene ontologies, and BD2 was the only significantly up-regulated antibacterial protein identified by gene ontology analysis using Venn diagram (Fig. 2D). These findings suggest that the antibacterial effects of MSCs might be mediated by the secretion of BD2 via TLR signalling pathway.

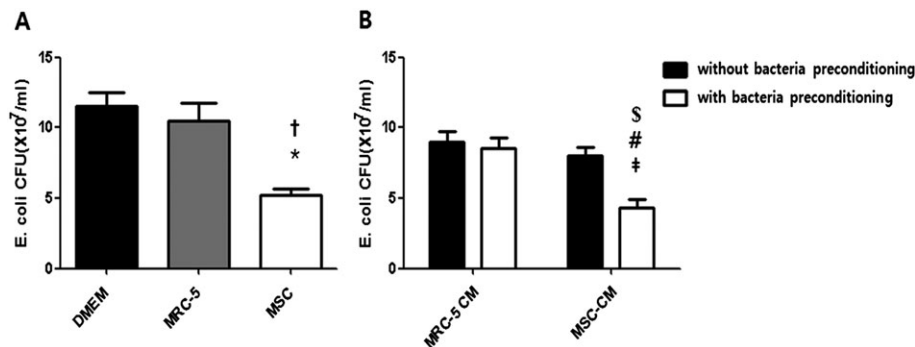


Fig. 1. Bacterial growth inhibition assay *in vitro*.

A. Bacterial colony forming units (CFUs) in the culture media (DMEM) only, human fibroblasts (MRC-5), or human umbilical cord blood-derived mesenchymal stem cells (MSC) after infection with 10^3 colony-forming unit (CFU) *E. coli* and incubation for 6 h. B. Bacterial CFUs in the conditioned media (CM) obtained from MRC-5 or MSC with or without bacterial preconditioning. Note the antibacterial effects are specific to MSC and MSC CM only with bacterial preconditioning. Data are presented as mean \pm SEM. The asterisk indicates the $P < 0.05$ compared with *E. coli* control group, the dagger sign the $P < 0.05$ compared with MRC-5 group, the number sign the $P < 0.05$ compared with MSC CM without bacterial preconditioning group, the double dagger sign the $P < 0.05$ compared to MRC-5 CM without bacterial preconditioning group, and the dollar sign the $P < 0.05$ compared with MRC-5 CM with bacterial preconditioning group ($n = 6$ per each analysis).

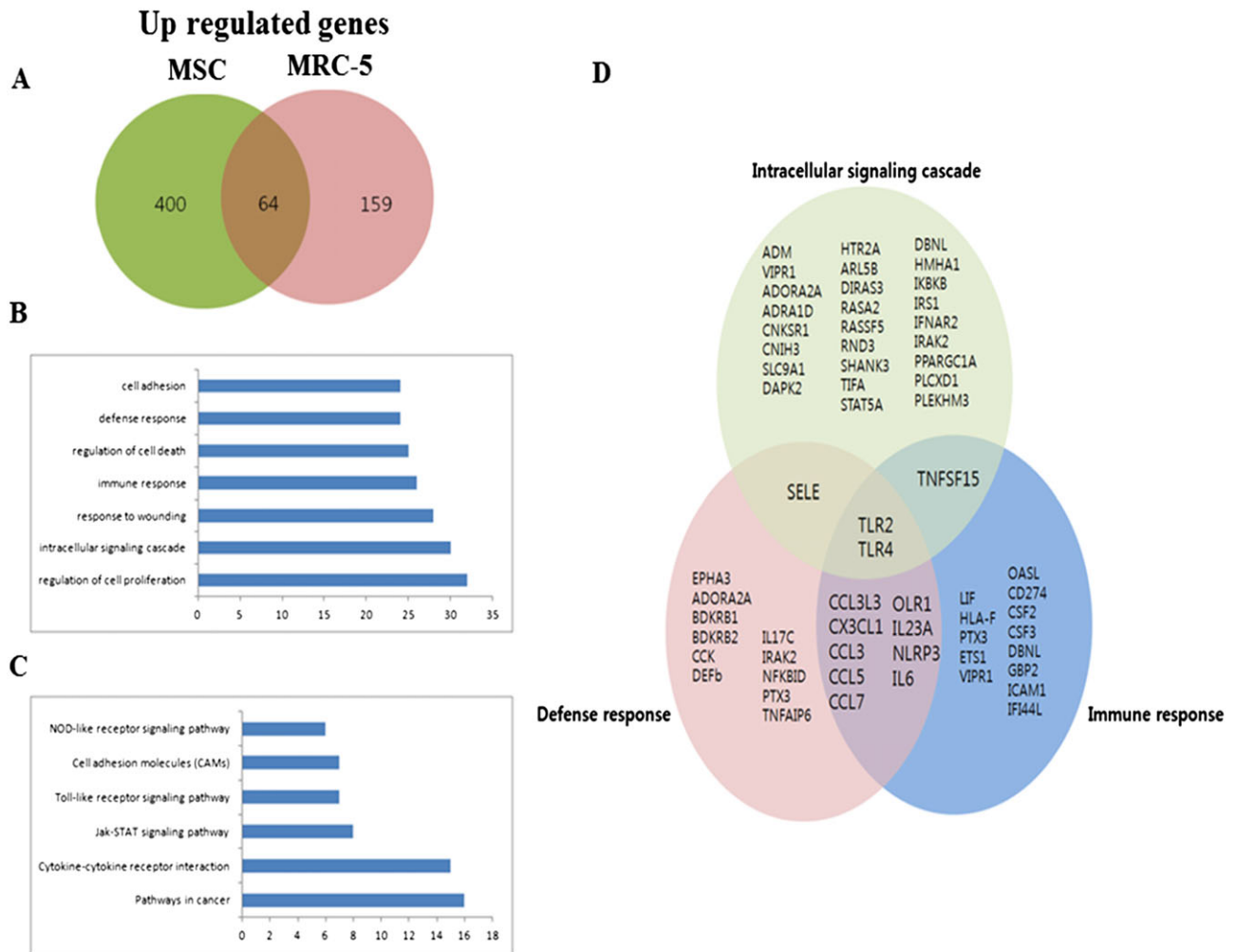


Fig. 2. Venn diagram, functional annotation, and KEGG molecular pathway analysis of microarray.

A. Venn diagram analyses showing significantly up-regulated genes in the human umbilical cord blood-derived mesenchymal stem cells (MSC) and/or human fibroblast (MRC-5) in the microarray analyses performed after infection with 10^3 CFU *E. coli* and incubation for 6 h.

B. Enriched functional categories and the number of genes in each category among the 400 significantly up-regulated genes in the MSC compared with MRC-5 after infection with 10^3 CFU *E. coli* and incubation for 6 h.

C. KEGG molecular pathway analysis and the number of genes in each pathway among the 400 significantly up-regulated genes in the MSC compared with MRC-5 after infection with 10^3 CFU *E. coli* and incubation for 6 h.

D. Gene ontology analysis up-regulated in MSCs using Venn diagram. Note that both the toll-like receptor (TLR)-2 and TLR-4 genes are commonly involved in the intracellular signaling cascade, defence response, and immune response, and β -defensin (BD)2 is the only significantly up-regulated antibacterial protein observed in the MSC, but not in the MRC-5, only after infection with 10^3 CFU *E. coli* and incubation for 6 h ($n=3$ per MSC or MRC-5 respectively).

