Molecular Therapy
Original Article

Antibacterial Fusion Protein BPI21/LL-37 Modification Enhances the Therapeutic Efficacy of hUC-MSCs in Sepsis

Zhan Li,[1,2,6] Yuqing Song,[1,5,6] Peisong Yuan,[1,2,6] Wei Guo,[1,2] Xueting Hu,[1,2] Wei Xing,[1,2] Luoquan Ao,[1,2] Yan Tan,[1,2] Xiaofeng Wu,[1,2] Xiang Ao,[1,2] Xiao He,[1,2] Dongpo Jiang,[4] Huaping Liang,[3] and Xiang Xu[1,2]

[1]Department of Stem Cell and Regenerative Medicine, State Key Laboratory of Trauma, Burn and Combined Injury, Daping Hospital, Army Medical University, Chongqing 400042, PR China; [2]Central Laboratory, State Key Laboratory of Trauma, Burn and Combined Injury, Daping Hospital, Army Medical University, Chongqing 400042, PR China; [3]Department of Wound Infection and Drug, State Key Laboratory of Trauma, Burn and Combined Injury, Daping Hospital, Army Medical University, Chongqing 400042, PR China; [4]Department of Critical Care Medicine, Daping Hospital, Army Medical University, Chongqing 400042, PR China; [5]Department of Critical Care Medicine, Jinling Hospital, Nanjing 210000, PR China

Sepsis, which is characterized by multiple organ dysfunctions as a result of an unbalanced host-inflammatory response to pathogens, is potentially a life-threatening condition and a major cause of death in the intensive care units (ICUs). However, effective treatment or intervention to prevent sepsis-associated lethality is still lacking. Human umbilical cord mesenchymal stem cell (hUC-MSC) transplantation has been shown to have potent immunomodulatory properties and improve tissue repair yet lacks direct antibacterial and endotoxin clearance activities. In this study, we engineered hUC-MSCs to express a broad-spectrum antibacterial fusion peptide containing BPI21 and LL-37 (named BPI21/LL-37) and confirmed that the BPI21/LL-37 modification did not affect the stemness and immunoregulatory capacities of hUC-MSCs but remarkably, enhanced its antibacterial and toxin-neutralizing activities *in vitro*. Furthermore, we showed that administration of BPI21/LL-37-engineered hUC-MSCs significantly reduces serum levels of tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β) , and IL-6, whereas increases that of IL-10 in cecal ligation and puncture (CLP)-induced sepsis mouse model. Administration of BPI21/LL-37-engineered hUC-MSCs significantly reduced systemic endotoxin (lipopolysaccharide [LPS]) levels and organ bacterial load, ameliorated damage to multiple organs, and improved survival. Taken together, our study demonstrates that BPI21/LL-37-engineered hUC-MSCs might offer a novel therapeutic strategy to prevent or treat sepsis via enhanced antimicrobial and anti-inflammatory properties to preserve organ functions better.

INTRODUCTION

Sepsis is a syndrome that is defined by widespread inflammation, host immune dysfunction, the dysregulation of the coagulation cascade, and endothelial dysfunction in response to invading pathogens.[1] It is common and is the leading cause of morbidity and mortality among critically ill patients and postoperative patients in intensive care units. Even with the appropriate antibiotic and resuscitative therapies, the worldwide incidence of sepsis is estimated to be 18 million cases per year, and the death rate of severe sepsis ranges from 30% to 50%, despite advances in critical care.[2–6] Furthermore, there is an increasing awareness that patients who survive sepsis often have long-term physical, psychological, and cognitive disabilities with significant health care and social implications. Thus, an effective treatment regimen is an unmet need.

The pathophysiological mechanism of sepsis is believed to be that invading pathogen components, such as lipopolysaccharide (LPS), induce the widespread activation of the inflammatory response, host immune dysfunction, dysregulation of the coagulation cascade, and endothelial dysfunction; these dysfunctions then progress to multiple organ dysfunction, the collapse of the circulatory system (septic shock), and death.[7] Thus, the implementation of preventive measures against infections is the first key step to reduce the occurrence of sepsis. However, with the increase in the incidence of both drug-resistant bacteria and immunocompromised septic patients, antibiotic therapies are ineffective and may even be detrimental. Furthermore, once a patient progresses into septic shock, pathogens are killed by antibiotic therapy; this causes the pathogens to release more toxic components, which then deteriorate the patient's clinical condition.[8]

Received 20 August 2019; accepted 12 May 2020;
https://doi.org/10.1016/j.ymthe.2020.05.014.

[6]These authors contributed equally to this work.

Correspondence: Xiang Xu, Department of Stem Cell and Regenerative Medicine, State Key Laboratory of Trauma, Burns and Combined Injury, Daping Hospital, Army Medical University, Chongqing 400042, PR China.
E-mail: xiangxu@tmmu.edu.cn
Correspondence: Huaping Liang, Department of Wound Infection and Drug, State Key Laboratory of Trauma, Burns and Combined Injury, Daping Hospital, Army Medical University, Chongqing 400042, PR China.
E-mail: 13638356728@163.com
Correspondence: Dongpo Jiang, Department of Critical Care Medicine, Daping Hospital, Army Medical University, Chongqing 400042, PR China.
E-mail: 13594231239@163.com

 © 2020 The American Society of Gene and Cell Therapy.

In addition, other therapeutic methods target the key points of pathophysiological downstream events in sepsis (for example, anti-thrombosis therapy of activated protein C,[9] anti-inflammation therapy using tumor necrosis factor α [TNF-α] monoclonal antibodies,[10] and neutralizing LPS using anti-LPS antibodies[11]); these therapies can only lead to temporary medical relief instead of curing the disease. The reason is that these approaches do not remove live and dead pathogens, which release toxins into the bloodstream. In fact, the clinical trials of the above-mentioned therapeutic strategies show disappointing outcomes.[9–11] At present, no drugs have been approved to treat sepsis specifically.

Mesenchymal stem cells (MSCs) could be a promising approach to treat sepsis, because they have multiple functions, including tissue repair and immune modulation.[12–14] Many preclinical studies have shown that MSCs can improve a number of pathophysiological processes that are central to sepsis and greatly reduce the rates of organ failure and death.[12,15] Our single-center clinical trial (ChCTR-TRC-14005094) data also show that a single intravenous infusion of allogeneic MSCs was safe and well tolerated in patients with severe sepsis.[16] In addition, accumulating evidence suggests that naive MSCs have a partial antibacterial effect by secreting the antimicrobial peptide LL-37.[17] Indeed, LL-37 is an antimicrobial peptide with a broad spectrum of antibacterial activity against gram-negative bacteria and gram-positive bacteria; LL-37 prevents the formation of LPS-cluster of differentiation 14 (CD14) complexes and blocks the LPS-induced inflammatory response by directly binding to both LPS and CD14 molecules.[18] However, the amount of LL-37 secreted by naive MSCs is limited and is not sufficient to prevent microbial infection or to remove the pathogen-released toxins in sepsis.[17]

In addition, bactericidal/permeability-increasing protein (BPI) is a 55- to 60-kDa positively charged antimicrobial peptide found in the primary granule of human neutrophils; BPI is capable of neutralizing LPS, directly killing gram-negative bacteria, and enhancing bacterial phagocytosis by phagocytes (opsonization).[19] These properties of BPI make this molecule an attractive agent to prevent and/or treat sepsis. Indeed, the use of a recombinant 21-kDa bioactive fragment of BPI (rBPI21) has been demonstrated to be a safe and effective approach that was developed to treat sepsis in an animal model.[20] However, like many other immunotherapies of recombinant proteins, the maintenance of an effective dose *in vivo* is difficult due to the high cost and the short half-life of recombinant proteins, resulting in disappointing outcomes in phase I/II/III clinical trials.[21,22] To solve this problem, a recombinant, replication-deficient adenoviral vector expressing secreted human BPI (AdhBPI) was developed.[23] This AdhBPI-mediated gene transfer markedly reduced the circulating level of inflammatory mediators, such as TNF-α and macrophage inflammatory protein 2 (MIP-2), and improved the survival of lethally septic mice.[23] However, it takes 10 to 14 days for a single dose of adenoviral gene delivery to achieve the elevated levels of transgene products.[23] Thus, it is unlikely to be practicable and effective to use the AdhBPI gene-transfer approach to treat patients who have already been diagnosed with sepsis.

MSCs have the potential to inhibit the inflammatory cascade and to improve injured tissue repair and regeneration. MSCs are also an ideal gene therapy tool. In this study, we aimed to extend the half-lives of the BPI and LL-37 antimicrobial peptides, to expand their antibacterial spectrum, and to enhance the capacity of MSCs to remove pathogens and their toxins; therefore, we engineered MSCs to express a fusion protein composed of BPI21 and LL-37. The BPI21/LL-37-modified MSCs significantly impaired the uncontrolled inflammatory response and reduced the rates of organ failure; this led to the improved survival rates of septic mice.

