Gelatin-9 Mediates the Therapeutic Effect of Mesenchymal Stem Cells on Experimental Endotoxemia

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Abstract

Background: Endotoxemia, mediated by uncontrolled immunocytes activation toward Lipopolysaccharide, could deteriorate into severe septic shock, but with limited treatment effect. Mesenchymal stem cells (MSCs), with excellent immune regulatory capacities, have displayed potential in multiple inflammatory disease treatment. Gelactin-9 (Gal-9), a newly discovered immune checkpoint, has been demonstrated to mediate immunomodulatory effect of MSCs *in vitro*. However, its *in vivo* role in alleviating endotoxemia remains to be elucidated.

Methods: MSCs (2.5×10^5/ml) were obtained and stimulated with IFN-γ (20ng/ml) for 72 hours. Gal-9 expression on MSCs were measured by ELISA, RT-PCR, flow cytometry and immunofluorescence respectively. Then, experimental endotoxemia was induced by LPS injection (10mg/kg, i.p.), followed by the treatment with Gal-9-MSC (20ng/ml, 72 hours), MSCs and MSC+α-lactose (10.8mg/mL, 500ul, i.v.). Therapeutic effects of MSC-based treatments were assessed by monitoring murine sepsis score, survival rate, splenocyte proportion, phenotype polarization, inflammatory mediator levels and pathological manifestations. Furthermore, Gal-9 expression in multiple organs was also detected after administering the treatments.

Results: It has been found that MSCs expressed Gal-9 and its level was increased in a dose-dependent manner after being stimulated by IFN-γ. Moreover, adoptive transferred of IFN-γ pre-stimulated MSCs into endotoxemia mice was found with relieved symptoms and increased survival rate. Flow cytometry analysis indicated that
Gal-9-MSC could promote macrophage polarization to M2-subtype and increase Treg ratios in spleen. Further results also demonstrated that, Gal-9-mediated MSC therapy could assist in attenuating local and circulating pro-inflammatory mediators expression (TNF-α, IL-1β, IFN-γ and iNOS), but increasing anti-inflammatory mediators expression (T-SOD and IL-35). Additionally, after administrating Gal-9-MSC, it was also found there was a significant relief in pathological manifestations, and with a higher expression of Gal-9 in liver, kidney and lung homogenate.

**Conclusions:** This study revealed that Gal-9 mediated therapeutic effects of MSCs in alleviating endotoxemia injury, which provides a novel idea for supplementing the research of MSC immunoregulatory mechanism, and offers an excellent candidate to be used in treatment of endotoxemia in the clinical settings.

**Keywords:**

Gelactin-9, Mesenchymal Stem Cells (MSCs), Lipopolysaccharide (LPS), Endotoxemia, Immunoregulation
Introduction

Aberrant activation of inflammatory cells, attributed to detrimental response to the circulating endotoxin, has been recognized as the main reason for the life-threatening diseases, such as endotoxemia, sepsis and septic shock [1]. Sepsis, a major public health concern, accounted for 19.7% of all global deaths annually [2, 3]. While endotoxemia, as an important pathophysiological process of sepsis, is mediated by uncontrolled immunocytes activation toward Lipopolysaccharide (LPS), which is also found in outer membrane of Gram-negative bacteria and known as endotoxins [4, 5]. Endotoxemia could lead to the severe septic shock with production of a large number of biologically active substances, including cytokines, bioactive amines and a variety of reactive oxygenspecies, thus lethal to the hosts [6].

Until now, majority clinical therapies implemented were mainly focused on supporting treatments, including incentive removal, antibiotics application, circulatory resuscitation, mechanical ventilation and renal replacement [7]. Although effective when implemented timely and appropriately, but these measures were often considered inadequate due to the lack of immunomodulatory in alleviating the overwhelming inflammatory response. Several clinical trials have adopted monoclonal antibodies to neutralize cytokines or block the receptors of inflammatory factors [8]. However, these attempts were proven with limited effect due to targeting the few receptors [9, 10]. Therefore, seeking a novel therapeutic method to supplement the existing immunomodulatory strategy is in urgent need.
Mesenchymal stem cells (MSCs), a newly discovered pluripotent stem cell, with multiple differentiation ability, self-renewal capacity and immunoregulation specialties, have drawn more and more attention in multiple disease treatment. Due to its outstanding immune modulated effects, MSCs are recognized as an attractive candidate for the treatment of immune imbalance disorders [11]. Furthermore, MSCs have also been reported with low expression of histocompatibility complex (MHC) I and deficiency in MHC II, resulting in its low immunogenicity and well accepted by recipients [12, 13]. It has also been reported that MSCs could mobilize from bone marrow and influenced by the chemokines, then gathering and accumulating in the impaired tissues and organs. Through proliferation, directional differentiation and modulators secretion, MSCs could mediate in preventing continuous damage and promoting the repairment [14]. Given these encouraging characters, MSC mediated treatment have been explored in various pre-clinical and clinical studies, such as multiple sclerosis, rheumatoid arthritis, Crohn's disease, systemic sclerosis, ischemic kidney injury and systemic lupus erythematosus [15-17].

Of interest, although the immunoregulation ability of MSCs has been widely recognized, the specific molecular mechanism behind this is not fully understood. Growing evidence have suggested MSCs could modulate the immune response through direct cell-to-cell contact or regulators secretion, such as NO, IL-10, TGF-β, VEGF, PGE2 and Indoleamine-2,3-dioxygenase (IDO) [11, 18, 19]. Meanwhile, series of studies
have also revealed that IFN-γ was required for MSC activation. But the vital phenotype changes in IFN-γ stimulated MSCs remain to be elucidated [20, 21].

Inspired by previous reports and preliminary data, we found that MSCs could express galectin-9 and this expression increased in a dose-dependent manner after being stimulated by IFN-γ. Galectin-9 (Gal-9), an important member of the galectin family, with two carbohydrate recognition domains (CRDs) joined by a linker peptide, is a natural ligand for T-cell immunoglobulin mucin-3 (Tim-3)[22]. While tim-3, one of the wildly studied immune checkpoint, expressed in Th1/17 cells, CD8+ cytotoxic T cells, regulatory T cells (Treg), macrophage and natural killer (NK) cells [23]. When Galectin-9 binds to Tim-3+ cells, it can negatively regulate Th1 and Th17 immunity [24], promote macrophage polarization to M2 subtype [25, 26], and enhance the development of FoxP3+ regulatory T cells (Treg) in a Tim-3-independent manner [27]. In addition, Gal-9 was also reported to suppress T and B cell response and involved in immunosuppression effect mediated by MSCs in vitro [28, 29].