Validation of TLR-2, TLR-4, and BD2 up-regulation

To validate the microarray data of significantly up-regulated TLR-2, TLR-4, and BD2 in MSCs but not in fibroblasts only after infection with 10^3 *E. coli* and incubation for 6 h, the expression levels of TLR-2, TLR-4, and BD2 were examined using semi-quantitative RT-PCR, Western blot, ELISA, or immunofluorescence staining (Figs 3 and 4).

In concordance with the microarray data showing significant up-regulation of both TLR-2 and TLR-4 in MSCs but not in fibroblasts only after *E. coli* exposure,

TLR-2 and TLR-4 mRNA expression by semi-quantitative RT-PCR analyses (Fig. 3A–D) and TLR-2 and TLR-4 protein expression by Western blot analyses (Fig. 3E–H) were significantly increased in MSCs but not in fibroblasts only after *E. coli* exposure. Despite somewhat weaker expression of TLR-2 compared with the RT-PCR and Western data, positive immunofluorescence staining of TLR-2 and TLR-4 in the MSCs but not in fibroblasts observed only after bacterial exposure also support the microarray data of up-regulated TLR-2 and TLR-4 in MSCs but not in fibroblasts only after *E. coli* exposure (Fig. 3I and J).

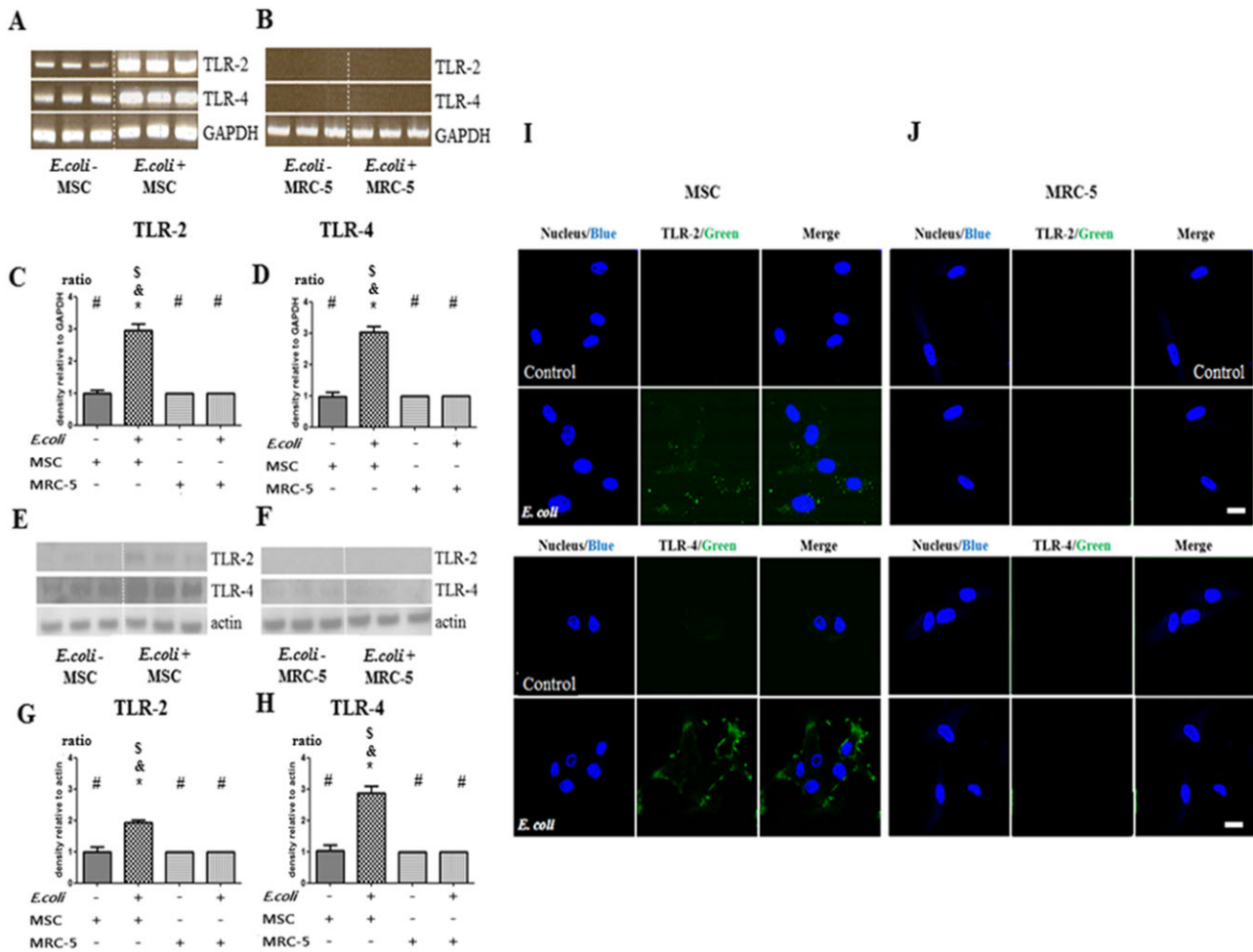


Fig. 3. Validation of the TLR-2 and TLR-4 up-regulation by reverse transcription-polymerase chain reaction (RT-PCR) (A–D), Western blotting (E–H) and immunofluorescence staining (I and J) in the human umbilical cord blood-derived mesenchymal stem cells (MSC) or fibroblasts (MRC-5) with or without infection with 10^3 CFU *E. coli* and incubation for 6 h. Note increased TLR-2 and TLR-4 expression by RT-PCR, Western blot, and immunofluorescence staining (green colour denotes TLR-2 or TLR-4; blue colour denotes nuclei stained by DAPI) in the MSC, but not in MRC-5 only after infection with 10^3 CFU *E. coli* and incubation for 6 h. Data are presented as mean \pm SEM. The asterisk indicates the $P < 0.05$ compared with MSC without *E. coli* group, the number sign the $P < 0.05$ compared with MSC with *E. coli* group, the dollar sign the $P < 0.05$ compared with MRC-5 without *E. coli* group, and the ampersand the $P < 0.05$ compared with MRC-5 with *E. coli* group ($n = 7$ per each analysis).

In concordance with the microarray data of significantly up-regulated BD2 genes in the MSCs but not in fibroblasts only after bacterial exposure, BD2 levels measured by semi-quantitative RT-PCR analysis (Fig. 4A) and ELISA (Fig. 4B) were significantly increased only in the MSCs but not in fibroblasts only after *E. coli* exposure. The positive immunofluorescence staining of BD2 in MSCs but not in fibroblasts only after bacterial exposure also supports the microarray data of up-regulated BD2 in MSCs, but not in fibroblasts, only after *E. coli* exposure (Fig. 4C and D).