RESULTS

Generation of Human Umbilical Cord (hUC)-MSCs Engineered with the BPI21/LL-37 or LL-37/BPI21 Antibacterial Fusion Protein

BPI21/LL-37 or LL-37/BPI21 was cloned into a lentiviral vector. They are in tandem with the BPI21 signal peptide and the LL-37 signal peptide, respectively. GGGGS was designed as a flexible peptide linker between BPI21 and LL-37 (Figure 1A). To identify the secretory expression of the BPI21/LL-37 or LL-37/BPI21 fusion protein in hUC-MSCs, BPI21/LL-37 or LL-37/BPI21 was detected in the culture supernatant and in the cytoplasms of engineered hUC-MSCs using western blotting and ELISA, respectively, 3 days after infection with lentivirus particles. The results show that two types of secretory fusion proteins were overexpressed in hUC-MSCs, and the expression level of BPI21/LL-37 was slightly higher than that of LL-37/BPI21 (Figures 1B and 1C). *In vivo*, BPI21/LL-37 expression of BL-hUC-MSCs showed that the expression curve of secretory fusion proteins peaks in 12–24 h (Figure 1D). These findings indicate that BPI21/LL-37 or LL-37/BPI21 fusion protein could be *in vitro* and *in vivo* overexpressed and secreted in the engineered hUC-MSCs.

BPI21/LL-37 and LL-37/BPI21 Fusion Proteins Enhance the Antibacterial Activity and Endotoxin-Neutralizing Activity of hUC-MSCs *In Vitro*

The results showed that the supernatants from both the engineered hUC-MSCs significantly inhibited the growth of nine bacteria compared with that from the control (wild-type [WT])-hUC-MSCs, and the inhibitory effect on *Staphylococcus aureus* was more robust than it was on other strains. The supernatants from BL-hUC-MSCs had better antibacterial effects *in vitro* than those from LB-hUC-MSCs (Figures 2A and S1). In addition, the endotoxin-neutralizing activities analyzed by the limulus amebocyte lysate test also showed that the supernatants from both the engineered hUC-MSCs had significantly higher levels of endotoxin-neutralizing activities compared with those from WT-hUC-MSCs, and these neutralizing activities were dose dependent. The neutralizing activities of BL-hUC-MSCs are superior to those of LB-hUC-MSCs (Figure 2B). These findings indicate that the secretion of engineered hUC-MSCs had remarkably enhanced antibacterial and endotoxin-neutralizing activity *in vitro*, and BL-hUC-MSCs are superior to LB-hUC-MSCs.

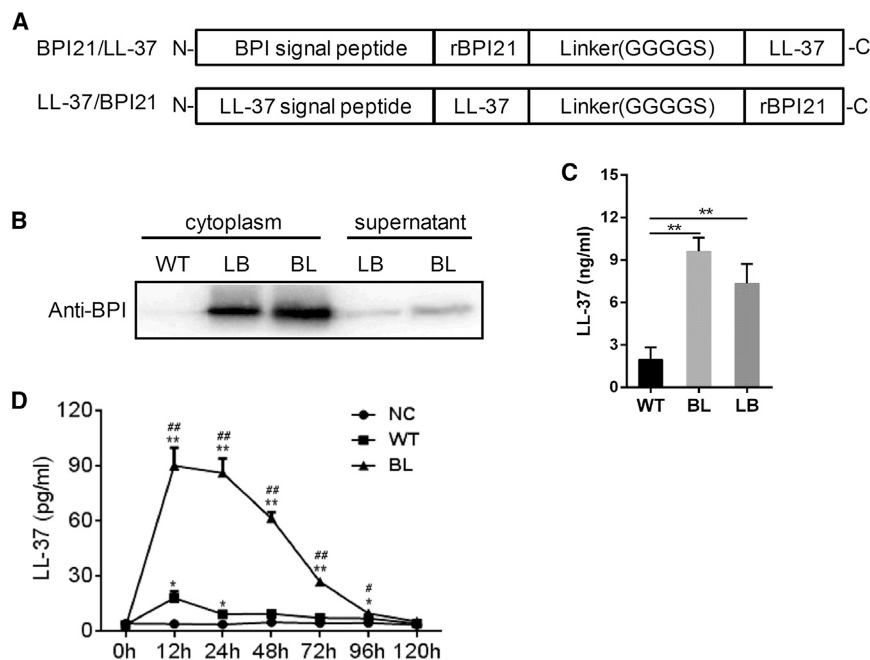

Figure 1. Design and Expression Function Identification of hUC-MSCs Engineered by an Antibacterial Fusion Protein Containing BPI21 and LL-37

(A) The design of an antibacterial fusion protein containing BPI21 and LL-37. (B) The expression of antibacterial fusion proteins in the culture supernatant and cytoplasm of engineered hUC-MSCs was detected by western blots. (C) The secretory protein levels of antibacterial fusion proteins in the culture supernatant of engineered hUC-MSCs were quantitatively analyzed by ELISA, and the data are the mean ± SEM of one representative experiment. Similar results were observed in at least three independent experiments. $^{**}p < 0.01$ compared with WT-hUC-MSCs. (D) BPI21/LL-37 expression and longevity of engineered hUC-MSCs were detected from mice serum. The serum was collected at 5 time points (0, 12, 24, 48, 96, and 120 h) after MSC administration. The experiments were performed in quintuplicate, and the data are presented as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with NC; $\#p < 0.05$, $\#\#\ p < 0.01$ compared with WT-hUC-MSCs. WT, wild-type hUC-MSCs; BL, BPI21/LL-37-engineered hUC-MSCs; LB, LL-37/BPI21-engineered hUC-MSCs, NC, negative control.

The *In Vitro* and *In Vivo* Immunomodulatory and Anti-inflammatory Effects of Engineered hUC-MSCs

As previously studied, MSCs had strongly immunosuppressive activity *in vitro*.[24] We tested the immunosuppressive effect, and peripheral blood mononuclear cell (PBMC) proliferation was inhibited, as indicated by the reduction in carboxyfluorescein diacetate succinimidyl ester (CFSE) intensity by MSCs. The results show that BL-hUC-MSCs and LB-hUC-MSCs, similar to WT-hUC-MSCs, still have inhibitory functions on phytohemagglutinin (PHA)-stimulated PBMC proliferation, but they were not significantly different among the three groups (Figures 3A and 3B). In addition, the anti-inflammatory effects of engineered hUC-MSCs were investigated in LPS-induced macrophages *in vitro*. The results show that the conditioned media from the two engineered cell lines hUC-MSCs and WT-hUC-MSCs remarkably inhibited TNF-α, interleukin 1β (IL-1β), and IL-6 expression but increased IL-10 expression in LPS-induced mouse macrophages in a dose-dependent manner, and the anti-inflammatory effects of conditioned media from the two engineered hUC-MSCs were better than those of the equal volume conditioned media from wild-type hUC-MSCs (Figure 3C). These findings indicate that three types of hUC-MSCs had similarly potent immunomodulatory and anti-inflammatory abilities *in vitro*.

Furthermore, the cecal ligation and puncture (CLP)-induced septic mice were treated with BL-hUC-MSCs and WT-hUC-MSCs, and the serum levels of inflammatory cytokines in each group were detected by ELISA. The results show that compared with those in the sham group, the serum levels of TNF-α, IL-1β, IL-6, and IL-10 were significantly increased in the other experimental groups (Figure 3D) at 24 h after treatment. However, compared with the levels in the PBS control cell group, the WT-hUC-MSCs moderately decreased the levels of TNF-α, IL-1β, and IL-6 and increased the level of IL-10; BL-hUC-MSCs displayed a significant anti-inflammatory effect compared to those exerted by WT-hUC-MSCs and the PBS control cells (Figure 3D). These findings indicate that BL-hUC-MSCs displayed *in vivo* the more potent immunomodulatory and anti-inflammatory abilities.

BL-hUC-MSC Transplantation Enhances Bacterial and Endotoxin Clearance Capabilities and Improves the Survival of Septic Mice

Compared to the characteristics of LB-hUC-MSCs, BL-hUC-MSCs have higher expression levels, better *in vitro* anti-inflammation activities, and superior antibacterial and endotoxin neutralization activities; therefore, BL-hUC-MSCs were selected for the subsequent experiments. The results of bacterial clearance and serum LPS level *in vivo* show that the colony-forming unit (CFU) counts of the organs and serum LPS level were significantly reduced in BL-hUC-MSC-treated mice compared with those of WT-hUC-MSC (and certainly PBS-treated) mice after treatment at 24 h (Figures 4A and 4B). These findings indicate that BL-hUC-MSCs *in vivo* also displayed the more potent bacterial clearance and endotoxin-neutralizing abilities.