Given together, Gal-9 has been demonstrated to mediate immunomodulatory effect of MSCs in vitro. However, its in vivo role in alleviating endotoxemia in still need to be explored. Therefore, this experiment was designed to explore whether MSCs express Gal-9 and whether Gal-9 is involved in the therapeutic effects of MSCs in alleviating endotoxemia.
Materials and Methods

Animals

Male C57BL/6 mice weighing 18-20g and aged 6-8 weeks were purchased from the China Food and Drug Inspection Institute (Beijing, China). All the mice were fed with a standard diet and acclimated in the animal care facility for 7 days before experiments. Experiments involving animals were all complied with the standard protocols approved by the Animal Care and Use Committee of Tianjin Medical University (Tianjin, China), according to the Chinese Council on Animal Care guidelines.

MSC preparation

Mesenchymal stem cells were obtained in accordance with the methods described previously [30]. Briefly speaking, 6-week-old mouse was sacrificed firstly and sterilized in 75% alcohol for 5 minutes. Then, bilateral inguinal fat were extracted and shred on the ultra-clean workbench. Fragment fat tissues were soaked in DMEM medium (Hyclone Laboratories Inc, USA) containing 1 mg/ml collagen type I (Solarbio, Beijing, China) and digested in a shaker (37°C, 5% CO2) at 300rpm for 30 minutes. After digestion, pellet cells were harvested and suspended in the DMEM-F12 culture medium with 10% FBS and 1% penicillin/streptomycin. The culture medium was changed every two days to remove the non-adherent cells. When the cells expand to 80% of the plate, the adherent cells were passaged down at a ratio of 1:3, and finally the MSCs were harvested for purity test and subsequent experiments. Adipose derived mesenchymal stem cells did
not express CD45 or CD31, but expressed CD105 and CD73 surface markers, and could differentiate into osteogenic and adipogenic cells under the stimulation.

**Detection of Gal-9 expression in MSCs**

The expression of secreted Gal-9 in MSCs was detected in p2-p5 cells (2.5 × 10⁵/ml) culture supernatant. Meanwhile, third-generation MSCs (2.5 × 10⁵/ml) were collected and stimulated with different concentrations of IFN-γ (PeproTech, Rocky Hill, USA) for 72 hours, and then the total protein of MSCs was extracted to determine Gal-9 expression. Furthermore, the third generation MSCs (2.5 × 10⁵/ml) were obtained and stimulated with 20ng/ml IFN-γ for 72 hours. Flow cytometry analysis was applied to measure Gal-9 expression on MSC surface, and RT-PCR was carried out for Gal-9 mRNA detection.

**Immunofluorescence staining**

Third-generation MSCs with or without IFN-γ stimulation (20ng/ml) were obtained and washed with PBS for three times. Then, these crawled cells were fixed in a 4% (w/v) paraformaldehyde solution on the slide for 15 minutes, followed by permeabilizing in 0.1% Triton X-100 solution for 5 minutes. After blocking with BSA, anti-mouse Gal-9 primary antibody (1:1000 dilution) was adopted to incubate the slides at 4°C overnight. On the other day, Alexa Fluor® 488-conjugated secondary antibody (Jackson ImmunoResearch Inc, USA) was further used to incubate the slides in a dark cassette for
60 minutes, followed by mounted with antifade mountant containing DAPI (SouthernBiotech, USA). Finally, these slides were obtained and photographed under fluorescence microscope.

**Experimental groups and endotoxemia induction**

Mice were randomly divided into five experimental groups (n=6) and received separate lipopolysaccharide (LPS)(10 mg/kg, i.p.) and MSCs (500ul, i.v.) injection at time 0h and time 1h, respectively: (1) Normal control group: Receive an equal amount of PBS as other groups. (2) Untreated group: Endotoxemia was induced with injection of LPS (10 mg/kg, i.p.) (Solarbio, Beijing, China), following by PBS injection containing no MSCs. (3) MSC group: After model establishment, 10⁶ MSCs diluted in 500ul PBS were injected through penile dorsal vein in each mouse. (4) Gal-9-MSC group: MSCs were co-cultured with IFN-γ solution (20ng/ml) (PeproTech, Rocky Hill, USA) for 72 hours in advance. When it is time to inject, MSCs were accurately counted at 10⁶ and dissolved in 500ul PBS for intravenous injection. (5) MSC+α-lactose group: 10⁶ MSCs were diluted in 500ul PBS containing 10.8mg/mL α-lactose (Sigma-Aldrich), and then used for i.v. injection. The mice were monitored each 6 hours after model establishment. While 24 hours later, all the mice were sacrificed. Spleen were ground for flow cytometry analysis, and serum, liver, lung and kidney were frozen in -80°C or immersed in formalin solution separately for further analysis.
Survival observation and clinical symptom evaluation

Monitoring of the health state of the mice was conducted by two investigators every 6 hours. Both investigators were blinded with the treatment information, and were requested for assessing the condition of the mice in accordance of the criteria reported previously [31]. Briefly speaking, Murine Sepsis Score (MSS) evaluation system mainly includes the following aspects: appearance, spontaneous activity, eyes condition, level of consciousness, response to stimuli, respiration rate and respiration quality. Each of these variable was given a score between 0 and 4, while the total score summed up was 0 to 28.

T-SOD activity and MDA content measurement

Serum T-SOD activity and MDA content were assayed by the hydroxylamine and modified 2-thiobarbituric acid (TBA) spectrophotometry method. The procedures performed were all in according with the manufacturer’s instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Each assay included three parallel samples.

Enzyme-linked immunosorbent assay (ELISA)

The culture supernatant of p2-p5 MSCs and MSC with or without INF-γ stimulation were collected to test for Gal-9 expression. Furthermore, the liver, lung and kidney tissue homogenate were also obtained to detect the expression level of Gal-9 (Bao Lai biological technology, Jiangsu, China). In addition, the serum and liver homogenate
were gathered respectively, to detect the expression level of TNF-α and IL-1β (DAKEWE, Shenzhen, China). According to the manufacturer’s instructions, the detection antibody, HRP conjugate, chromogenic substrate and stop solution were added in orders. Finally, the absorbance of each well was detected at 450 nm, and the concentration of each sample was obtained by comparing with the standard curve. All tests were performed in duplicate to eliminate the error.

**Flow cytometry analysis**

Cell suspensions of MSCs with or without IFN-γ stimulation were prepared. Among which, half of the MSC suspensions were incubated with fixation & permeabilization buffer (Thermo Fisher Scientific, USA) in advance. Then all the specimens were incubated with Gal-9 primary antibody for 30 minutes, followed by staining Alexa Fluor® 488-conjugated secondary antibody for 30 minutes in the dark place. After finishing the staining, these samples were analyzed on the FACS cytometer.

In addition, the spleens from each group were obtained separately, and then ground with a 100-mesh filter to make a single-cell suspension. After disposing with blood cells lysing buffer, these suspensions were washed and dispensed in tubes for further use. Fluorescent monoclonal antibodies (ebioscience Inc., San Diego, CA, USA) were used for staining to analyze the proportion of M2-type macrophage (CD68⁺CD206⁺), Total Macrophage (CD68⁺) and Treg (CD4⁺CD25⁺Foxp3⁺). All the data acquired were
analyzed by flowjo V10 software, in which forward angle and side light scatter were used to exclude dead cells.