BD2 secreted by MSCs via TLR-4, but not TLR-2, mediates the antibacterial effects

We used an antagonist or a siRNA against TLR-2 or TLR-4 to further elucidate the molecular signalling pathway mediating the antibacterial effects of the MSCs. The

increased BD2 levels after bacterial exposure and the inhibition of bacterial growth *in vitro* by MSCs were abolished with the TLR-4 antagonist (Figs. 5A and B). However, the TLR-2 antagonist or control peptide did not abolish the antibacterial effect of MSCs *in vitro*. Moreover, supplementation with 1 ng of BD2 restored the antibacterial effects of MSCs abolished by the TLR-4 antagonist.

Knockdown of TLR-4 by transfection with TLR-4 siRNA, but not transfection with TLR-2 siRNA nor scrambled siRNA, also abolished the increased BD2 levels after bacterial exposure, and the antibacterial effects of MSCs *in vitro* (Fig. 5C and D). Supplementation with 1 ng of BD2 restored the antibacterial effects of MSCs abolished by the TLR-4 siRNA. Overall, these findings suggest that the antibacterial effects of MSCs are mediated by secretion of BD2 via TLR-4, but not TLR-2, signalling pathway.

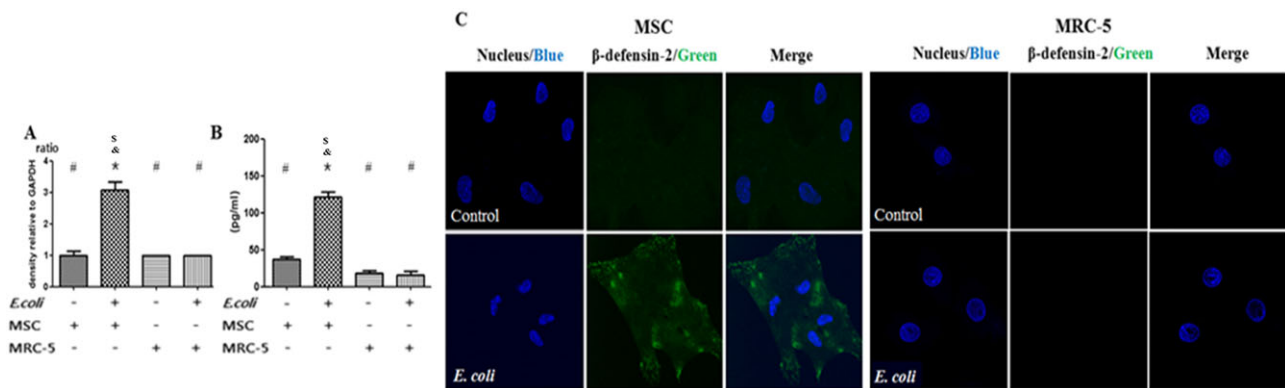


Fig. 4. Validation of the β -defensin (BD)2 up-regulation by reverse transcription-polymerase chain reaction (RT-PCR) (A), ELISA (B), and immunofluorescence staining (C and D) in the human umbilical cord blood-derived mesenchymal stem cells (MSC) or fibroblasts (MRC-5) with or without infection with 10^3 CFU *E. coli* and incubation for 6 h. Note increased BD2 expression by RT-PCR, ELISA, and immunofluorescence staining (green colour denotes BD2; blue colour denotes nuclei stained by DAPI) in the MSC, but not in MRC-5 only after infection with 10^3 CFU *E. coli* and incubation for 6 h. Data are presented as mean \pm SEM. The asterisk indicates the $P < 0.05$ compared with MSC without *E. coli* group, the number sign the $P < 0.05$ compared with MSC with *E. coli* group, the dollar sign the $P < 0.05$ compared with MRC-5 without *E. coli* group, and the ampersand the $P < 0.05$ compared with MRC-5 with *E. coli* group ($n = 7$ per each analysis).

In a dose titration study, supplementation of 0.5 or 1 ng, but not 0.1 ng, of BD2 significantly restored the antibacterial effects of MSCs abolished by the TLR-4 siRNA along with the simultaneous comparable, significantly higher and lower respective BD2 levels compared with those of naïve MSCs (Fig. 5E and F). These findings exclude the non-specific antibacterial effects of BD2 because of its inappropriately large amounts of supplementation to mask the effects of other antimicrobial peptides or mechanisms active in these cells.

Conditioned medium obtained from MSCs with TLR-4 siRNA transfection, but not with scrambled siRNA transfection, with bacterial preconditioning, abolished the increased BD2 levels and the antibacterial effects of conditioned medium obtained from MSCs with bacterial preconditioning *in vitro* (Fig. 5G and H). These findings also support that BD-2 secreted by MSCs via TLR-4 signalling pathway is the prime paracrine mediator of their microbicidal effects.

Lung histology and injury scores

Figure 6A–F presents representative photomicrographs showing histopathological differences observed by optical microscopy in each experimental group obtained 1 day after intratracheal inoculation of 1×10^7 CFU *E. coli*. The increased alveolar congestion, haemorrhage, neutrophil infiltration, and wall thickening observed 1 day after *E. coli* inoculation were significantly attenuated with intratracheal transplantation of MSCs, but not with fibroblasts transplantation (Fig. 6G–J). Furthermore, the protective effects of MSCs against *E. coli*-induced ALI were abolished with TLR-4 siRNA, but not with scrambled siRNA transfections. These findings suggest that the *in vivo* protective effects of MSCs transplantation against *E. coli*-induced ALI are associated with the TLR-4 signalling pathway.

Bacterial counts, BD2, protein, and cytokines in the BAL fluid

To evaluate the bacterial burdens *in vivo*, the number of CFUs in BAL fluids of mice was counted at 24 h after intratracheal inoculation of 1×10^7 CFU *E. coli*. The antibacterial effects were observed only with MSCs, but not with fibroblasts, transplantation, and the antibacterial effects of MSCs were abolished with TLR-4 siRNA, but not with scrambled siRNA, transfection of MSCs (Fig. 7A). Moreover, the human BD2 levels secreted from the transplanted MSCs in the concentrated BAL fluid were significantly higher with the naïve MSCs or scrambled siRNA transfection compared with the TLR-4 siRNA transfection of MSCs (Fig. 7G). Overall, these findings suggest that BD2 secreted by transplanted MSCs plays a pivotal role in mediating the *in vivo* antibacterial effects of MSCs.

The increased protein concentrations and cytokine levels such as IL-1 α , IL-1 β , IL-6, and TNF- α in the BAL fluid measured at 24 h after *E. coli*-induced ALI, were significantly attenuated only with the MSCs, but not with the fibroblasts, transplantation, and the anti-inflammatory effects of MSCs were abolished with TLR-4 siRNA, but not with scrambled siRNA, transfection of MSCs (Fig. 7B–F).