Furthermore, the septic mouse-survival results show that BL-hUC-MSCs significantly improved the CLP-induced septic mouse, 120-h survival rates (BL-hUC-MSCs versus WT-hUC-MSCs versus PBS, 56.7% versus 20.7% versus 10%, respectively) when septic mice were treated with BL-hUC-MSCs or WT-hUC-MSCs (3 h after the CLP or sham surgery) combined with Tienam, 24 h after the CLP

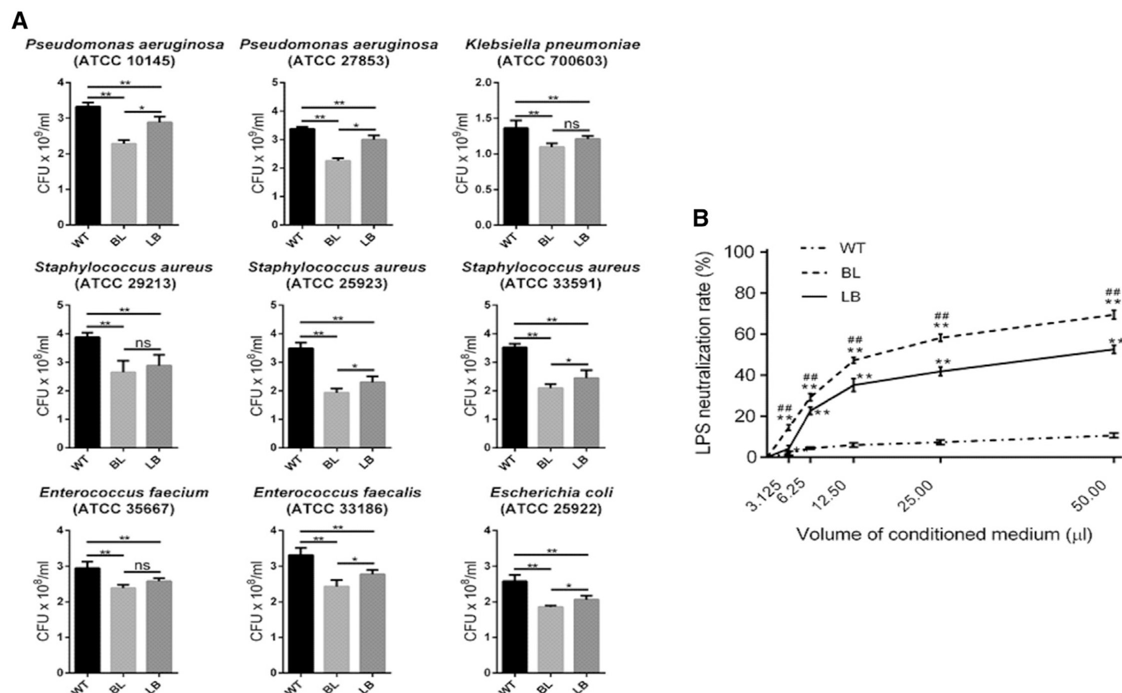

Figure 2. Antibacterial Fusion Proteins Enhanced the Antibacterial and Endotoxin-Neutralizing Activities of hUC-MSCs *In Vitro*

(A) The antibacterial activities of antibacterial fusion proteins secreted by engineered hUC-MSCs on gram-negative bacteria and gram-positive bacteria. (B) The neutralizing effect of culture supernatant from hUC-MSCs engineered by antibacterial fusion proteins on LPS *in vitro*. The experiments were performed in quintuplicate, and the data are presented as the mean ± SEM. *$p < 0.05$, **$p < 0.01$ compared with WT-hUC-MSCs. #$p < 0.05$, ##$p < 0.01$ compared with LB-hUC-MSCs. WT, wild-type hUC-MSCs; BL, BPI21/LL-37-engineered hUC-MSCs; LB, LL-37/BPI21-engineered hUC-MSCs; ns, not significant.

or sham surgery (Figure 4C). However, if Tienam were used 6 h after the CLP or sham surgery, both the WT-hUC-MSCs and the BL-hUC-MSCs also remarkably improved the septic mouse survival rates; however, the BL-hUC-MSCs were not better than the WT-hUC-MSCs, but there is no statistical significance (Figure 4D). Similar results were also found in the other two septic models, where BL-hUC-MSCs significantly increased the survival rates (BL-hUC-MSCs versus WT-hUC-MSCs versus PBS, 60% versus 26.7% versus 16.7%, respectively) in the bacteremia model (Figure 4E) and (BL-hUC-MSCs versus WT-hUC-MSCs versus PBS, 46.7% versus 30% versus 13.3%, respectively) in the endotoxemia model (Figure 4F). These results indicate that BL-hUC-MSCs significantly improve the survival of various types of septic mice.

Protection of BL-hUC-MSCs on Organ Function in CLP-Induced Lethal Septic Mice

To confirm the protection of BL-hUC-MSCs from multiple organ dysfunction syndrome (MODS) in CLP-induced septic mice, laboratory indicators and pathological examinations of various vital organ dysfunctions and injuries were tested. The results show that the CLP surgery significantly increased the serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), amylase (AMS), creatinine (crea), and urea, but the treatment of both BL-hUC-MSCs and WT-hUC-MSCs remarkably attenuated the increase of these indicators; in addition, BL-hUC-MSCs exerted better protective effects from MODS than the protective effects exerted by WT-hUC-MSCs (Figure 5A). Furthermore, histological examinations also showed damage to the lungs, liver, and kidneys in the CLP-induced septic mice, the infiltration of inflammatory cells into the lung interstitium and alveolar spaces, alveolar wall thickening, intra-alveolar exudation, the atrophy and degeneration of renal tubular epithelial cells, and necrotic lesions in the liver parenchyma, which were all observed in the photomicrographs of hematoxylin and eosin (H&E)-stained tissue sections (Figure 5B). In addition, immunohistochemical staining also showed that cleaved caspase-3-positive cells were frequently observed in the above three types of tissue sections in the PBS-vehicle group (Figure 5C). However, both BL-hUC-MSC and WT-hUC-MSC treatment significantly attenuated these histological changes and the number of cleaved caspase-3-positive cells, and BL-hUC-MSC treatment exerted significantly better protection than the protection exerted by WT-hUC-MSCs. Semi-quantitative assessment using a lung, liver, and kidney histological injury or cell apoptosis score demonstrated that the degrees of organ injury in the BL-hUC-MSC and WT-hUC-MSC treatment groups were significantly lower than that in the PBS-vehicle group and that BL-hUC-MSC treatment exerted better protective effects than the protective effects exerted by WT-hUC-MSC treatment (Figures 5B and 5C). These results indicate that transplanted BL-hUC-MSCs can significantly reduce the apoptosis rate of organ cells in septic mice and exert organ-protective functions.

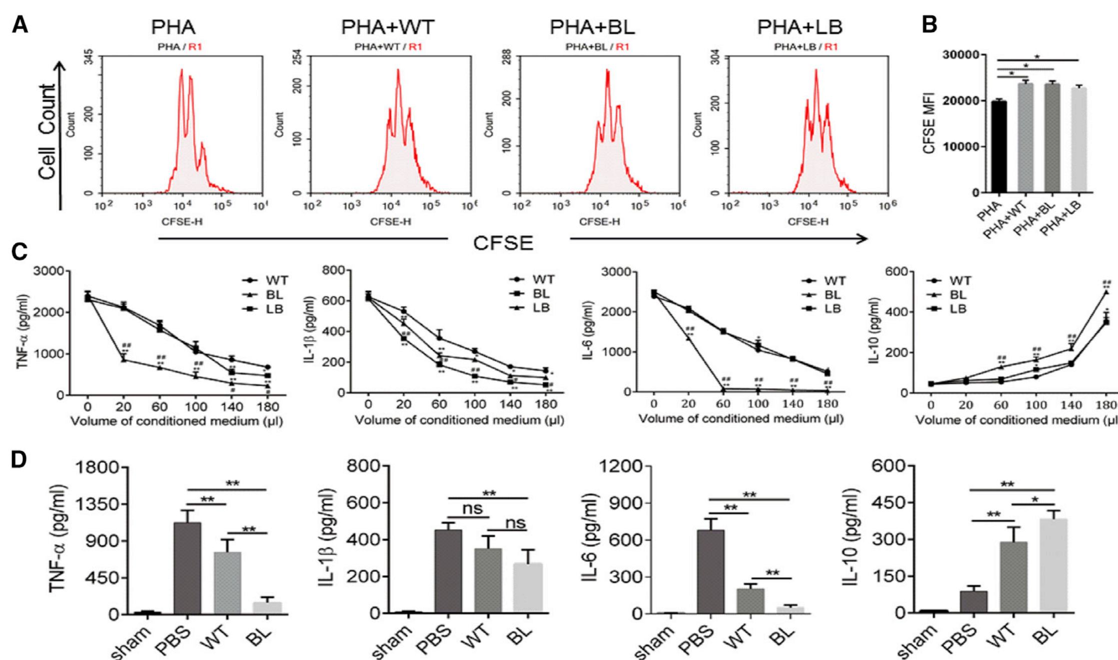

Figure 3. Immunomodulatory and Anti-inflammatory Effects of hUC-MSCs Engineered with an Antibacterial Fusion Protein

(A) The inhibitory effect of engineered-hUC-MSCs on PHA-induced peripheral blood mononuclear cell (PBMC) proliferation using flow cytometry and CFSE staining. CFSE fluorescence intensity reduction of PBMCs was detected by flow cytometry. Data are representative of three independent experiments. (B) The mean fluorescent intensity (MFI) of the CFSE dilution in the cells from the data in (A) was quantified. Similar experiments were performed at least in triplicate. The data are mean ± SEM. *$p < 0.05$. (C) Conditioned medium from engineered hUC-MSCs regulated the *in vitro* proinflammatory and anti-inflammatory mediator expression in LPS-induced mouse peritoneal macrophages. Similar experiments were performed at least in triplicate. The data are mean ± SEM. *$p < 0.05$, **$p < 0.01$ compared with wild-type hUC-MSCs. #$p < 0.05$, ##$p < 0.01$ compared with LB-hUC-MSCs. (D) The inhibitory effect of BL-hUC-MSCs on the inflammatory response in CLP-induced septic mice at 24 h after treatment. Similar experiments were at least performed in quintuplicate. The data are mean ± SEM. *$p < 0.05$, **$p < 0.01$. WT, wild-type hUC-MSCs; BL, BPI21/LL-37-engineered hUC-MSCs; LB, LL-37/BPI21-engineered hUC-MSCs; CFSE, carboxyfluorescein diacetate succinimidyl ester.