**Pathological analysis**

Liver, lung and kidney tissues fixed in 10% formalin were obtained respectively. After undergoing paraffin embedding, sectioning, dehydration and HE staining, all the specimens were sealed with neutral gum and observed under microscope. All sections were scored and evaluated by two pathologists after double-blind reviewing. The scoring criteria for evaluating liver damage are as follows: necrosis, sinus congestion and edema, lipid vacuoles and infiltration of hyperemia and inflammatory cells. Each of which has a score of 0-4, and the total score is 0-16 [32]. While the criteria for assessing kidney injury are as follows: tubular dilatation/flattening, tubular casts and tubular degeneration/vacuolization (cortex and medulla, 0-3, total: 0-18) [33]. In addition, criteria for evaluating lung damage are as follows: edema, alveolar and interstitial inflammation, alveolar and interstitial hemorrhage, and necrosis (0-4, total: 0-16) [34].

**Real-time polymerase chain reaction (RT-PCR):**

The above-obtained MSCs, in addition with liver, lung and kidney tissue homogenate, were collected and extracted total RNA by the existing commercial RNA extraction kits (DP430, DP431, Tiangen Biotech Co. Ltd.). To determine purity and concentration, the extracted RNA was evaluated with an UV spectrophotometer at the
spectrum of 260 and 280nm. Then, cDNA was acquired from the obtained RNA respectively by reverse transcribed method with a FastKing one-step kit (KR106, Tiangen Biotech Co. Ltd). RT-PCR reaction was carried out according to the manufacturer's recommended instruction by SuperReal Color Premix kit (FP216, Tiangen Biotech Co. Ltd.). The primer sequences of all reactions were designed as follows:

| Gene      | Primers (5'-3')                                                                 |
|-----------|--------------------------------------------------------------------------------|
| Gal-9     | Forward: ATGCCCTTTTGAGCTTTTGCTTC  
Reverse: AACTGGACTGGCTGAGAGAAC                                |
| TNF-α     | Forward: CCCTCACACTCAGATCATTTCT  
Reverse: GCTACGACGTGGGCTACAG                                |
| IL-1β     | Forward: TTCAGGCAAGCAGTATCCTC  
Reverse: GAAGGTCCAAGGGAAAGACAC                                |
| IFN-γ     | Forward: ATGAACGCTACACACTGCATC  
Reverse: CCATCCTTTTGCCAGTTTCCTC                                |
| IL-35 (EBI3) | Forward: CTTACAGGCTCGGTGTCGC  
Reverse: GTGACATTAGCATGTAGGGCA                                |
| SOD       | Forward: CAGACCTGCCCTTACAGCTATGG  
Reverse: CTCGTTGGCGTTGAGATTGT                                   |
| iNOS      | Forward: GTTCTCTACCCCAACATACAGAGA  
Reverse: GTGGACGGTGTCATGTCAC                                 |
| GAPDH     | Forward: AGGTCGGTGTGAACCGGATTTG  
Reverse: TGTAGACCATGTAGTTGAGGTC                                 |

Each experiment was repeated twice, and the gene expression differences between different groups were analyzed by the relative quantitative $2^{-\Delta\Delta CT}$ method.

Statistical analysis
Majority Data obtained were analyzed by SPSS 22.0 and presented in Mean ± SD. Data variance was assessed by using one-way analysis of variance (ANOVA) (groups ≥ 3) or unpaired t test (groups=2). For the analysis of survival rate, the Kaplan-Meier cumulative survival method and the differences among groups were analyzed by Log-rank (Mantel-Cox) test. Differences with p values less than 0.05 were considered statistically significant.

Results

The expression of Gal-9 on murine MSCs was enhanced by IFN-γ stimulation

Gal-9 expression in p2-p5 MSCs were measured by ELISA. By comparing with the standard sample, it was confirmed that Gal-9 indeed expressed in the supernatant (Figure 1A, n=3) and lysate of MSCs (data not shown). However, there was no statistical difference observed between different generations. While intriguingly, by introducing IFN-γ, it was found that the expression of Gal-9 was increased in a dose dependent manner with the stimulation of IFN-γ (Figure 1B, n=3, * p < 0.05, ** p < 0.01).

To further clarify the expression difference of Gal-9, single-cell suspensions of MSCs with or without IFN-γ stimulation (20ng/ml IFN-γ, 72h) were prepared. Through flow cytometry analysis, it was found that the fluorescence of Gal-9 on MSCs increased apparently after IFN-γ stimulation (Figure 1C, n=3). Moreover, the fluorescence intensity of MSCs was presented further improved by fixation & permeabilization process (Figure 1C, * indicate samples were processed with fixation & permeabilization
buffer before staining). Meanwhile, through RT-PCR analysis, it was verified that the mRNA expression of Gal-9 in IFN-γ pre-stimulated MSCs was also significantly improved (Figure 1D, n=3, * p < 0.05, ** p < 0.01). The quantitative-based detection method is still not intuitive and explicit enough. Therefore, we performed immunofluorescence staining for further detection. As shown, it was demonstrated that MSCs had a small amount of Gal-9 expression. But after IFN-γ stimulation, the expression of Gal-9 has a marked increase, both in the cytoplasm and cell surface (Figure 1E).

**Gal-9 mediates MSC-based therapy in ameliorating the symptoms of endotoxemia**

To explore the involvement of Gal-9 in mediating the therapeutic effect of MSCs on endotoxemia, two investigators were required to monitor the health condition of the mice and evaluate murine sepsis score (MSS) every six hours. As shown in Figure 2A, the survival rate in the untreated group was the lowest, and at the end of the observation point, it dropped down to 50%. While the survival rate in MSC+α-lactose group was about 67%, which was lower than that of the MSC group, and no death was found in the Gal-9-MSC treated group at the end of observation. It was worth noticing that α-lactose, the specific antagonist of Gal-9, was described previously with ability in blocking Gal-9 activity and abolishing Gal-9 suppressive effect independently [29, 35].

When it turns to murine sepsis score (MSS), it was evaluated mainly based on the following aspects: appearance, spontaneous activity, eyes condition, level of
conscious, response to stimuli, respiration rate and respiration quality. As described in Figure 2B, it showed that Gal-9-MSC group had the lowest score, which was statistically different with the MSC treated group at different time points (n=6, #p<0.5, ## p<0.01), while the MSC+α-lactose group had a higher average score than the MSC treated group (n=6, *p<0.5, ** p<0.01). Given together the above findings, it indicated that Gal-9 was required for MSCs to exert an alleviated effect on the symptoms of endotoxemia. While after introducing α-lactose, the therapeutic effect of MSCs tended to be antagonized.