Discussion

Little is currently known about the precise mechanism underlying the antibacterial effects of MSCs. In the present study, human UCB-derived MSCs, but not fibroblasts, specifically inhibited bacterial growth *in vitro*, and the antibacterial activity of the MSCs was conserved in their conditioned media after bacterial preconditioning. These findings indicate that the antibacterial effects are

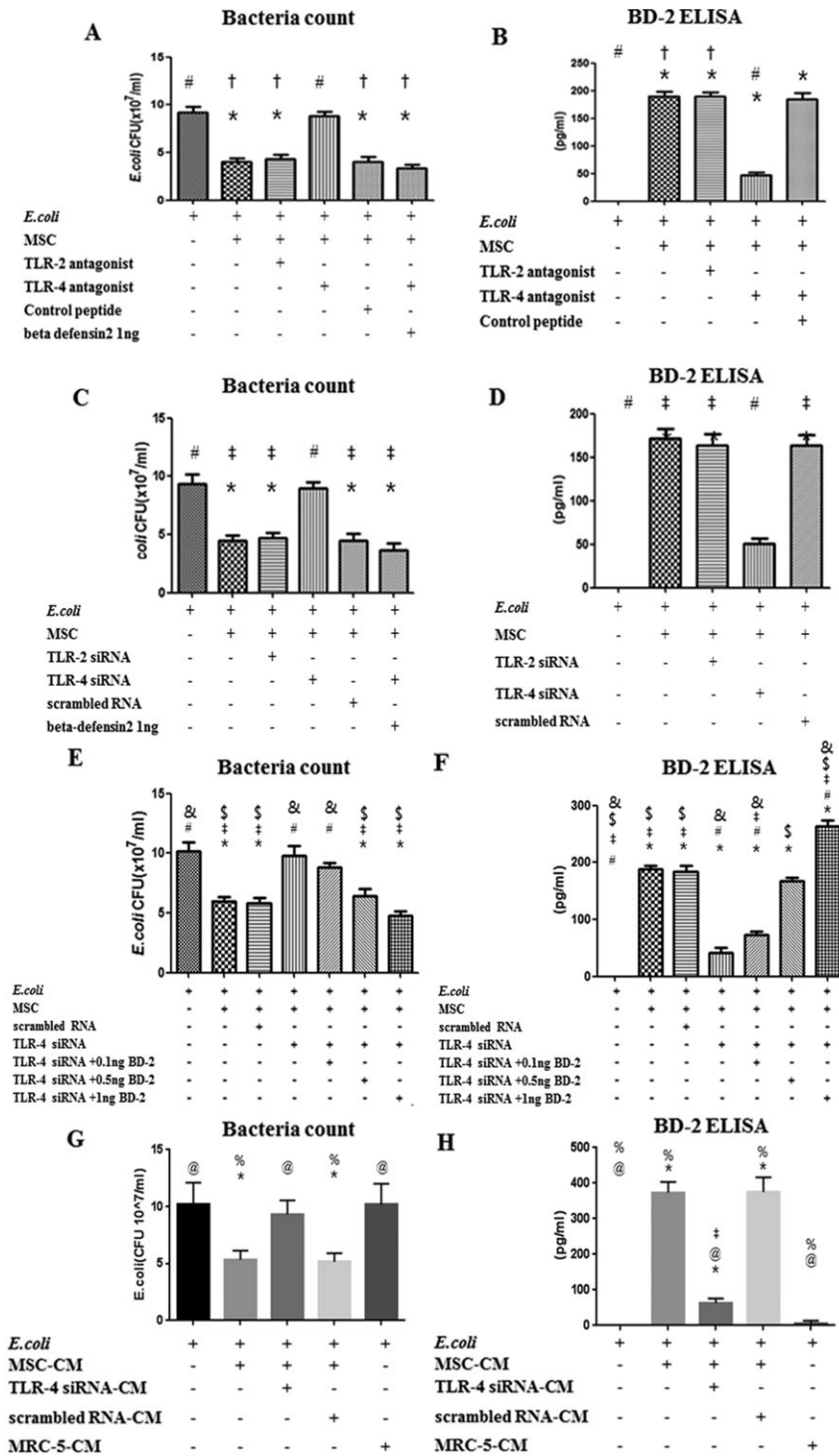


Fig. 5. Bacterial quantification and β -defensin (BD)2 levels *in vitro* in the MSC with toll-like receptor (TLR)-2/TLR-4 antagonist (A and B), or with TLR-2/TLR-4 siRNA transfection (C and D), or with dose titrated supplementation of BD2 (E and F), or with bacterial preconditioned media (CM) (G and H) after infection with 10^3 CFU *E. coli* and incubation for 6 h. Note the abolishment of antibacterial effects and the simultaneously increased BD2 levels of MSC or its bacterial preconditioned media only with TLR-4 antagonist or siRNA transfection, but not with TLR-2 antagonist or siRNA transfection, and the restoration of the antibacterial effects with dose titrated supplementation of BD2. Data are mean \pm SD. The asterisk indicates the $P < 0.05$ versus *E. coli* treated group, the number sign the $P < 0.05$ versus *E. coli* + MSC treated group, the dagger sign the $P < 0.05$ versus *E. coli* + MSC + TLR-4 antagonist treated group, the double dagger sign the $P < 0.05$ versus *E. coli* + MSC + TLR-4 siRNA treated group, the dollar sign the $P < 0.05$ versus *E. coli* + MSC + TLR-4 siRNA +0.1 ng BD treated group, the ampersand the $P < 0.05$ versus *E. coli* + MSC + TLR-4 siRNA +0.5 ng BD treated group, the commercial at sign the $P < 0.05$ versus *E. coli* + MSC-CM, and the percent sign the $P < 0.05$ versus *E. coli* + MSC + TLR4 siRNA-CM. By ANOVA (Tukey) ($n = 5$ per each analysis).