DISCUSSION

Sepsis is an excessive systemic inflammatory response syndrome that is followed by a compensatory anti-inflammatory response syndrome.[25,26] Excessive inflammatory reactions can lead to extensive host damage, which is sometimes greater than that caused by the infection alone. Whereas the majority of therapeutic strategies, such as anti-thrombosis therapy of activated protein C, anti-inflammation therapy using TNF-α monoclonal antibodies, and neutralizing LPS using anti-LPS antibodies, are designed to treat a single key point of the pathophysiological events in sepsis, they have failed.[9–11] At present, no drugs have been specifically approved to treat sepsis. Therefore, a comprehensive therapy designed for the control of the infection, anti-inflammation, scavenging pathogen microorganisms and their toxic substances, and organ function protection or repair can be an effective therapeutic strategy for sepsis. MSC transplantation has been considered a promising comprehensive therapeutic strategy with multiple functions (including anti-inflammation, tissue repair, and regeneration) to treat sepsis.[12,16,27–29] In addition, MSC-derived vesicles, whether exosomes or microvesicles, have been shown to be as potent as the parent stem cell as a therapeutic in various organ-injury models.[30] In 2007, the first study of bone marrow-derived mesenchymal stem cells (BMSCs) for the treatment of sepsis was reported.[31] Moreover, another study also showed that BMSCs are more effective in treating sepsis in mice than adipose-derived mesenchymal stromal cells (ADMSCs).[32] Another study has shown that human mesenchymal stem cells from umbilical cord blood reduce proinflammatory cytokines more significantly than BMSCs and ADMSCs.[33] Additionally, some research has also shown that hUC-MSCs showed similar or superior characteristics to MSCs from other sources and displayed better treatment effect in sepsis.[34,35] Therefore, hUC-MSCs were selected as seed cells in this study. However, mesenchymal stem cells usually lack effective antimicrobial and endotoxin-neutralizing functions.

The establishment of control of an infection is the first key step to reduce the occurrence of sepsis. However, the incidence of both drug-resistant bacteria and the number of immunocompromised septic patients is increasing; this suggests that antibiotic therapies are ineffective or even detrimental, as they have been shown to deteriorate a patient's clinical condition.[8] Therefore, the activation of the body's natural anti-infection immunity may also help to control sepsis. BPI is a natural, potent bactericidal protein and is considered as an attractive agent to be used for preventing and/or treating sepsis. Notably, rBPI21 was developed as a novel agent to treat sepsis.[36] However, the

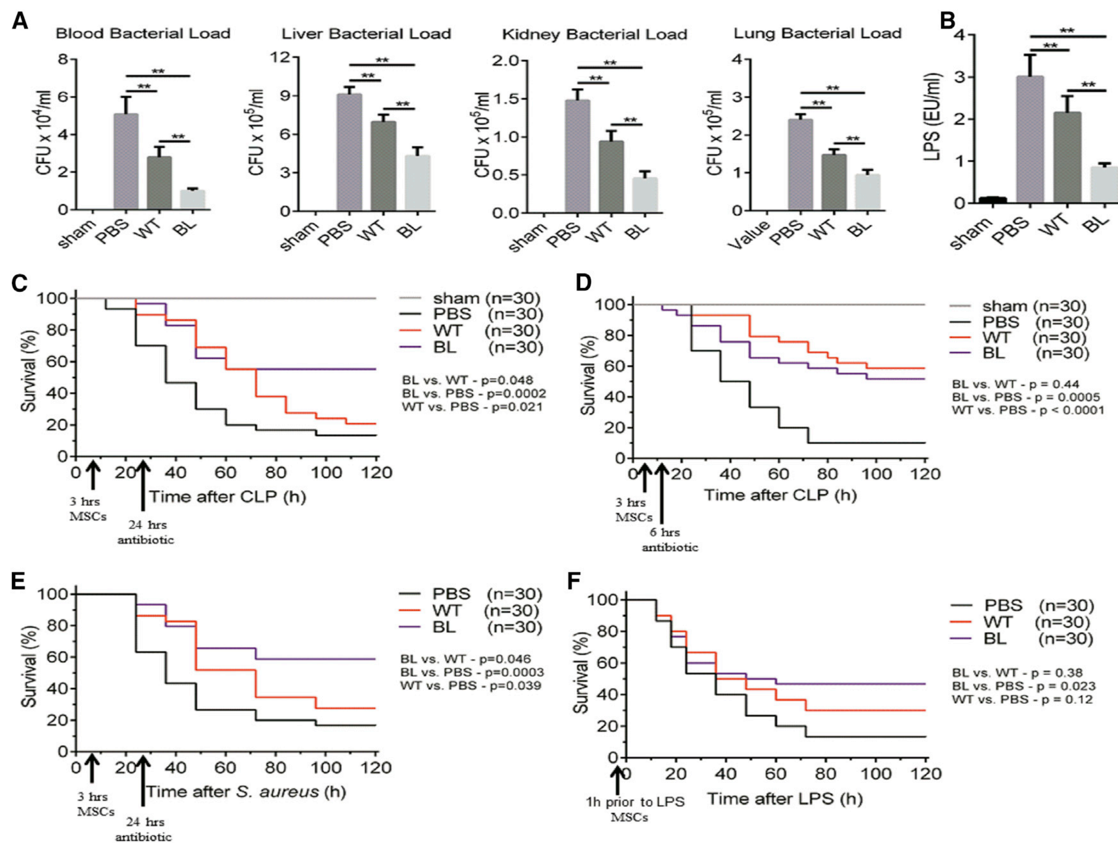

Figure 4. BL-hUC-MSC Transplantation Enhances Bacterial and Endotoxin Clearance Capabilities and Improves the Survival Rates in Septic Mice

(A) The bacterial clearance in blood, kidney, liver, and lung was detected after hUC-MSCs treatment at 24 h in the CLP-induced septic mice. The CFU were counted in the organs. The experiments were performed in quintuplicate, and the data are presented as the mean ± SEM. *p < 0.05, **p < 0.01. WT, wild-type hUC-MSCs; BL, BPI21/LL-37-engineered hUC-MSCs. (B) The serum LPS level was measured after hUC-MSC treatment at 24 h in the CLP-induced septic mice. Similar experiments were performed in quintuplicate, and the data are presented as the mean ± SEM. *p < 0.05, **p < 0.01. (C) BL-hUC-MSCs were combined with Tienam (7 mg/kg), 24 h after the CLP surgery and significantly improved the 120-h survival rates of CLP-induced septic mice. (D) BL-hUC-MSCs were combined with Tienam (7 mg/kg), 6 h after the CLP surgery, and significantly improved the 120-h survival rates of CLP-induced septic mice but not better than the WT-hUC-MSCs. (E) BL-hUC-MSCs were combined with Tienam (7 mg/kg), 24 h after the bacterial injection and significantly improved the 120-h survival rates of *Staphylococcus aureus*-induced septic mice. (F) BL-hUC-MSCs significantly improved the 120-h survival rates of LPS-induced septic mice. WT, wild-type hUC-MSCs; BL, BPI21/LL-37-engineered hUC-MSCs. Survival curves of different treatment groups were plotted according to the Kaplan-Meier method and were compared using the log-rank tests.

administration of rBPI21 was unable to maintain a constant optimal therapeutic level, due to its high cost and the short *in vivo* half-life of the recombinant protein. These defects caused disappointing clinical trial outcomes.[21] LL-37 is a broad-spectrum antibacterial and can be secreted by naive MSCs; however, the amounts secreted are limited.[17,37] Therefore, in the current study, a broad-spectrum antibacterial fusion peptide (BPI21/LL-37; based on BPI21 and LL-37) was developed to modify hUC-MSCs using a lentiviral vector. This novel secreted BPI21/LL-37 anti-bacterial fusion peptide significantly enhanced hUC-MSCs, the broad-spectrum antibacterial functions, and the LPS neutralization activities and its half-life, whereas we could not further address whether the mechanism of action is bacteriostatic or bactericidal at this stage. These results support the use of BL-hUC-MSCs as an adjunct therapeutic antibacterial agent for sepsis. Although lentivirus-mediated gene modification has used chimeric antigen receptor (CAR) T cell therapies,[38] a gene-modified stem cell was not still used in the clinical treatment. Therefore, more preclinical safety of BL-hUC-MSCs, such as its tumorigenesis, needs to be assessed before clinical trials. Fortunately, hUC-MSCs display low immunogenicity, but it still is allogeneic and can be cleared by the recipient's immune system, 4 months after transplantation.[39] In addition, the safety experiments show that the level of BPI21/LL-37 in the blood circulation restored to baseline level 4–5 days after BL-hUC-MSC transplantation, and BL-hUC-MSC transplantation did not affect the survival rate, body weight, organ weight, and appetite of the C57BL/6J mice (Tables S2 and S3). The above results suggested that the short-term safety of BL-hUC-MSCs is manageable, but its long-term safety needs to perform more experiments in the future.