**Gal-9 mediates MSC-based therapy in alleviating inflammatory mediators release in endotoxemia**

Based on the symptom divergence observed above, to further analyze oxidative stress and inflammatory factor secretion changes, we collected and measured the expression of inflammatory mediators in serum. As shown in Figure 2C, Malondialdehyde (MDA) is a metabolic product of lipid peroxidation, whose expression could represent the degree of cell damage. It was found that MDA decreased significantly in Gal-9-MSC group, and increased in MSC+α-lactose group (Figure 2C, Gal-9-MSC group vs. MSC group, p < 0.01; MSC group vs. MSC+α-lactose group, p < 0.01), while the expression decreased to some extent in MSC group, when compared with untreated group (p < 0.01). Total Superoxide Dismutase (T-SOD), with the ability to scavenge the oxygen free radicals released by damage cells, was recognized as the vital free radical scavenger. To further evaluate T-SOD levels, serum in each group was also
measured. As shown in Figure 2D, T-SOD increased significantly in the Gal-9-MSC group, but decreased in the MSC+α-lactose group (Figure 2D, Gal-9-MSC group vs. MSC group, p < 0.01; MSC group vs. MSC+α-lactose group, p < 0.01).

Meanwhile, we analyzed the secretion level of IL-1β and TNF-α in the serum, both of which are pro-inflammatory factors that play a vital role in promoting sepsis. The results demonstrated there was a lower level of pro-inflammatory factors in Gal-9-MSC group (Figure 2C and 2D, Gal-9-MSC group vs. MSC group, IL-1β, p < 0.01; TNF-α, p < 0.01). but after α-lactose antagonism, the mitigation effect in reducing pro-inflammatory factors was suppressed (Figure 2C and 2D, MSC group vs. MSC+α-lactose group, IL-1β, p < 0.01; TNF-α, p < 0.01). In summary, it suggested that Gal-9 was actively involved in the modulating effects of MSCs, in regulation of oxidative metabolites and inflammatory factors at the system level.

**Gal-9 mediates MSC-based therapy in regulation of M1- and M2-type macrophage polarization**

Macrophages are centrally involved in the pathogenesis of endotoxemia. However, whether Gal-9-MSC could affect the ratio of macrophage subtype still unclear. Thus, we detected the percentages of M2-type macrophage (CD68+CD206+) and M2/M (CD68+CD206+/CD68+) ratio changes in splenocytes. Representative dot plots were shown in Figure 3A, and percentages of M2-type macrophage were shown in Figure 3C. The proportion of M2-type macrophage in the MSC-treated group was significantly
higher than that in the untreated group ($p < 0.01$), and when the expression of Gal-9 was increased by IFN-γ stimulation, or antagonized by α-lactose respectively, the proportion of M2 cells was shown higher or lower than that in the MSC group (Figure 3C, Gal-9-MSC group vs. MSC group, $p < 0.01$; MSC group vs. MSC+α-lactose group, $p < 0.01$).

Furthermore, the percentage of CD68$^+$ cells (data not shown) and M2/M (CD68$^+$CD206$^+$/CD68$^+$) ratio changes in each group were also analyzed. As shown in Figure 3D, the polarization level of M2/M in the MSC group was higher than that in the untreated group ($p < 0.01$), and when Gal-9 expression was increased, the polarization level tend to improve ulteriorly (Gal-9-MSC group vs. MSC group, $p < 0.01$). Given the above results, it suggested that Gal-9-MSC participated in the regulation of macrophages and promoted macrophage polarization towards M2-subtype.

**Gal-9 mediates MSC-based therapy in promotion of Tregs expansion**

Regulatory T (Treg) cells have a role in inhibiting T cell proliferation and cytokine production, thereby mediating the maintenance of immunologic homeostasis. The proportion of Treg cells (CD4$^+$CD25$^+$Foxp3$^+$) derived from spleen was also detected in our experiment. As shown in Figure 3B, representative dot plots were displayed, while the percentage of Treg with statistical difference was shown in Figure 3E. The percentage of Treg in untreated group was a little bit higher than that in normal control group, which is in accordance with previous reports. Whereas, in the MSC-treated group,
the proportion of Treg cells was shown higher than that in the untreated group ($p < 0.01$). While after antagonizing Gal-9 by lactose, the modulate effect of MSC tend to be inhibited (MSC group vs. MSC+α-lactose group, $p < 0.05$). But after stimulating Gal-9 expression with IFN-γ, Treg ratio was increased (Gal-9-MSC group vs. MSC group, $p < 0.05$). In summary, Gal-9 might be involved in mediating MSC effect on regulating Treg proportion changes.

**Gal-9 mediates MSC-based therapy in attenuating hepatic damage**

Liver is one of the most vulnerable organs in systemic inflammatory response syndrome (SIRS). We analyzed the pathological manifestations of the liver, and representative picture (100×) of each group was displayed in Figure 4A. As shown, in the untreated group, the pathological manifestations of hepatic damage were the most severe, which were displayed with central venous sinus congestion, lipoid vacuoles, hyperemia cells infiltration and lobular inflammation. However, the pathological manifestation seemed with relief in MSC treated group, which was manifested as the disappearance of central venous sinus congestion and the reduction of lipid vacuoles, but there were still hyperemia cells and inflammatory cells infiltration between the hepatic lobules. This pathological manifestation was markedly relieved in the Gal-9-MSC treated group. But after blocking with lactose, sinus congestion and hepatocyte vacuolation injury displayed again.

Liver damage was further verified by analyzing cytokine mediator changes in the
tissue homogenate. TNF-α and IL-1β were considered as essential factors for the function of M1-type macrophage, and were detected in serum previously. In order to further explore these cytokine changes in the specific functional organ, we measured relative mRNA expression levels of the following factors. As shown in Figure 4, TNF-α and IL-1β mRNA expression increased significantly in the untreated group, but decreased after MSC treatment (Figures 4C and 4D, Untreated group vs. MSC group, \( p < 0.01 \)). While after giving Gal-9-MSC treatment, TNF-α and IL-1β decreased further when compared with MSC group (TNF-α, \( p < 0.05 \); IL-1B, \( p < 0.01 \)), but increased again after antagonising with lactose (MSC group vs. MSC+α-lactose group, TNF-α, \( p < 0.05 \); IL-1B, \( p < 0.01 \)). Furthermore, liver homogenate was extracted and tested by ELISA. Through analysis, it was found that the protein expression changes of TNF-α and IL-1β were consistent with the mRNA alternation (Figure 4F and 4G).