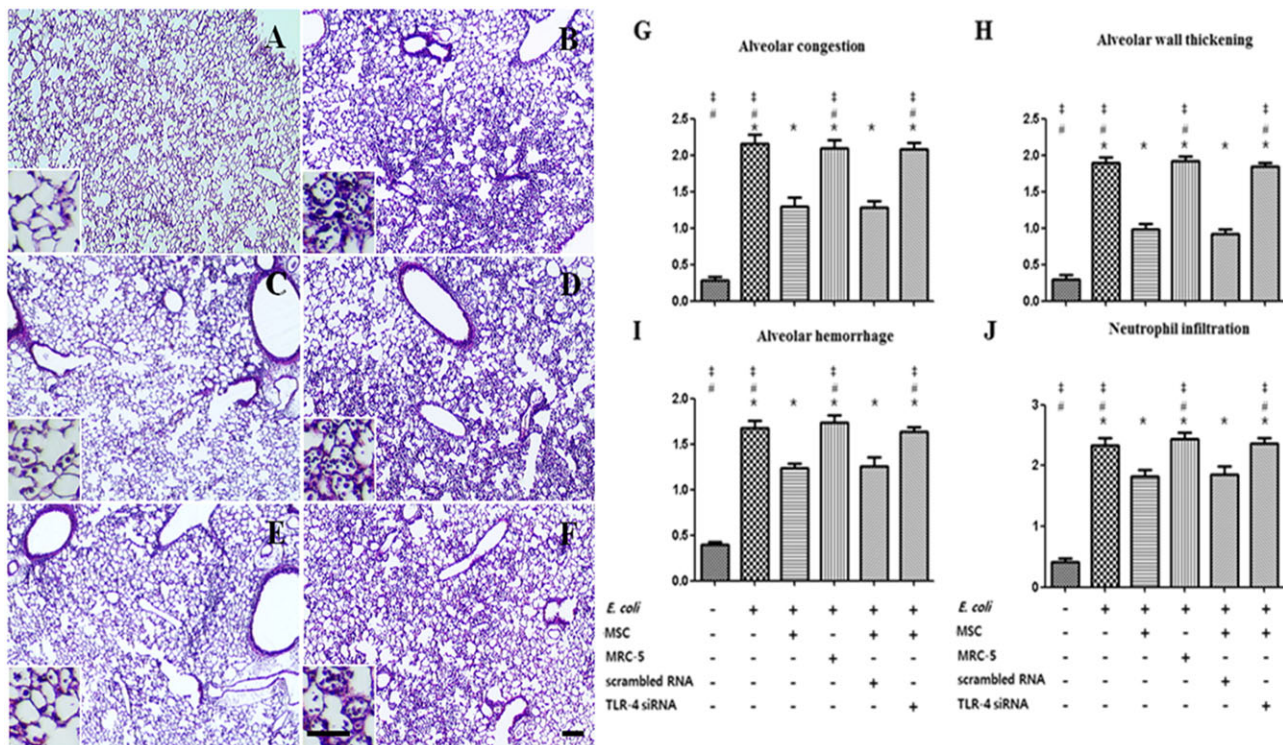


Fig. 6. Representative optical microscopy photomicrographs of the mice lungs stained with hematoxylin and eosin (A–F), and lung injury scores such as alveolar congestion, alveolar wall thickening, alveolar haemorrhage, and neutrophil infiltration (G–J) in each experimental group at 24 h after intratracheal inoculation of 10^7 *E. coli*. Note that the significant attenuation of the increase in *E. coli*-induced acute lung injury scores observed with human umbilical cord blood-derived mesenchymal stem cells (MSC) transplantation was abolished with TLR-4 siRNA transfection, but not with scrambled siRNA transfection, of MSC. Data are presented as mean \pm SEM. Data are mean \pm SD. The asterisk indicates the $P < 0.05$ versus *E. coli* treated group, the number sign the $P < 0.05$ versus *E. coli* + MSC treated group, and the double dagger sign the $P < 0.05$ versus *E. coli* + MSC + TLR4 siRNA treated group. By ANOVA (Tukey) ($n = 5$ per each analysis).

specific to MSCs and might be primarily mediated by soluble paracrine factors secreted by MSCs.

In contrast to the traditional knowledge-based approaches used to identify the molecular mechanisms of the antibacterial effects of MSCs, our microarray analysis enabled us to simultaneously interrogate the transcriptional response of entire MSC genomes after exposure to bacteria, independent of any prior knowledge. In this study, microarray analysis was performed to identify networks of genes or signalling pathways that might be associated with the antibacterial effects of MSCs. Significant increases in the levels of TLR-2, TLR-4, and BD2 mRNAs were identified by microarray analysis after *E. coli* exposure in MSCs compared with the levels seen in fibroblasts. The accuracy of the microarray data was verified by RT-PCR, Western and ELISA analyses, and immunofluorescence staining. Overall, these findings suggest that these significantly up-regulated TLR-2, TLR-4, and BD2 genes might be associated with the MSCs specific antibacterial paracrine effects. However, further studies will be necessary to elucidate other antimicrobial mechanism of MSCs including effects on phagocytosis.

The gene expression profiles of other antimicrobial proteins, such as LL-37 (Krasnodembskaya *et al.*, 2010) or lipocalin2 (Gupta *et al.*, 2012), which have previously been shown to mediate the antibacterial effects of MSCs, were not significantly up-regulated in the present study. However, as small changes in gene expression often result in biologically important differences (Soverchia *et al.*, 2005), our data of insignificant up-regulation, i.e. less than the arbitrarily set three-fold change, in the gene expression profiles of these factors do not exclude their role in mediating the antibacterial effects of MSCs. Therefore, as our data do not guarantee that BD2 is the only one exclusive antimicrobial mechanism of MSCs, further studies will be necessary that take account of the diverse antibacterial mechanism of MSCs, including other potentially important antimicrobial peptides such as LL-37 and lipocalin2, and effects on the macrophages to enhance phagocytosis.

Despite some controversy regarding TLR specificity, LPS from Gram-negative bacteria and peptidoglycan from Gram-positive bacteria are known to bind to TLR-4 and TLR-2 respectively and can induce the secretion of defensins, particularly of BD2 (Amlic-Lefond *et al.*, 2005;