The stemness phenotype of hUC-MSCs is the basis of its tissue repair and regeneration function.[40] In this study, we found that the engineered hUC-MSCs still have differentiation capacity similar to the

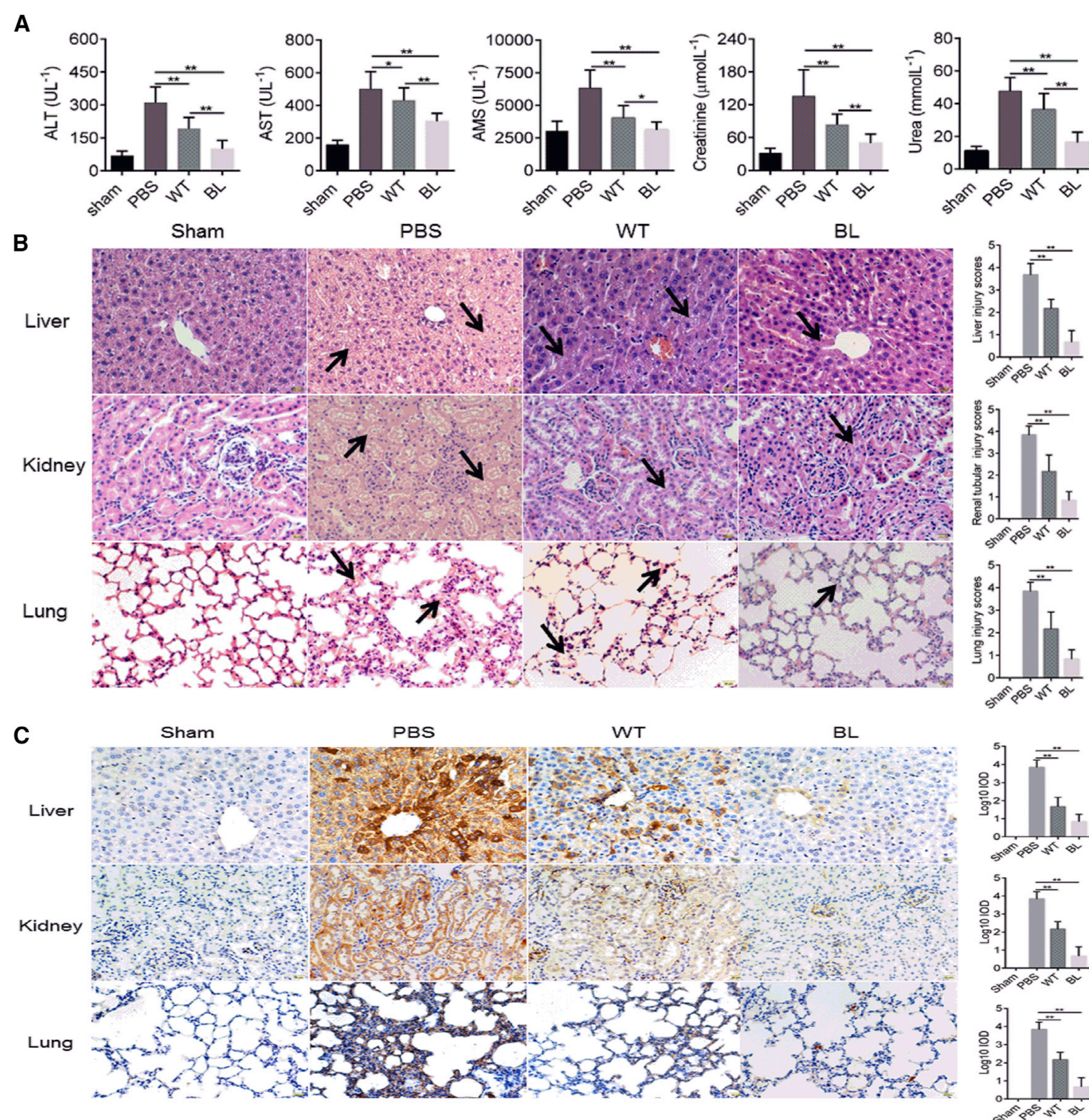

Figure 5. Protection of BL-hUC-MSCs on the Organ Functions of CLP-Induced Septic Mice

(A) The serum levels of the liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were remarkably decreased in the BL-MSC-treated mice ($p < 0.01$ versus the CLP + PBS mice) but only slightly decreased in the MSC-treated mice. Similar results were also shown in the serum levels of amylase (AMS), urea, and creatinine (crea). These findings indicate that BL-MSCs can effectively enhance the therapeutic effect of MSCs in sepsis. (B) H&E staining of livers, kidneys, and lungs. The arrows indicate damage sections of livers, kidneys, and lungs. Histological injury scores are shown at right. n = 5 for each group. Bar, 20 μm. *$p < 0.05$, **$p < 0.01$. (C) Immunocytochemistry staining showing that apoptosis was colocalized in the livers, kidneys, and lungs. We examined cleaved caspase-3 protein in the liver, kidney, and lung tissue of each group at 48 h after CLP. Bar, 20 μm. *$p < 0.05$, **$p < 0.01$. IOD, integrated optical density.

wild-type hUC-MSCs (Figures S2A and S2B); the engineered hUC-MSCs also express stemness genes, including OCT-4, INHBA, and c-MYC (Figures S2C and S2D). Our results indicate that the ectopic expression of BPI21/LL-37 does not affect the tissue repair and regeneration functions of hUC-MSCs.

Furthermore, the addition of antibacterial and endotoxin neutralization functions to MSCs is critical, but retaining its anti-inflammatory and immunosuppressive functions is also important for the successful treatment of sepsis. In this study, we confirmed that engineered hUC-MSCs retain the inhibitory ability against PHA-induced PBMC proliferation at a similar level as that of the wild-type hUC-MSCs. In addition, the conditioned medium from both types of engineered hUC-MSCs, especially from the BL-engineered hUC-MSCs, significantly downregulated the expression of proinflammatory cytokines and upregulated the expression of anti-inflammatory cytokines in

LPS-induced mouse peritoneal macrophages. Therefore, our data indicate that BL- or LB-engineered hUC-MSCs retain anti-inflammatory functions.

In addition, our *in vivo* results also indicate that WT-hUC-MSC and BL-hUC-MSC transplantation significantly improved the survival rate of septic mice and that BL-hUC-MSCs displayed better protective effects in CLP-induced septic mice that received antibiotics 24 h after operation (Figure 4C); this is true, except for when the antibiotic Tienam was used jointly in CLP-induced septic mice, 6 h after CLP induction, and the wild-type hUC-MSCs display slightly well-protective effects but no statistical significance. In our opinion, when potent antibiotics were used at the early stage after CLP induction, the infection was effectively controlled, and the leading lethal cause may be a systemic inflammatory response. However, the comprehensive therapy capacities of BL-hUC-MSCs for sepsis were enhanced by its bacterial and endotoxin clearance activities, but we thought that its anti-inflammatory or other activities might be impaired by the lentivirus infection. Therefore, the early clinical use of antibiotics, in combination with hUC-MSCs, can timely and effectively treat sepsis patients. However, in clinical practice, there is a lack of a diagnostic "gold standard," due to an insidious sepsis status in the early stages;[41] in addition, there is an increasingly strict clinical use of antibiotics. Up to one-third of patients has negative blood cultures and lack the strong evidence needed for the use of third-line antibiotics; so once sepsis is diagnosed in clinical practice, patients often have missed the best time to use antibiotics to rescue their condition. Research has shown that for every 1 h that antibiotic administration is delayed, the patient's mortality will increase by 12%.[42] Fortunately, the concentration of BPI21/LL-37 continuously maintained at a high level in blood circulation for 4–5 days by BL-hUC-MSC transplantation-mediated constitutive overexpression and its extended half-life (Figure S3), which could significantly improve the survival rate by exerting their bacterial and endotoxin clearance capabilities, as was seen in severe septic mice. In addition, we also observed in each septic model that BL-hUC-MSCs did not show more advantages in the early stages after the injection of cells and antibiotics. However, the protective effects of BL-hUC-MSCs gradually appeared over time with severe infection, and the difference between the wild-type and BL-engineered hUC-MSCs changed more significantly in the later stages. In our opinion, bacteria and endotoxin clearance therapies that are continuously supported by BL-hUC-MSCs, which impair the excessive systemic inflammatory response by reducing the existence of pathogen-associated molecular patterns (PAMPs), play an important role in severe sepsis and in the late stages of sepsis.

In sepsis, the body's immune system is overactivated and interacts to create an "inflammatory waterfall effect" and an imbalance in homeostasis.[25,26] Previous studies have shown that in sepsis models or patients, a number of septic cytokines, including TNF-α, IL-1β, and IL-6 and the anti-inflammatory cytokine IL-10 in serum, are elevated.[25,26] In the current study, compared with those of PBS and hUC-MSCs, BL-hUC-MSC treatment significantly improved this imbalance inflammatory response; this indicates that BL-hUC-MSCs can play an immunosuppressive role in an excessive inflammatory response, leading to a balance of "proinflammatory" and "anti-inflammatory" effects.

Multiple organ failure is the leading cause of death in patients with sepsis, whereas the liver, kidneys, and lungs are the most commonly damaged organs early in sepsis.[42–45] Our study found that after treatment with BL-hUC-MSCs and hUC-MSCs, the liver function (AST and ALT), renal function (crea and urea), and pancreatic function (AMS) were markedly improved, and BL-hUC-MSCs displayed a better protective role. Histopathological examination also showed similar results. In addition, immunohistochemical staining showed that cleaved caspase-3-positive apoptotic cells were remarkably reduced in liver, kidney, and lung tissue sections after treatment with WT-hUC-MSCs and BL-hUC-MSCs, but BL-hUC-MSCs displayed more protective effects. Sepsis is often accompanied by organ damage, of which apoptosis is closely related to the injury induced by inflammatory mediators, such as TNF-α[46]. Some studies indicate that MSCs attenuate injury induced with LPS through reducing the infiltration of inflammatory cells in various target organs and by reducing cell death.[12,47] Our study suggested that fusion proteins BPI21/LL-37 could be continuously overexpressed *in vivo*, which enhanced bacteria clearance and endotoxin-neutralizing activities of MSCs *in vivo*. Therefore, the decrease of LPS would reduce endotoxin-induced systemic response and prevent apoptosis progression in sepsis.