To explore the level of oxidative stress, we also analyzed the changes of iNOS and SOD in liver. We found that the change of SOD was identical with the change in serum, which was significantly higher in Gal-9-MSC group (Gal-9-MSC group vs. MSC group, \( p < 0.05 \)). However iNOS, which indicates NO synthesis level to promote injury, decreased in the MSC treated group (vs. untreated group, \( p < 0.01 \)), and further reduced in the Gal-9-MSC group (vs. MSC group, \( p < 0.05 \)). Combined with the above results, it suggested Gal-9-mediated MSC therapy could reduce the levels of inflammatory and oxidative stress factors in liver tissues, in consistent with the change trend in serum, and could alleviate the pathological damage in liver.
Gal-9 mediates MSC-based therapy in alleviating pulmonary injury

In order to further evaluate the effect of Gal-9 mediated MSC treatment in different organs, we obtained pulmonary tissues, and then analyzed the pathological changes and inflammatory mediator expressions. As displayed in Figure 5A, in the untreated group, it appeared with obvious alveolar and interstitial inflammation, alveolar and interstitial hemorrhage, and necrosis. However, after treatment with MSCs, it was obvious that the inflammatory infiltration and tissue necrosis have relieved. This injury was further exacerbated in the MSC+lactose group, and was significantly alleviated in Gal-9-MSC group. The pathological score based on pulmonary pathological manifestation can more intuitively explain the differences of structure damages in different groups (Figure 5B).

In addition, total RNA of the lung tissue was extracted and used for evaluating changes in inflammation mediators. As expected, TNF-α, IL-1β, IFN-γ and iNOS were decreased in the MSC treated group, when compared with untreated group (p < 0.01). While these factors were further decreased in the Gal-9-MSC treatment group (vs. MSC group, TNF-α, p < 0.05; IL-1β, IFN-γ and iNOS, p < 0.01), but increased in the MSC+lactose group (vs. MSC group, p < 0.01). Meanwhile, IL-35 and SOD were also measured. It turned out that IL-35 and SOD mRNA expression level were little higher in the Gal-9-MSC group (vs. MSC group p < 0.01), and lower in the MSC+lactose group (vs. MSC group, IL-35, p < 0.05; SOD, p < 0.01). In total, the above results indicated that Gal-9 mediates the effect of MSCs in relieving lung pathological damage in sepsis, and
facilitates the effect of MSCs in reducing related damaging factors expression.

**Gal-9 plays a role in MSC-mediated therapy in lightening renal damage**

Furthermore, we obtained the kidneys and evaluated the damage among different groups. As shown in Figure 6A, the kidneys in the untreated group showed obvious tubular casts, inflammatory cell infiltration, tubular degeneration and vacuolization in cortex and medulla. While the pathological injury tended to be remission in the MSC treated group and normalize in Gal-9-MSC group, with a small amount of tubular dilatation and flattening appearing. The above pathological pictures were independently reviewed by two pathologists, and the obtained pathological scores were shown in Figure 6B.

Total RNA of kidney tissue was extracted and measured to assess the changes of the following inflammatory factors. As shown, TNF-α, IL-1β and IFN-γ mRNA expression were significantly lower ($p < 0.01$), while IL-35 was significantly higher ($p < 0.01$) in the MSC group than those in the untreated group. Moreover, in Gal-9-MSC group, TNF-α, IL-1β and IFN-γ mRNA expression were further reduced ($p < 0.01$), while IL-35 expression was increased ($p < 0.01$), as compared with those of MSC group. The above immunomodulatory effect was weakened when Gal-9 effect was antagonized by lactose (vs. MSC group, $p < 0.01$). Meanwhile, iNOS and SOD were also measured and evaluated in renal tissues. In the MSC treated group, iNOS decreased (Figure 6H, $p < 0.01$) and SOD increased (Figure 6G, $p < 0.01$), compared with the untreated group.
While in the Gal-9-MSC group, iNOS was further reduced (vs. MSC group, $p < 0.01$) and SOD (vs. MSC group, $p < 0.01$) was increased, but after treated by MSC+lactose, the above effects were antagonized (vs. MSC group, $p < 0.01$). Given together the above results, It suggested that Gal-9 played an important role in mediating the therapeutic effect of MSCs in alleviating renal damage and reducing inflammatory factors expression.

**Gal-9 is highly expressed in liver, lung and kidney after Gal-9-MSC treatment**

We hypothesized that MSC-based therapy is mainly mediated by Gal-9. Thus, to verify Gal-9 expression differences after administrating different treatments, we designed and measured Gal-9 expression in liver, kidney and lung. As shown in Figure 7A-7E, Gal-9 expression tend to increase after administrating the MSCs, verified by homogenate protein detection (Untreated group vs. MSC group, liver, $p < 0.05$; kidney, $p < 0.01$; lung, $p < 0.01$) and mRNA expression evaluation (Untreated group vs. MSC group, liver, $p < 0.01$; kidney, $p < 0.01$; lung, $p < 0.05$). Moreover, the expression level of Gal-9 was further improved after implementing Gal-9-MSC treatment (MSC group vs. Gal-9-MSC group, Homogenate: liver, $p < 0.01$; kidney, $p < 0.01$; lung, $p < 0.05$; mRNA expression: liver, $p < 0.01$; kidney, $p < 0.01$; lung, $p < 0.05$). Based on the above results, it suggested there was a higher expression of Gal-9 after administrating the Gal-9-MSC in vulnerable and injured organs.
Discussion

Endotoxemia, induced by lipopolysaccharide (LPS) release into the bloodstream, which was derived from bacteria outer membrane, could cause irregular and aberrant inflammatory response, and then resulting in the multiple organ dysfunction syndrome (MODS) [36, 37]. Current treatment strategies for treating endotoxemia are limited and not completely satisfactory. MSC has been used in the treatment of endotoxemia and exhibited promising advantages, which possess an ability to modulate the inflammatory response and assist in alleviating organogenic injury in endotxemia mice [38]. Besides, multiple investigations have reported lower mortality and proinflammatory cytokines secretion were found after administration with MSCs [39, 40].

However, mechanisms govern MSC capability in modulating immune response is still incomprehensive. Growing evidence have suggested MSCs could modulate and suppress the immune response through cell-to-cell contact or cytokines secretion, such as IL-10, TGF-β, VEGF, PGE2 and indoleamine-2,3-dioxygenase (IDO) [18, 19]. There are also reports that IFN-γ could improve the immunoregulation efficacy of MSCs, but the mechanism is rarely explored [20, 21]. Based on in vitro experiments and preliminary data, we designed this experiment to detect whether IFN-γ stimulation could promote in Gal-9 expression in MSCs and whether Gal-9 high expression could mediate alleviating damage on experimental endotoxemia.