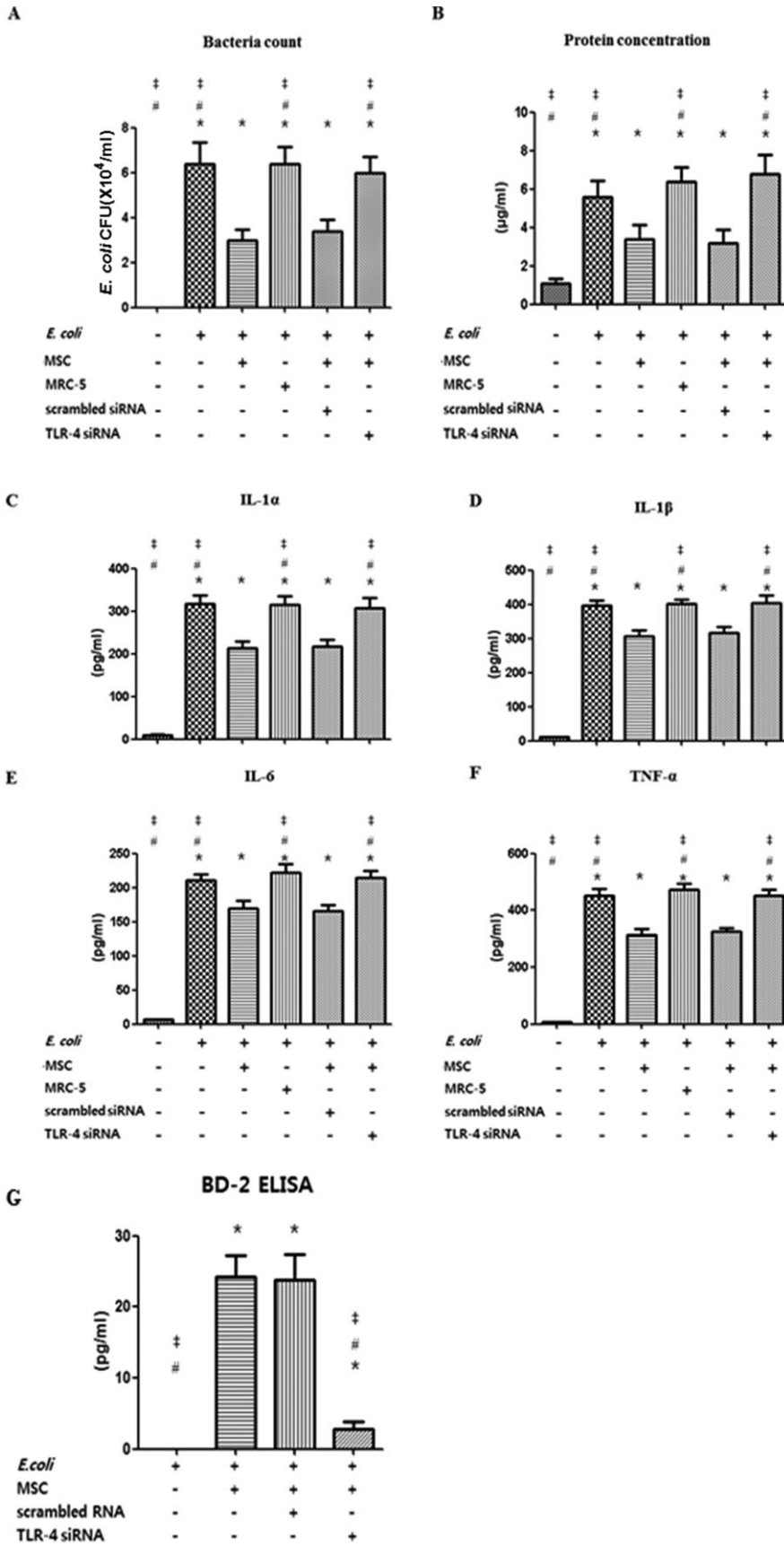


Fig. 7. Bacterial counts (A), protein (B), cytokine (C–F) and β -defensin (BD)2 (G) concentrations in the bronchoalveolar lavage (BAL) fluid of mice at 24 h after intratracheal inoculation of 10^7 CFU *E. coli*. Note that the significant attenuation of the *E. coli*-induced increase in protein and cytokine concentrations along with the simultaneous increase in BD2 levels in the BAL fluid observed with human umbilical cord blood-derived mesenchymal stem cells (MSC) transplantation was abolished with TLR-4 siRNA transfection, but not with scrambled siRNA transfection, of MSC. Data are presented as mean \pm SEM. Data are mean \pm SD. The asterisk indicates the $P < 0.05$ versus *E. coli* treated group, the number sign the $P < 0.05$ versus *E. coli* + MSC treated group, and the double dagger sign the $P < 0.05$ versus *E. coli* + MSC + TLR4 siRNA treated group. By ANOVA (Tukey) ($n = 5$ per each analysis).

MacRedmond *et al.*, 2005; Lai *et al.*, 2010). In the present study, although both TLR-2 and TLR-4 were significantly up-regulated in MSCs after *E. coli* exposure, increased BD2 levels and inhibition of bacterial growth *in vitro* by MSCs or their bacterial conditioned media were abolished only with TLR-4, but not with TLR-2, antagonist, or siRNA transfection. Furthermore, the abolished antibacterial effects of MSCs resulting from TLR-4 antagonist/siRNA were restored by the supplementation of BD2. Taken together, these findings suggest that the antibacterial effects of MSCs after exposure to *E. coli* are mediated by secretion of BD2 via the TLR-4, but not TLR-2, signalling pathway. Furthermore, as BD2 displays potent antibacterial activity against many Gram-negative bacteria and less potent bacteriostatic activity against Gram-positive bacteria (Zasloff, 2002), our data on the secretion of BD2 by MSCs through the TLR-4 signalling pathway after *E. coli* exposure might be the specific response of MSCs to produce the most effective antimicrobial peptide against the Gram-negative *E. coli* challenge. Further studies will be necessary to clarify whether there is bacterial pathogen type dependent variation in the TLR signalling pathways and the resultant production of antimicrobial peptides by MSCs.

As the antimicrobial resistance to antibiotics has increased rapidly, thereby greatly limiting the medications available to treat bacterial infections in clinical practice, the development of a new antimicrobial agent, especially one effective against multi-drug resistant pathogens, is an urgent issue (Li and Vederas, 2009). BD2 exerts its microbicidal effect via binding to plasma membranes, disrupting their integrity, leading to a leakage of intracellular components and inhibition of DNA, RNA, and protein syntheses (Pazgier *et al.*, 2006; Lavery *et al.*, 2011; Mendez-Samperio, 2013). Therefore, as its antibacterial mechanisms differ from conventional antibiotics, our data suggest the potential of BD2 as a novel adjuvant microbicidal agent in addition to antibiotics especially against multi-drug resistant Gram-negative pathogens (Lavery *et al.*, 2011).

Both infection and inflammation can be detrimental during bacterial pneumonia and the ensuing ALI. Given the potent anti-inflammatory action of MSCs (Uccelli *et al.*, 2008), there has been concern over their deleterious effects on the host defence against a live bacterial infection. In our previous and present studies, we have demonstrated that intratracheal transplantation of UCB-derived MSCs attenuates symptoms of *E. coli*-induced ALI, such as leukocyte infiltration, alveolar congestion, haemorrhage, and wall thickening, not only by enhancing bacterial clearance but also by down-modulating the inflammatory responses (Kim *et al.*, 2011). Overall, our data indicate that intratracheal transplantation of MSCs could be a novel adjunctive therapeutic modality to reduce *E. coli*-induced ALI, which functions by simultaneously enhancing bacterial clearance and attenuating the inflammatory responses.

In the present study, the *in vivo* protective anti-inflammatory and antibacterial effects of MSCs against the *E. coli*-induced ALI along with simultaneous increased BD2 levels in the BAL fluid were abolished with knockdown of TLR-4 with siRNA transfection. However, considering the pro-inflammatory phenotype of TLR-4 primed MSCs (Waterman *et al.*, 2010), our conflicting data showing that TLR-4 from MSCs exerts an anti-inflammatory property in *E. coli*-induced ALI *in vivo* are difficult to explain. There may be several factors that could account for the critical role of TLR4 in mediating the *in vivo* anti-inflammatory effects of MSCs. First, reduced pathogenic burden resulting from the antimicrobial effects of MSCs primarily mediated by secretion of BD2 via the TLR-4 signalling pathway might be responsible for the down-regulation of the inflammatory responses. Second, LPS could protect MSCs from oxidative stress-induced apoptosis via the TLR-4 pathway (Lombardo *et al.*, 2009; Wang *et al.*, 2009). A third mode of action would be the creation a negative feedback loop whereby bacterial toxins, such as lipopolysaccharide, acts on the TLR-4 of MSCs to activate nuclear factor- κ B signalling, up-regulate cyclooxygenase 2, and secrete the anti-inflammatory paracrine mediator prostaglandin E2 that augments IL-10 production by macrophages (Németh *et al.*, 2009; Le Blanc and Mougiakakos, 2012). Taken together, these findings suggest that MSCs sense and control host inflammation by switching between their dual roles as pro-inflammatory or anti-inflammatory mediators (Bernardo and Fibbe, 2013), and TLR4 of MSCs plays a critical role as its plasticity enables the MSCs to trade-off between eliminating pathogens by augmenting antimicrobial immunity and reducing host injury by attenuating the inflammatory responses (Auletta *et al.*, 2012; Balan *et al.*, 2014).

In conclusion, BD2 secreted by human UCB-derived MSCs via TLR-4 signalling pathway is the key paracrine factor mediating the *in vitro* antibacterial effects of MSCs 6 h after *E. coli* exposure. Furthermore, TLR-4 from the transplanted MSCs and subsequent secretion of BD2 is at least one of the critical factors mediating the protective effects of MSCs against the *in vivo* *E. coli*-induced ALI through both its antibacterial and anti-inflammatory effects.