In summary, the BPI21/LL-37 modification enhanced the antibacterial function and endotoxin neutralization of hUC-MSCs but did not affect its stemness, tissue repair and regeneration, anti-inflammatory abilities. BPI21/LL-37 could be continuously overexpressed *in vivo* as a broad-spectrum antibacterial fusion protein, which significantly enhanced the bacterial and LPS clearance effect of organs and blood in the CLP-induced septic mice. Therefore, the BPI21/LL-37 modification provides hUC-MSCs with an additional, multifaceted therapeutic potential for sepsis and can significantly improve the protective effects of hUC-MSCs on survival and multiple organ dysfunction in severe septic mice. Thus, we believe that BL-hUC-MSCs are a promising therapeutic approach for severe sepsis and septic shock, and the future validation of our studies in the clinic is warranted.

MATERIALS AND METHODS

Design and Construction of the BPI21/LL-37 Fusion Peptide

Two kinds of BPI21/LL-37 (BL) and LL-37/BPI21 (LB) fusion peptides that were linked by a GGGGS oligopeptide linker were designed. The fusion genes that contained a DNA sequence of the BPI or LL-37 signaling peptide in the N terminus of the antibacterial fusion peptide were artificially synthesized; then, they were ligated into lentiviral vectors to construct a lentiviral expression system of the BPI21/LL-37 or LL-37/BPI21 (Figure 1A).

Animals, Septic Models, and Improving the Effects of Engineered hUC-MSCs on the Survival Rate of Septic Mice

Male C57BL/6J mice, weighing 22–25 g, were purchased from Vital River Laboratory Animal Technology (Beijing, China). The mice

were housed at a controlled temperature of 22°C–24°C with 12 h light-dark cycles. The mice were offered standard laboratory tap water and chow *ad libitum*. All mice were kept in quarantine for 1 week before our experiment. The experiments were performed according to the protocols approved by the Ethics Review Committee for Animal Experimentation of Daping Hospital, Army Medical University. All mice were treated humanely throughout the experimental period.

The CLP-induced severe septic model was induced by an operation in male C57BL/6J mice, as described previously.[10] Two other septic models were induced by injecting a 10-mg/kg dose of LPS (Sigma, St. Louis, MO, USA) via an intraperitoneal injection or by administering 1×10^9 CFU *Staphylococcus aureus* (ATCC 25923) per mouse via an intraperitoneal injection. MSCs were administered to the septic mice by tail intravenous injections at 2×10^5 cells per mouse after 3 h of CLP surgery or the bacterial injection or 1 h prior to LPS injection. The survival curves of the mice were calculated during 120 h after the surgery, bacterial injection, or LPS injection.

Construction of Engineered hUC-MSCs Using an Antibacterial Fusion Peptide Lentiviral Expression System

MSCs from human umbilical cords were separated, as previously described,[48] and provided by Chongqing Fumei Stem Cell Biotechnology Development. For the subcultures of hUC-MSCs, the culture media were replaced with DMEM/F12 (HyClone, Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA).[49] Human umbilical cords were obtained from full-term caesarian section births from Daping Hospital, Army Medical University. hUC-MSCs, between 4 and 6 passages, were used for the *in vitro* and *in vivo* experiments.

Lentivirus particles were produced in 293T cells, as previously described.[50] An equal number of viruses containing the antibacterial fusion peptide gene were used to infect hUC-MSCs to transform them into BPI21/LL-37-engineered hUC-MSCs (BL-hUC-MSCs) and LL-37/BPI21-engineered hUC-MSCs (LB-hUC-MSCs). After the engineered hUC-MSCs were cultured for 48 h, the culture supernatants and cells were collected, and aliquots were stored at −80°C for the antibacterial activity assay, the endotoxin-neutralizing activity assay, and other subsequent experiments.

In Vivo Expression and Longevity of BPI21/LL-37 Assays

To detect the expression and longevity of BPI21/LL-37 in C57BL/6J mice, the animals were randomly divided into three groups (PBS versus WT versus BL). *In-vitro*-cultured BL-hUC-MSCs and WT-hUC-MSCs were used in this study, and MSCs were administered to mice by tail intravenous injections (2×10^5 cells per mouse in 200 μL of PBS). Mice blood and serum were collected at 5 time points (0, 12, 24, 48, 96, and 120 h) after MSC administration, and the serum samples were stored at −80°C. The protein level of BPI21/LL-37 in the mice serum was detected using the corresponding mouse ELISA kit, according to the manufacturer's instructions (TSZ, USA).

In Vitro Antibacterial Activity Assay

To test the broad-spectrum antibacterial activity of conditioned medium from engineered hUC-MSCs, nine microbial strains, including *Klebsiella pneumoniae* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 10145, ATCC 27853), *Staphylococcus aureus* (ATCC 29213 and ATCC 25923), methicillin-resistant *Staphylococcus aureus* (MRSA; ATCC 33591), *Enterococcus* (ATCC 35667), *Enterococcus faecalis* (ATCC 33186), and *Escherichia coli* (ATCC 25922), were obtained from the clinical laboratory of Daping Hospital, Army Medical University of China. Then, 100 μl Mueller-Hinton broth medium containing 1×10^4 CFU of the above bacteria was mixed with conditioned medium from three types of hUC-MSCs in a ratio of 1:1 in a sterile, 96-well culture plate; the plates were incubated for 12–18 h at 37°C. The microplate reader detected the absorbance of each well at 600 nm and compared the optical density of a 600-nm wavelength (OD600) difference of each group. In addition, culture media were serially diluted (1:10) using PBS, 50 μL of each dilution was then plated and cultured for 24 h at 37°C on Mueller-Hinton agar, and then CFUs were counted, as previously described.[17] Each group experiment was performed in quintuplicate.

Endotoxin Neutralization Assay

To test the ability of BL- and LB-hUC-MSCs to neutralize endotoxins, the endotoxin-neutralizing activity of conditioned media from engineered hUC-MSCs was detected by the dynamic colorimetric method (Xiamen Reagent Biotech, Xiamen, China), according to the manufacturer's instructions. Then, 150 μL of the test solution containing 0.25 endotoxin unit (EU) endotoxin was mixed with 50 μL fresh cell medium containing 0 μL, 3.125 μL, 6.25 μL, 12.5 μL, 25 μL, or 50 μL conditioned medium from three kinds of hUC-MSCs, and the mixtures were incubated for 2 h at 37°C. The absorbance was detected at 405 nm. Each group experiment was performed in quintuplicate.

Assay of the Inhibitory Effect of Engineered hUC-MSCs on PBMC Proliferation

To test the inhibitory effect of engineered hUC-MSCs on immunocyte proliferation, MSCs and PBMCs were cocultured in a Transwell-48 system. Freshly isolated PBMCs (10^6 cells/mL in phosphate-buffered saline) from fresh human blood were labeled with 5 mM CFSE (BD Horizon, USA) for 8 min at room temperature, and the reaction was terminated by adding 2% fetal calf serum in PBS. Labeling PBMCs (2×10^5 cells/well) were cocultured with their corresponding MSCs at a 5:1 ratio (PBMCs:MSCs) in 48-well plates. After the coculture system was stimulated with 20 μg/mL PHA for 48 h, the PBMCs were collected, and the remaining cell-associated CFSE fluorescence was analyzed by the NovoCyte flow cytometer (ACEA Biosciences, Hangzhou, China). PBMC proliferation was detected by flow cytometry by reduction of CFSE fluorescence intensity. A lower amount of CFSE per cell indicates increased proliferation of the respective cells.

Anti-inflammatory Ability Assays of Engineered hUC-MSCs

The preparation of the conditioned medium was as follows: after WT-hUC-MSCs, BL-hUC-MSCs, and LB-hUC-MSCs were cultured for 48 h, 10 mL of culture supernatant was collected and concentrated to 1 mL of conditioned medium used with the Amicon Ultra-15 Centrifugal Filter (molecular weight cutoff of 10 kDa). Then, aliquots were stored at −80°C for later use.

The mouse peritoneal macrophages were seeded into 6-well plates at a density of 1×10^6 cells/well and were cultured for 24 h. After macrophages were treated with 10 ng/mL of LPS for 3 h, the medium was replaced with 1 mL of fresh medium containing 0 μL, 20 μL, 60 μL, 100 μL, 140 μL, and 180 μL of conditioned medium from three kinds of hUC-MSCs, respectively. After treatment for 9 h, the supernatant of each well was collected. Each group experiment was performed in triplicate. The concentrations of IL-10, TNF-α, IL-1β, and IL-6 in the supernatant were measured by ELISA, according to the instructions of the Wuhan Boster Bio-ELISA kit.

Bacterial Clearance Assays in Blood, Kidney, Liver, and Lung and Serum LPS-Level Determinations in the CLP-Induced Septic Mice

BL-hUC-MSCs or WT-hUC-MSCs were administered by tail intravenous injections at 2×10^5 cells per mouse after 3 h of CLP surgery. Then, the blood, kidney, liver, and lung samples were collected at 24 h after MSC treatment for analysis. Total blood and homogenized snips of each organ were serially diluted (1:10) using PBS; 50 μL of each dilution was then plated on 5% sheep blood agar plates until the number of CFUs was counted on blood agar. The serum LPS level was detected by the dynamic colorimetric method (Xiamen Reagent Biotech, Xiamen, China), according to the manufacturer's instructions.