Gal-9, a member of the β-galactoside-binding galectin family, with two carbohydrate recognition domains (CRDs) joined by a linker peptide, was
recognised as the Tim-3 ligand [22]. While T cell immunoglobulin-3 (TIM-3), expressed on Th1, Th17, CD8 T, macrophage and natural killer (NK) cells, has been identified as a regulatory molecule and plays a crucial role in immune tolerance [23]. Recent studies have shown that Gal-9 signaling could negatively regulates Th1 and Th17 immunity, dependent on Gal-9/Tim-3 interaction, resulting in the suppression of Th1- and Th17-related cytokine production [24, 27]. Moreover, there were other reports demonstrated that Gal-9 could promote macrophage polarization to M2 subtype [25, 26], and enhance the development of Foxp3+ regulatory T cells (Tregs) in a Tim-3-independent manner [27]. In addition, Gal-9 was also reported to suppress T and B cell response and involved in immunosuppression effect mediated by MSCs in vitro [28]. However, the in vivo studies concerning Gal-9-mediated immunoregulation of MSCs are still scanty. Although recombinant human Gal-9 protein have been employed to examine their role on alleviating endotoxemia in mice [41], but we hold that systemic administration may not be as effective as MSCs, which could migrate to damaged organs by chemotaxis and generate Gal-9 continuously. Therefore, exploring the therapeutic effect of Gal-9-mediated MSC therapy in endotoxemia was warranted.

In order to detect the expression of Gal-9 in MSCs, we designed to compare the expression of Gal-9 in the supernatant and lysate of different generations of MSCs. However, we found no statistical difference there. Then, we pre-stimulated MSCs with IFN-γ for 72h, we found that the expression of Gal-9 on MSCs could increase in a dose-dependent manner by IFN-γ stimulation. Furthermore, we selected the third
generation of MSCs, and stimulated them with 20ng/ml IFN-γ for 72 hours. The protein and mRNA expression levels of Gal-9 were detected respectively, and supplemented with immunofluorescence staining, it was found that Gal-9 was indeed highly expressed in IFN-γ stimulated MSCs. Above all, it can provide a preliminary experimental foundation for later in vivo experiments to verify the enhancement of Gal-9 expression in MSCs and to explore their role in endotoxemia.

Endotoxemia in mice was induced by LPS intraperitoneal (i.p.) injection (10mg/kg) according to the methods reported previously [42]. The use of MSCs to improve the survival rate of endotoxemia has been reported previously [43]. Whereas, the immunomodulatory effect of Gal-9-mediated MSC therapy in endotoxemia model still need to be verified. To evaluate the survival rate, Gal-9-MSC, MSC and MSC+α-lactose were given respectively. Strongly evidence have introduced that α-lactose could abrogate Gal-9 effect and present as a competitive inhibitor of galectin carbohydrate-binding activity [29, 35]. By comparing with the survival rate, it is clear that Gal-9-mediated MSC treatment does have certain positive significance in improving endotoxemia mice survival condition. Furthermore, two observers recorded and evaluated the endotoxemia mice performance every six hours according to the existing evaluation criteria [31]. All the process were double-blinded, and the analysis suggested that MSS score was the lowest in the Gal-9-mediated MSC treatment group, indicating with the best therapeutic effect.

High levels of circulating proinflammotory factors were demonstrated in
endotoxemia. Among which, TNF-α and IL-1β, majorly secreted by macrophages, were recognized as essential components in endotoxemia shock [44]. When administration of anti-TNF-α Abs or knock-out of TNFR, it could greatly diminished or abrogated mortality in endotoxic models [45-47]. In our experiment, circulating TNF-α, and IL-1β were also detected to evaluate the inflammatory cytokines level. Through comparison, it could be found that Gal-9-MSC treatment group has a overall lower inflammation level than that in Gal-9 antagonistic group.

In addition, oxidative stress also participated in the pathogenesis of endotoxemia and contribute to multiple organ failure in septic patients [48, 49]. Antioxidant management focusing on mimic the activity of human superoxide dismutase enzymes, have been shown with univocal and promising role in preventing cellular energetic failure associated with shock [50]. Thus, we supposed whether improving Gal-9 expression in MSCs could also have a modulating role in relieving the oxidative stress damage. Based on this, we measured the levels of SOD and MDA in serum. As expected, after increasing the expression of Gal-9 on MSCs, the expression of T-SOD increased significantly, and the level of circulating MDA decreased.

Circulating monocyte and macrophage play an important role in eliminating bacteria [51]. It is now generally believed that macrophages can be at least categorized into two phenotypic subsets: M1 pro-inflammatory sub-type and M2 selectively activated anti-inflammatory sub-type [52]. Macrophages are highly versatile phagocytic cells whose diverse effector functions can selectively be reprogrammed by an array of
environmental signals [53]. IFN-γ, TNF-α, or substimulatory LPS could promote macrophages differentiate into M1 sub-type. Whereas, exposure to IL-4 or IL-10 could promote generate M2 sub-type macrophages [54, 55]. There are also reports suggesting that treating septic mice with MSCs could promote macrophage polarization to IL-10 production type, rather that proinflammatory subtype [56].

Moreover, further investigations revealed that increasing Gal-9 expression in RAW264.7 cell could also promote macrophage polarization to M2 phenotype instead of M1 phenotype [26]. There were also investments indicating that Gal-9 could alleviate LPS-induced preeclampsia-like impairment via switching decidual macrophage polarization to M2 subtype [25]. However, whether the high expression of Gal-9 in MSCs could also affect macrophage polarization in endotoxemia is still unclear. By analyzing the changes of macrophage proportion and subtype in each group, it can be concluded that Gal-9-mediated MSC therapy could promote macrophage polarization into M2 subtype. Meanwhile, trough detecting the changes of TNF-α and IL-1β in serum and specific organ homogenate, it also indirectly reflect that the pro-inflammatory function of M1 type macrophages was inhibited. On the contrary, the anti-inflammatory effect of M2 type tended to be manifested.

Multiple literatures have suggested increased percentages of Tregs have been observed in the blood of septic patients, and their presence does not contribute significantly to overall survival [57, 58]. In other investigations, Treatment with GITR agonistic antibody to block Treg function was demonstrated to accompany with
improved immune function and microbial killing [59]. While controversially, other reports demonstrated that adoptive transfer of CD4⁺CD25⁺ regulatory T cells which were stimulated in advance [59], could promote bacterial clearance and improve survival rate in polymicrobial sepsis mice [60]. Further experiments concerning deleting functional Tregs (CD25 KO mice) or implementing anti-CD25 monoclonal antibody respectively, lead to acute death in an original nonlethal LPS administration[61].