Experimental procedures

Cell preparation

In the present study, MSCs were isolated and cultivated from human UCB, collected from umbilical veins after neonatal delivery with informed consent from pregnant mothers, as previously reported (Rocha *et al.*, 2000; Yang *et al.*, 2004). The cells expressed CD105 and CD73 (99.6% and 96.3% respectively) but not CD34, CD45, or CD14 (0.1%, 0.2%, and 0.1% respectively). The cells were positive for HLA-AB (96.8%) but not for HLA-DR (0.1%) (Kim *et al.*, 2011). The osteogenic, chondrogenic,

and adipogenic differentiation of MSCs have also been previously described (Yang *et al.*, 2004; Jang *et al.*, 2006; Lee *et al.*, 2007; Kim *et al.*, 2011; Ahn *et al.*, 2015). We confirmed the differentiation potential and karyotypic stability of the human UCB-derived MSCs up to the 11th passage (Chang *et al.*, 2011; Kim *et al.*, 2011). In this study, fifth passage human UCB-derived MSCs from a single donor was used for the transplantations.

Human fibroblasts (MRC-5; Korean Cell Line Bank No.10171) were purchased from the Korean Cell Line Bank (Seoul, Korea) and cultured in DMEM medium supplemented with 10% foetal bovine serum.

Bacterial preparation

We used *E. coli* as the source of infection in this study because it is a common cause of Gram-negative bacterial lung infections (Cordero *et al.*, 2004). The *E. coli* strain E69 was generated by PI transduction of the *E. coli* K12 outer membrane protein A (Omp A) gene into an Omp A mutant of RS 218, isolated from the CSF of a newborn with *E. coli* meningitis (a gift from Dr Kwang Sik Kim, Johns Hopkins University, Baltimore, MD, USA) (Park *et al.*, 2001). *E. coli* was cultured overnight in 10 ml of brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI, USA) at 37°C. The bacteria were then diluted in BHI media and grown for 1 h to mid-logarithmic phase. The suspension was centrifuged for 10 min at 5000 r.p.m. and washed in phosphate-buffered saline (PBS). Optical density was measured, and the bacterial samples were adjusted to the desired concentration.

Preparation of bacterial preconditioned media

For preparation of bacterial preconditioned media, human UCB-derived MSCs, scrambled siRNA-transfected MSCs, TLR-4 siRNA-transfected MSCs, or fibroblasts (MRC-5), with 5×10^5 cells in 50 μ l of PBS per well, or equal volume of PBS for normal control group per well, were seeded in each well of 12-well plates pre-filled with 1 ml of serum-free low-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NJ, USA). After then, each well was infected with 10^3 colony-forming unit (CFU) *E. coli* and incubated for 6 h in a humidified CO₂ incubator. The bacterial preconditioned media from each well were filtered with a 0.2- μ m pore-size syringe filter (Nalgene, Rochester, NY, USA) to remove *E. coli*, centrifuged at 6000 r.p.m. for 10 min at 4°C, and the supernatants were stored at -80°C for later use of testing for inhibition of bacterial growth or measuring BD2.

Inhibition of bacterial growth in vitro

For bacterial growth inhibition assay *in vitro*, MSCs, scrambled siRNA-transfected MSCs, TLR4 siRNA-transfected MSCs, fibroblasts (MRC-5), with 5×10^5 cells in 50 μ l of PBS per well, or equal volume of PBS for normal

control group per well, were firstly seeded in each well of 12-well plates pre-filled with 1 ml of DMEM. For the bacterial inhibition assay of conditioned media, 1 ml of DMEM with or without bacterial preconditioning was pre-filled per well. After then, each well was infected with 10^3 CFU *E. coli* and incubated for 6 h in a humidified CO₂ incubator.

Bacterial quantification

Assessment of direct inhibition of bacterial growth by MSCs, scrambled siRNA-transfected MSCs, TLR4 siRNA-transfected MSCs, or their bacterial preconditioned media was performed by counting CFUs *in vitro*. DMEM was used as a control medium, and human fibroblasts (MRC-5) were used as control cells. Briefly, after infection with 10^3 CFU *E. coli* in each well and incubation for 6 h in an humidified CO₂ incubator, aliquots of media were taken from each well, serially diluted with sterile PBS, and plated on BHI agar, and colonies were counted after overnight incubation at 37°C.

Bacterial concentrations in the bronchoalveolar lavage (BAL) fluids were measured by counting CFU levels at dilutions of 10^{-3} – 10^{-7} plated on BHI agar after overnight incubation at 37°C.

Microarray analysis and functional analysis

To identify gene expression changes in MSCs responsible for their antibacterial action, microarray analyses were performed using an Agilent Beadchip assay on RNA from MSCs and fibroblasts after infection with 10^3 CFU *E. coli* and incubated for 6 h. Total RNA of MSCs or fibroblasts after infection with 10^3 CFU *E. coli* and incubation for 6 h in an humidified CO₂ incubator was isolated using the Trizol kit (Invitrogen Carlsbad, CA, USA). For microarray analysis, RNA was hybridized to Agilent Human Oligo Microarray (60 K) chips, and the hybridized images were scanned using an Agilent DNA microarray scanner and quantified with Feature Extraction Software (Agilent Technology, Palo Alto, CA, USA). All data normalization and selection of fold-changed genes were performed using GeneSpringGX 7.3 (Agilent Technology, Palo Alto, CA, USA). Changes in gene expression greater than threefold with *P* values less than 0.05 in each group were considered to be significant changes in gene expression (Voehringer *et al.*, 2000; Lu *et al.*, 2004). Genes meeting all the criteria were further analysed by means of hierarchical clustering and K-means clustering using the GENOWIZ software. To assay functional annotation, we used the list of relevant genes in the DAVID online database (<http://david.abcc.ncifcrf.gov/>), Medline (<http://www.ncbi.nlm.nih.gov/>) and the KEGG database (<http://www.genome.jp/kegg/>).

TLR-2 and TLR-4 siRNA transfection of MSCs or antagonist treatment

TLR-2 and TLR-4 siRNAs were purchased from Santa Cruz (Santa Cruz, CA, USA). MSCs were transfected with siRNA oligonucleotides using Oligofectamine (Invitrogen Carlsbad, CA, USA), according to the manufacturer's instructions. All assays or transplants were performed 24 h after RNA transfection. MSCs were pre-incubated with TLR-4 specific antagonist VIPER or control peptide (Imgenex, San Diego, CA, USA), TLR-2 specific antagonist CU-CPT22 (Sigma-Aldrich St. Louis, MO, USA), diluted in the PBS, 30 min prior to infection with *E. coli*.

RNA isolation and RT-PCR

RNA was isolated from MSCs or fibroblasts using the TRIZOL kit (Qiagen Inc, CA). The quality of the RNA was assessed with the NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE) according to the manufacturer's instructions. The 260/280 and 260/230 nm absorbance ratios of 1.8–2.0 indicated a pure RNA sample. Primers for TLR-2, TLR-4, BD2 and GAPDH were custom made (Bioneer Co., Taejeon, Korea). The sequences were as follows: TLR-2 (forward 5'-ATGTTACAAGTTCCGTGGAATG-3'; reverse 5'-TGAAACAACTTTTCATCGGTG-3'), TLR-4 (forward 5'-CATTGCTTCTTGCTAAATGCTG-3'; reverse 5'-GGATTAAAGCTCAGGTCAGG-3'), BD2 (forward 5'-GAGGAGGCCAAGAAGCTGC-3'; reverse 5'-CGCACGTCTCTGATGAGGG-3'), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward 5'-TGAGCGATGTGGCTCGGCT 3'; reverse 5'-CTCTCTGCTCCTCCTGTTTCGAC-3'). RT-PCR was performed using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase protocol from Invitrogen, according to manufacturer's instructions. The specificity of the PCR products was confirmed by DNA sequencing. The resulting amplified DNA products were run on a 1% agarose gel, and bands were visualized with the use of ethidium bromide.