ELISA

The protein levels of TNF-α, IL-10, IL-6, and BPI21/LL-37 in the cell culture supernatants or mouse serum were detected using the corresponding mouse ELISA kit, according to the manufacturer's instructions (Boster, Wuhan, China).

Histological Examination of Liver, Lung, and Kidney Damage

Fresh mouse liver, lung, and kidney tissues were randomly selected from six mice per experimental group. The samples were fixed with 4% paraformaldehyde (PFA; pH 7.4) and were then gradually dehydrated, embedded in paraffin, cut into 4 μm sections, and stained with H&E for light microscopy. To evaluate the degree of organ injury, a liver injury grading score (grade 0–4), based on the severity of necrotic lesions in the liver parenchyma, was carried out, as previously reported;[51] a lung injury grading score (grade 0–4) was based on the four following categories: interstitial inflammation, neutrophil infiltration, congestion, and edema, which were carried out, as previously reported;[52] and then, the renal tubular injury score was assessed according to the percentage of cortical tubules with epithelial necrosis, as previously reported.[53] The degree of organ injury was evaluated with blind procedure, and injury grading score was carried out in five random microscopic fields per organ.

Immunohistochemical Staining for Apoptosis

The paraffin-embedded sections of liver, lung, and kidney tissues from histopathological evaluations (above) were employed for immunohistochemical experiments. The embedded tissue sections were deparaffinized and gradually dehydrated; then, antigen retrieval was performed, and the nonspecific antigens were blocked. For cleaved caspase-3 staining, the specimens were incubated with a rabbit anti-mouse cleaved caspase-3 antibody (1:200; Cell Signaling Technology [CST], Danvers, MA, USA), as previously reported, at 4°C overnight,[54] followed by a 30-min incubation with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (ZSGB-BIO, China). Subsequently, the samples were stained with the 3,3′-diaminobenzidine (DAB) Immunohistochemistry Color Development Kit (ZSGB-BIO, China), followed by hematoxylin. Then, the slides were washed using deionized water and mounted in xylene-based mountant. Then, the immunohistochemical staining of cleaved caspase-3 in liver, lung, and kidney tissues was analyzed by Image Pro-Plus (IPP), and the results were compared with visual assessment, as described previously.[55] The integrated optical density (IOD) was log10 transformed, and the analysis of cleaved caspase-3 staining was semiquantitatively evaluated in the tissues.

Statistical Analysis

All data are expressed as the mean ± SEM unless otherwise indicated. The statistical significance between the groups was analyzed using a t test or one-way ANOVA, followed by Tukey's post hoc test, to correct for multiple comparisons in GraphPad Prism 6 (San Diego, CA, USA). Survival curves of different treatment groups were plotted, according to the Kaplan-Meier method, and were compared using the log-rank tests. The statistical significance was set at $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.ymthe.2020.05.014.

AUTHOR CONTRIBUTIONS

X.X. and H.L. conceived and designed the research. Z.L., Y.S., P.Y., W.G., X.H., W.X., L.A., Y.T., X.W., X.A., and X.H. performed the research. D.J. and H.L. analyzed the data. Z.L., D.J., and X.X. designed the experiments and wrote the manuscript. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

ACKNOWLEDGMENTS

This study was supported by the Military Biosafety Project (no. 19SWAQ18); Military Research and Development Program (nos. 2019CXJSB017 and 2019XYY21); National Natural Science Foundation of China (nos. 81801585 and 81372027); and Military Medicine Frontier Innovation Capability Training Program (nos. 2019CXJSC033 and 18CXZ002).

REFERENCES

1. Bone, R.C., Grodzin, C.J., and Balk, R.A. (1997). Sepsis: a new hypothesis for pathogenesis of the disease process. Chest *112*, 235–243.
2. Angus, D.C., Birmingham, M.C., Balk, R.A., Scannon, P.J., Collins, D., Kruse, J.A., Graham, D.R., Dedhia, H.V., Homann, S., and MacIntyre, N. (2000). E5 murine monoclonal antiendotoxin antibody in gram-negative sepsis: a randomized controlled trial. E5 Study Investigators. JAMA *283*, 1723–1730.
3. Lyle, N.H., Pena, O.M., Boyd, J.H., and Hancock, R.E. (2014). Barriers to the effective treatment of sepsis: antimicrobial agents, sepsis definitions, and host-directed therapies. Ann. N Y Acad. Sci. *1323*, 101–114.
4. Rangel-Frausto, M.S., Pittet, D., Costigan, M., Hwang, T., Davis, C.S., and Wenzel, R.P. (1995). The natural history of the systemic inflammatory response syndrome (SIRS). A prospective study. JAMA *273*, 117–123.
5. Annane, D., Sébille, V., Troché, G., Raphaël, J.C., Gajdos, P., and Bellissant, E. (2000). A 3-level prognostic classification in septic shock based on cortisol levels and cortisol response to corticotropin. JAMA *283*, 1038–1045.
6. (1990). From the Centers for Disease Control. Increase in National Hospital Discharge Survey rates for septicemia–United States, 1979–1987. JAMA 263, 937–938.
7. Venet, F., and Monneret, G. (2018). Advances in the understanding and treatment of sepsis-induced immunosuppression. Nat. Rev. Nephrol. *14*, 121–137.
8. Wiessner, W.H., Casey, L.C., and Zbilut, J.P. (1995). Treatment of sepsis and septic shock: a review. Heart Lung *24*, 380–392, quiz 392–393.
9. Angus, D.C. (2012). Drotrecogin alfa (activated)...a sad final fizzle to a roller-coaster party. Crit. Care *16*, 107.
10. Abraham, E., Wunderink, R., Silverman, H., Perl, T.M., Nasraway, S., Levy, H., Bone, R., Wenzel, R.P., Balk, R., Allred, R., et al. (1995). Efficacy and safety of monoclonal antibody to human tumor necrosis factor alpha in patients with sepsis syndrome. A randomized, controlled, double-blind, multicenter clinical trial. TNF-alpha MAb Sepsis Study Group. JAMA *273*, 934–941.
11. Derkx, B., Wittes, J., and McCloskey, R.; European Pediatric Meningococcal Septic Shock Trial Study Group (1999). Randomized, placebo-controlled trial of HA-1A, a human monoclonal antibody to endotoxin, in children with meningococcal septic shock. Clin. Infect. Dis. *28*, 770–777.
12. Németh, K., Leelahavanichkul, A., Yuen, P.S., Mayer, B., Parmelee, A., Doi, K., Robey, P.G., Leelahavanichkul, K., Koller, B.H., Brown, J.M., et al. (2009). Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. Nat. Med. *15*, 42–49.
13. Wannemuehler, T.J., Manukyan, M.C., Brewster, B.D., Rouch, J., Poynter, J.A., Wang, Y., and Meldrum, D.R. (2012). Advances in mesenchymal stem cell research in sepsis. J. Surg. Res. *173*, 113–126.
14. Laroye, C., Gibot, S., Reppel, L., and Bensoussan, D. (2017). Concise Review: Mesenchymal Stromal/Stem Cells: A New Treatment for Sepsis and Septic Shock? Stem Cells *35*, 2331–2339.
15. Krasnodembskaya, A., Samarani, G., Song, Y., Zhuo, H., Su, X., Lee, J.W., Gupta, N., Petrini, M., and Matthay, M.A. (2012). Human mesenchymal stem cells reduce mortality and bacteremia in gram-negative sepsis in mice in part by enhancing the phagocytic activity of blood monocytes. Am. J. Physiol. Lung Cell. Mol. Physiol. *302*, L1003–L1013.
16. He, X., Ai, S., Guo, W., Yang, Y., Wang, Z., Jiang, D., and Xu, X. (2018). Umbilical cord-derived mesenchymal stem (stromal) cells for treatment of severe sepsis: aphase 1 clinical trial. Transl. Res. *199*, 52–61.
17. Krasnodembskaya, A., Song, Y., Fang, X., Gupta, N., Serikov, V., Lee, J.W., and Matthay, M.A. (2010). Antibacterial effect of human mesenchymal stem cells is mediated in part from secretion of the antimicrobial peptide LL-37. Stem Cells *28*, 2229–2238.
18. Vandamme, D., Landuyt, B., Luyten, W., and Schoofs, L. (2012). A comprehensive summary of LL-37, the factotum human cathelicidin peptide. Cell. Immunol. *280*, 22–35.
19. Bülow, E., Gullberg, U., and Olsson, I. (2000). Structural requirements for intracellular processing and sorting of bactericidal/permeability-increasing protein (BPI): comparison with lipopolysaccharide-binding protein. J. Leukoc. Biol. *68*, 669–678.
20. Elsbach, P., and Weiss, J. (1998). Role of the bactericidal/permeability-increasing protein in host defence. Curr. Opin. Immunol. *10*, 45–49.
21. Giroir, B.P., Scannon, P.J., and Levin, M. (2001). Bactericidal/permeability-increasing protein–lessons learned from the phase III, randomized, clinical trial of rBPI21 for adjunctive treatment of children with severe meningococcemia. Crit. Care Med. *29* (7, Suppl), S130–S135.
22. Levin, M., Quint, P.A., Goldstein, B., Barton, P., Bradley, J.S., Shemie, S.D., Yeh, T., Kim, S.S., Cafaro, D.P., Scannon, P.J., and Giroir, B.P. (2000). Recombinant bactericidal/permeability-increasing protein (rBPI21) as adjunctive treatment for children with severe meningococcal sepsis: a randomised trial. rBPI21 Meningococcal Sepsis Study Group. Lancet *356*, 961–967.
23. Alexander, S., Bramson, J., Foley, R., and Xing, Z. (2004). Protection from endotoxemia by adenoviral-mediated gene transfer of human bactericidal/permeability-increasing protein. Blood *103*, 93–99.
24. Ren, G., Su, J., Zhang, L., Zhao, X., Ling, W., L'huillie, A., Zhang, J., Lu, Y., Roberts, A.I., Ji, W., et al. (2009). Species variation in the mechanisms of mesenchymal stem cell-mediated immunosuppression. Stem Cells *27*, 1954–1962.
25. Lewis, D.H., Chan, D.L., Pinheiro, D., Armitage-Chan, E., and Garden, O.A. (2012). The immunopathology of sepsis: pathogen recognition, systemic inflammation, the compensatory anti-inflammatory response, and regulatory T cells. J. Vet. Intern. Med. *26*, 457–482.
26. Novotny, A.R., Reim, D., Assfalg, V., Altmayr, F., Friess, H.M., Emmanuel, K., and Holzmann, B. (2012). Mixed antagonist response and sepsis severity-dependent dysbalance of pro- and anti-inflammatory responses at the onset of postoperative sepsis. Immunobiology *217*, 616–621.
27. Liles, W.C., Matthay, M.A., Dos Santos, C.C., Weiss, D.J., and Stewart, D.J. (2014). Mesenchymal stromal (stem) cell therapy: an emerging immunomodulatory strategy for the adjunctive treatment of sepsis. Am. J. Respir. Crit. Care Med. *189*, 363–364.
28. Alcayaga-Miranda, F., Cuenca, J., Martin, A., Contreras, L., Figueroa, F.E., and Khoury, M. (2015). Combination therapy of menstrual derived mesenchymal stem cells and antibiotics ameliorates survival in sepsis. Stem Cell Res. Ther. *6*, 199.
29. Saeedi, P., Halabian, R., and Fooladi, A.A.I. (2019). Mesenchymal stem cells preconditioned by staphylococcal enterotoxin B enhance survival and bacterial clearance in murine sepsis model. Cytotherapy *21*, 41–53.
30. Matthay, M.A., Pati, S., and Lee, J.W. (2017). Concise Review: Mesenchymal Stem (Stromal) Cells: Biology and Preclinical Evidence for Therapeutic Potential for Organ Dysfunction Following Trauma or Sepsis. Stem Cells *35*, 316–324.
31. Xu, J., Woods, C.R., Mora, A.L., Joodi, R., Brigham, K.L., Iyer, S., and Rojas, M. (2007). Prevention of endotoxin-induced systemic response by bone marrow-derived mesenchymal stem cells in mice. Am. J. Physiol. Lung Cell. Mol. Physiol. *293*, L131–L141.
32. Elman, J.S., Li, M., Wang, F., Gimble, J.M., and Parekkadan, B. (2014). A comparison of adipose and bone marrow-derived mesenchymal stromal cell secreted factors in the treatment of systemic inflammation. J. Inflamm. (Lond.) *11*, 1.
33. Jin, H.J., Bae, Y.K., Kim, M., Kwon, S.J., Jeon, H.B., Choi, S.J., Kim, S.W., Yang, Y.S., Oh, W., and Chang, J.W. (2013). Comparative analysis of human mesenchymal stem cells from bone marrow, adipose tissue, and umbilical cord blood as sources of cell therapy. Int. J. Mol. Sci. *14*, 17986–18001.
34. Romanov, Y.A., Svintsitskaya, V.A., and Smirnov, V.N. (2003). Searching for alternative sources of postnatal human mesenchymal stem cells: candidate MSC-like cells from umbilical cord. Stem Cells *21*, 105–110.
35. Watson, N., Divers, R., Kedar, R., Mehindru, A., Mehindru, A., Borlongan, M.C., and Borlongan, C.V. (2015). Discarded Wharton jelly of the human umbilical cord: a viable source for mesenchymal stromal cells. Cytotherapy *17*, 18–24.
36. Srivastava, A., Casey, H., Johnson, N., Levy, O., and Malley, R. (2007). Recombinant bactericidal/permeability-increasing protein rBPI21 protects against pneumococcal disease. Infect. Immun. *75*, 342–349.
37. van der Does, A.M., Bergman, P., Agerberth, B., and Lindbom, L. (2012). Induction of the human cathelicidin LL-37 as a novel treatment against bacterial infections. J. Leukoc. Biol. *92*, 735–742.