While from our perspective, we hold that Treg is beneficial and well needed before immune storm formation. When treatment with MSCs in the initial of endotoxemia, Treg is a prerequisite, similar with M2 subtype macrophages, to inhibit and modulate inflammation development. Whereas, in the late stage of endotoxemia, immune response tend to be exhausted and percentage of Treg and M2 seems with increase, thus transforming into suppressors and harmful to immune response. However, it was thought this number increase may due to the effector T helper cell loss from apoptosis rather than an absolute increase in Treg numbers [62]. In summary, Tregs was considered with positive therapeutic effect, at least in the initial stage of endotoxemia. Therefore, we recommend Gal-9-MSC should be injected at early times once endotoxemia tend to occur. In our experiment, we administrated Gal-9-MSC one hour later after modeling, and discovered that Treg ratio in MSC-treated group was higher than that in untreated group, whose effect was strengthened by elevating Gal-9 expression and inhibited by Gal-9 antagonist.
Besides evaluating circulating inflammatory factors and immune cell changes, we also designed to detect the pathological changes of liver, kidney and lung, and inflammatory factor mRNA expression changes in tissue homogenate. According to the standards criteria reported by the literatures [32-34], two pathologists double-blind evaluated the pathological samples of each group. The statistical results revealed that there were severe damages in the untreated group. While in the MSC-treated group, the total injury score tend to be a little lower. Furthermore, when it comes to the Gal-9-MSC group, the injury was further relieved, but the effect was significant abrogated by the α-lactose antagonist.

In addition, we also evaluated the changes in the levels of pro-inflammatory factors and oxidative stress mediators in liver, lung and kidney tissue homogenates. The trends of these results were consistent with the pathological findings, which further explained that Gal-9 is involved in the regulation effect of MSCs in alleviating organ damage. However, there are still lack of creatinine evaluation or blood gas analysis, to assess the function of kidney, lung or liver. We only evaluated the pathological damage, but it was expected that this experiment can provide a basis for the following specific and detailed functional assessment for the target organs.

Based on the hypothesis that Gal-9 mediates MSCs to alleviate organ damage, in order to further investigate whether there are differences in the expression of Gal-9 in different organs after treatment, we designed and evaluated Gal-9 protein and mRNA expression in liver, lung and kidney tissue homogenate. As expected, after administering
MSC, high expression of Gal-9 was discovered in the target organs, and this expression was further increased after giving Gal-9-MSC. Given above, we have reason to believe that MSC treatment assist in enhancing Gal-9 expression in the target organs, and the high expression of Gal-9 is supposed to participate in the repair of target organ damages.

Although this experiment results were inspiring and promising, the modeling method might not ideal enough, in which injecting LPS could only simulate the pathophysiological changes of the inflammatory response imbalance in clinical sepsis [5]. For better mimic the occurrence and development of clinical sepsis, the participation of bacteria is required, and cecum ligation and puncture (CLP) model seems with more advantages [63]. However, active exploration of immune imbalances in endotoxemia might provide sufficient theoretical support for further experimental verification. Meanwhile, whether Gal-9-mediated MSC therapy can be used in combination with antibiotics and play a role in eliminating bacteria still needs further exploration.

Furthermore, given the reports that immune exhaustion occurs in the late stage of endotoxemia [58], we recommend that MSCs should be given at early time, to limit and inhibit the initial inflammation response. At the same time, the experimental hypothesis, in which comparing the effects of MSC administration at the different time points of sepsis, has also been proposed.

Taken together, this experiment preliminarily verified the significance of Gal-9 mediated MSC therapy in relief of endotoxemia, which mainly manifested as: attenuating circulating pro-inflammatory mediators secretion, promoting macrophage
polarization to M2-subtype, inducing the increase of Treg ratio and facilitating the alleviation of multiple organ injury. Although the application of recombinant Gal-9 alone shows certain effect in multiple immune dysregulation diseases [25, 64], but due to the inflammatory site targeting and chemotaxis ability of MSCs, in addition with continuously release of new regulatory factors synthesized by itself, it is well believed that cell therapy based on MSCs has more potential and advantage [65, 66].

Even though MSCs regulate the immune response through cell-to-cell contact or regulatory factors secretion (IL-10, TGF-β, VEGF, PGE2 and IDO) has been continuously verified. While, focusing on Gal-9-mediated therapy in MSCs is still staying at the in vitro experimental stage. This experiment provides a novel idea for supplementing the research of MSC immunoregulatory mechanism. At the same time, it also lays a fundamental for the vivo experiments, which can provide the initial experimental basis for the later in vivo experiment verification.

**Conclusion**

In this study, we preliminary verified that MSCs could express Gal-9 and this expression increased in a dose-dependent manner after being stimulated by INF-γ. Giving the rarely study of Gal-9-mediated MSC immunomodulation in vivo, we for the first time to evaluate its role in experimental endotoxemia. The results demonstrated that Gal-9-mediated MSC therapy could assist in attenuating circulating pro-inflammatory mediators secretion, promoting macrophage polarization to M2-subtype, inducing the
increase of Treg ratio and facilitating the alleviation of multiple organ injury. Furthermore, we also discovered there was a higher expression of Gal-9 in liver, kidney and lung homogenate after receiving Gal-9-MSC. This experiment provides a novel idea for supplementing the research of MSC immunoregulatory mechanism, and offers an excellent candidate to be used in treatment of endotoxemia in the clinical settings.

**List of abbreviations**

Mesenchymal Stem Cells (MSCs), Gelactin-9 (Gal-9), Interferon gamma (IFN-γ), Lipopolysaccharide (LPS), Histocompatibility Complex I (MHC-I), Nitric Oxide (NO), Interleukin 10 (IL-10), Transforming Growth Factor Beta (TGF-β), Vascular Endothelial Growth Factor (VEGF), Prostaglandin E2 (PGE2), Indoleamine-2,3-dioxygenase (IDO), Carbohydrate Recognition Domains (CRDs), T-cell immunoglobulin mucin-3 (Tim-3), regulatory T cells (Treg), natural killer (NK), Dulbecco's Modified Eagle Medium (DMEM), Fetal bovine serum (FBS), Bovine serum albumin (BSA), Murine Sepsis Score (MSS), 2-thiobarbituric acid (TBA), 4',6-diamidino-2-phenylindole (DAPI), Tumor Necrosis Factor-α (TNF-α), Interleukin-1β (IL-1β), Horseradish Peroxidase (HRP), Fluorescence Activating Cell Sorter (FACS), Inducible Nitric Oxide Synthase (iNOS), interleukin-35(IL-35), Epstein-Barr Virus Induced 3 (EBI3), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Superoxide Dismutase (SOD), Multiple organ dysfunction syndrome (MODS), Malondialdehyde (MDA), Glucocorticoid-induced
tumor necrosis factor receptor (GITR), Cecum ligation and puncture (CLP).

**Ethics approval and consent to participate**

Experiments involving animals were all complied with the standard protocols approved by the Animal Care and Use Committee of Tianjin Medical University (Tianjin, China) (IRB2020-DW-02), according to the Chinese Council on Animal Care guidelines.