Western blot

After *E. coli* infection and incubation for 6 h, MSCs or MRC-5 were lysed by radioimmunoprecipitation assay (RIPA) buffer followed by 10 s of sonication. The samples were separated on sodium dodecyl sulfate (SDS)–polyacrylamide gels (12%) and transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The membranes were washed in PBST (phosphate-buffered saline, 0.1% Tween 20) for 5 min and incubated for in PBST-3% BSA (TBST supplemented with 3% bovine serum albumin). After two washes with PBST, the TLR-2 or TLR-4 antibodies (Santa Cruz 1:5000 in PBST-5% skim milk) were added either for overnight at 4°C. Then the secondary antibodies

conjugated with horseradish peroxidase (1:1000 in PBST-5% skim milk) were added for 1 h.

BD2 enzyme-linked immunosorbent assay (ELISA)

Because of its low BD2 concentrations, the obtained BAL fluid was concentrated 10-fold by using ultrafiltration units with a 3-kD molecular weight cutoff (Amicon Ultra-PL3; Millipors, Bedford, MA, USA) for BD2 measurements.

The β -defensin 2 in the media or concentrated BAL fluid was measured using an ELISA kit according to the manufacturer's protocol (Phoenix Pharmaceuticals, Belmont, CA, USA).

Fluorescent immunohistochemistry

For the immunofluorescence staining of TLR-2, TLR-4 or BD2 in cell cultures, MSCs or fibroblasts were seeded on cover-glass bottom dishes (SPL Lifescience, Seoul, Korea) at a density of 1×10^3 cells cm^{-2} . Cells were grown for 24 h and then infected with *E. coli* (10^3 CFUs) and incubated for 6 h. After then, the cell monolayer was washed three times with cold PBS and fixed in 4% paraformaldehyde for 30 min. The cells were then washed three times with PBS for 10 min in a gently shaking chamber at room temperature. The cells were then permeabilized by 0.2% Triton X-100 and blocked with 5% BSA for 2 h. The slides were incubated with a primary antibody for BD2 (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) at a dilution of 1:100, TLR-2 (Santa Cruz Biotechnology) or TLR-4 (Santa Cruz Biotechnology) at dilutions of 1: 500 in 3% BSA overnight at 4°C. The slides were washed three times with PBS for 10 min and then exposed to the goat Alexa-Fluor 488-labelled anti-mouse-IgG secondary antibody. After washing, slides were incubated with DAPI as a counter stain.

Animal model

Experimental protocols for animal procedures were approved by the Institutional Animal Care and Use Committee of Samsung Biomedical Research Institute. The study followed our institutional and National Institutes of Health guidelines for laboratory animal care.

Eight-week-old male ICR mice, purchased from Orient Co. (Seoul, Korea), were housed in individual cages with free access to water and laboratory chow. Animals were randomly divided into six experimental groups: sham control ($n=5$), *E. coli*-induced ALI control ($n=5$), ALI with fibroblasts transplantation ($n=5$), ALI with MSCs transplantation ($n=5$), ALI with scrambled siRNA-transfected MSCs transplantation ($n=5$), and ALI with TLR4 siRNA-transfected MSCs transplantation ($n=5$).

After anaesthesia with intraperitoneal injection of a mixture of ketamine and xylazine (45 and 8 mg kg^{-1} respectively), intubation was performed with a 20-gauge central catheter (Leader Cath 20 G \times 8 cm; Vygon, Paris,

France), and *E. coli* at 10^7 CFUs in 0.05 ml normal saline was administered (Kim *et al.*, 2011).

For cell transplantation, 1×10^5 cells (MSCs or fibroblasts) in 0.05 ml PBS were administered intratracheally 3 h after the *E. coli* instillation. Equal volume of normal saline was administered intratracheally in the control groups. Normal control and *E. coli* control groups received the same volume of normal saline intratracheally. For intratracheal transplantation, the animals were anesthetized, and the catheter was placed as described in the preceding texts. After intratracheal transplantation, the catheter was removed and the mice were allowed to recover, and subsequently returned to their dams.

Preparation of the BAL fluid

The BAL fluids was collected at post-infection day 1 by using three consecutive instillations of 1 ml sterile PBS at room temperature. For each instillation, the trachea was cannulated with a blunted 22-gauge needle, 1 ml of PBS was instilled, and aspirated after a gentle massage over the chest. After plating on the BHI agar for bacterial quantification, the remaining collected BAL fluid was centrifuged at 6000 r.p.m. at 4°C for 10 min, and stored at -80°C for estimation of protein, cytokines and BD2 levels.

Protein and cytokine assays of BAL fluid

BAL fluid protein concentration was measured by the Bradford method using a Bio-Rad protein assay kit. Interleukin (IL)-1 α , IL-1 β , IL-6, and TNF- α in the BAL fluid were measured using a mouse procarta immunoassay kit (Affymetrix, Santa Clara, CA, USA), according to the manufacturer's protocol.

Tissue preparation

Lung tissue was prepared from surviving animals at 24 h after bacterial infection. The mice were anesthetized with sodium pentobarbital (100 mg kg^{-1}), and the lungs and heart were exposed via thoracotomy, followed by transcardiac perfusion with ice-cold PBS. The lungs were fixed by tracheal instillation of 4% formaldehyde with a constant inflation pressure of 20 cm H₂O. The trachea was ligated, and the lungs were removed and immersed in 4% formaldehyde overnight at room temperature. Both lungs were embedded in paraffin, and transverse serial sections (4 μm thick) were prepared for hematoxylin and eosin staining.

Statistical analyses

The data are expressed as the mean \pm SEM. For continuous variables with a normal distribution, the groups were compared using one-way ANOVA followed by Tukey's multiple comparison test. All data were analysed using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). Values of $P < 0.05$ were considered statistically significant.

Conclusion

We describe in the work that BD2 secreted by the MSCs via TLR-4 signalling pathway is one of the critical paracrine factors mediating their microbicidal effects against *E. coli* both *in vitro* and *in vivo*, and TLR-4 of the transplanted MSCs plays a seminal role in attenuating the *in vivo* *E. coli*-induced ALI by both their antibacterial and anti-inflammatory effects.

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Conflicts of interest

The authors indicate no potential conflicts of interest.

Author contributions

D.K.S. and Y.S.C.: conception and design, collection and/or assembly of data, data analysis and interpretation, and manuscript writing; S.I.S.: data analysis and interpretation; H.S.Y.: data analysis and interpretation; S.Y.A.: collection and/or assembly of data and manuscript writing; W.S.P.: conception and design, data analysis and interpretation, manuscript writing, and final approval of manuscript.

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