**Consent for publication**

Not applicable

**Availability of data and materials**

All data generated or analysed during this study are included in this published article

**Competing interests**

The authors declare that they have no competing interests

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Authors' contributions

Y. Zhao, D. Yu, and H. Wang are co-first authors on this paper. Y. Zhao conceived, designed and carried out the research, performed data analysis and interpretation, and drafted the manuscript. D. Yu designed and carried out the research, performed data analysis, and helped in revising the manuscript. H. Wang carried out the research, performed data analysis and paper revision. W. Jin, X. Li, Y. Hu, Y. Qin, D. Kong and G. Li contributed to experimental procedures. H. Wang and A. Ellen helped to review the data and the manuscript. Hao Wang conceived and designed the study, provided financial and administrative support, helped in revising the manuscript and gave final approval of the manuscript. All authors read and approved the final manuscript.

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Figure legend

Figure 1. IFN-γ stimulation could enhance Gal-9 expression in MSCs. (A) Different concentrations of Gal-9 in p2-p5 MSC supernatant. n=3. (B) Expression of Gal-9 increased in a dose-dependent manner in the lysate of P3 MSCs by IFN-γ stimulation. n=3. * p < 0.05, ** p < 0.01. (C) Fluorescence intensity of Gal-9 in different samples (* indicates: permeabilization & fixation optimized before fluorescent antibody staining). (D) Relative mRNA expression of Gal-9 in IFN-γ pre-stimulated MSCs (20ng/ml IFN-γ, 72h) was higher than that in non-stimulated MSCs. n=3. * p < 0.05, ** p < 0.01. (E) Representative immunofluorescence figures of MSCs with IFN-γ pre-stimulation (20ng/ml, 72h) or not. The nucleus were presented as blue and Gal-9 protein appeared as green, merged figures represent the expression of Gal-9 on each cells. Bar graphs represent mean ± SD. Abbreviation: Gal-9, Galectin-9; p2-p5, passage 2-passage5; MSCs, mesenchymal stem cells.

Figure 2. Gal-9 mediates MSC-based therapy in alleviating symptoms and inflammatory mediators expression. (A) Changes in the survival rate of endotoxemia mice, n=6. (B) Variation levels of mean MSS (murine sepsis score) in different groups (Gal-9-MSC group vs. MSC group, n=6, * p < 0.05, ** p < 0.01 ; MSC group vs. MSC+α-lactose group, n=6, # p < 0.05, ## p < 0.01). (C-D) Expressions of MDA and T-SOD in serum, n=6. (E-F) Secretion levels of TNF-α and IL-β in serum, n=6. Data variance was assessed by using one-way analysis of variance (ANOVA), followed by
least significant difference (LSD) test. * p < 0.05, ** p < 0.01. Abbreviations: MDA, Malondialdehyde; T-SOD, Total Superoxide Dismutase.

Figure 3. Gal-9 mediates MSC-based therapy in Regulation of macrophage polarization and Tregs percentage. Spleen cells were collected 24 hours after LPS injection, followed by staining with anti-mouse CD68 antibody for total macrophage, CD68<sup>+</sup>CD206<sup>+</sup> for M2-type macrophage and CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> for Tregs. (A) Representative Dot plots of M2 (CD68<sup>+</sup>CD206<sup>+</sup>) macrophage. (B) Representative Dot plots of Treg (CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>). (C) Percentage of M2 (CD68<sup>+</sup>CD206<sup>+</sup>) macrophage. (D) M<sub>2</sub>/M<sub>Total</sub> Ratio (CD68<sup>+</sup>CD206<sup>+</sup>/CD68<sup>+</sup>). (E) Percentage of Tregs in spleen. Data variance was assessed by using one-way analysis of variance (ANOVA), followed by least significant difference (LSD) test, n=6. * p < 0.05, ** p < 0.01. Abbreviations: Treg: Regulatory T cells; M total: total macrophage.

Figure 4. Gal-9 mediates MSCs-based therapy in Attenuating Hepatic damage. (A) Photomicrographs of representative histological sections (H&E staining, original 100× magnification) of liver in each group. (B) Liver injury score. Double-blind reviewing was evaluated by the following criteria: necrosis, sinus congestion and edema, lipid vacuoles and infiltration of hyperemia and inflammatory cells (0-4, total: 0-16). (C) Liver TNF-α mRNA expression level. (D) Liver IL-1β mRNA expression level. (E) Liver
SOD mRNA expression level. (F) TNF-α protein concentration in liver homogenate. (G) IL-1β protein concentration in liver homogenate. (H) Liver iNOS mRNA expression level. Data variance was assessed by using one-way analysis of variance (ANOVA), followed by least significant difference (LSD) test, n=6. * p < 0.05, ** p < 0.01. Abbreviations: iNOS, inducible Nitric Oxide Synthase; SOD, Superoxide dismutase.

Figure 5. Gal-9 mediates MSCs-based therapy in Alleviating Pulmonary Injury. (A) H&E staining of representative histological sections of mouse lung in each group (100×magnification), and selected small square area was magnificently displayed on the right side (200×magnification). (B) Pulmonary injury score. Criteria for evaluating lung injury were as follows: edema, alveolar and interstitial inflammation, alveolar and interstitial hemorrhage, and necrosis (0-4, total: 0-16). (C-H) pulmonary mRNA expression levels of TNF-α (C); IL-1β (D); IFN-γ (E); EBI3 (IL-35) (F); SOD (G); iNOS (H). Data variance was assessed by using one-way analysis of variance (ANOVA), followed by least significant difference (LSD) test, n=6. * p < 0.05, ** p < 0.01. Abbreviations: EBI3, Epstein-Barr virus-induced gene3; iNOS, inducible Nitric Oxide Synthase; SOD, Superoxide dismutase.

Figure 6. Gal-9 Plays a Role in MSC-Mediated Therapy in Lightening Renal Damage. (A) H&E staining of representative histological sections of mouse kidney in each group (100×magnification). To specific observe the difference, selected small
square area was magnificently displayed on the right side (200×magnification). (B) Renal injury score. Criteria for assessing kidney injury are as follows: tubular dilatation/flattening, tubular casts and tubular degeneration/vacuolization (cortex and medulla, 0-3, total: 0-18). (C-H) Renal mRNA expression level of TNF-α (C); IL-1β (D); IFN-γ (E); EBI3 (IL-35) (F); SOD (G); iNOS (H). Data variance was assessed by using one-way analysis of variance (ANOVA), followed by least significant difference (LSD) test, n=6. * p < 0.05, ** p < 0.01. Abbreviations: EBI3, Epstein-Barr virus-induced gene3; iNOS, inducible Nitric Oxide Synthase; SOD, Superoxide dismutase.

**Figure 7. Gal-9 is highly expressed in liver, lung and kidney after Gal-9-MSC treatment.** (A-C) Gal-9 protein expression in the homogenate of Liver (A), Lung (B) and Kidney (C), n=6. (D-F) Relative Gal-9 mRNA expression levels in Liver (D), Lung (E) and Kidney (F), n=6. Data variance was assessed by using one-way analysis of variance (ANOVA), followed by least significant difference (LSD) test. * p < 0.05, ** p < 0.01.