38. Maude, S.L., Frey, N., Shaw, P.A., Aplenc, R., Barrett, D.M., Bunin, N.J., Chew, A., Gonzalez, V.E., Zheng, Z., Lacey, S.F., et al. (2014). Chimeric antigen receptor T cells for sustained remissions in leukemia. N. Engl. J. Med. *371*, 1507–1517.

39. Zangi, L., Margalit, R., Reich-Zeliger, S., Bachar-Lustig, E., Beilhack, A., Negrin, R., and Reisner, Y. (2009). Direct imaging of immune rejection and memory induction by allogeneic mesenchymal stromal cells. Stem Cells *27*, 2865–2874.

40. Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D.j., and Horwitz, E. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy *8*, 315–317.

41. Singer, M., Deutschman, C.S., Seymour, C.W., Shankar-Hari, M., Annane, D., Bauer, M., Bellomo, R., Bernard, G.R., Chiche, J.D., Coopersmith, C.M., et al. (2016). The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). JAMA *315*, 801–810.

42. Gotts, J.E., and Matthay, M.A. (2016). Sepsis: pathophysiology and clinical management. BMJ *353*, i1585.

43. Mei, S.H., Haitsma, J.J., Dos Santos, C.C., Deng, Y., Lai, P.F., Slutsky, A.S., Liles, W.C., and Stewart, D.J. (2010). Mesenchymal stem cells reduce inflammation while enhancing bacterial clearance and improving survival in sepsis. Am. J. Respir. Crit. Care Med. *182*, 1047–1057.

44. Cóndor, J.M., Rodrigues, C.E., Sousa Moreira, R.d., Canale, D., Volpini, R.A., Shimizu, M.H., Camara, N.O., Noronha, Ide.L., and Andrade, L. (2016). Treatment With Human Wharton's Jelly-Derived Mesenchymal Stem Cells Attenuates Sepsis-Induced Kidney Injury, Liver Injury, and Endothelial Dysfunction. Stem Cells Transl. Med. *5*, 1048–1057.

45. Rhodes, A., Evans, L.E., Alhazzani, W., Levy, M.M., Antonelli, M., Ferrer, R., Kumar, A., Sevransky, J.E., Sprung, C.L., Nunnally, M.E., et al. (2017). Surviving Sepsis Campaign: International Guidelines for Management of Sepsis and Septic Shock: 2016. Crit. Care Med. *45*, 486–552.

46. Aziz, M., Jacob, A., and Wang, P. (2014). Revisiting caspases in sepsis. Cell Death Dis. *5*, e1526.

47. Yagi, H., Soto-Gutierrez, A., Kitagawa, Y., Tilles, A.W., Tompkins, R.G., and Yarmush, M.L. (2010). Bone marrow mesenchymal stromal cells attenuate organ injury induced by LPS and burn. Cell Transplant. *19*, 823–830.

48. Liu, L., Zhao, X., Li, P., Zhao, G., Wang, Y., Hu, Y., and Hou, Y. (2012). A novel way to isolate MSCs from umbilical cords. Eur. J. Immunol. *42*, 2190–2193.

49. Zhu, M., He, X., Wang, X.H., Qiu, W., Xing, W., Guo, W., An, T.C., Ao, L.Q., Hu, X.T., Li, Z., et al. (2017). Complement C5a induces mesenchymal stem cell apoptosis during the progression of chronic diabetic complications. Diabetologia *60*, 1822–1833.

50. Lanitis, E., Poussin, M., Hagemann, I.S., Coukos, G., Sandaltzopoulos, R., Scholler, N., and Powell, D.J., Jr. (2012). Redirected antitumor activity of primary human lymphocytes transduced with a fully human anti-mesothelin chimeric receptor. Mol. Ther. *20*, 633–643.

51. Dai, C., Xiao, X., Li, D., Tun, S., Wang, Y., Velkov, T., and Tang, S. (2018). Chloroquine ameliorates carbon tetrachloride-induced acute liver injury in mice via the concomitant inhibition of inflammation and induction of apoptosis. Cell Death Dis. *9*, 1164.

52. Itoh, T., Obata, H., Murakami, S., Hamada, K., Kangawa, K., Kimura, H., and Nagaya, N. (2007). Adrenomedullin ameliorates lipopolysaccharide-induced acute lung injury in rats. Am. J. Physiol. Lung Cell. Mol. Physiol. *293*, L446–L452.

53. Sung, M.J., Kim, D.H., Jung, Y.J., Kang, K.P., Lee, A.S., Lee, S., Kim, W., Davaatseren, M., Hwang, J.T., Kim, H.J., et al. (2008). Genistein protects the kidney from cisplatin-induced injury. Kidney Int. *74*, 1538–1547.

54. Kobayashi, T., Masumoto, J., Tada, T., Nomiyama, T., Hongo, K., and Nakayama, J. (2007). Prognostic significance of the immunohistochemical staining of cleaved caspase-3, an activated form of caspase-3, in gliomas. Clin. Cancer Res. *13*, 3868–3874.

55. Wang, C.J., Zhou, Z.G., Holmqvist, A., Zhang, H., Li, Y., Adell, G., and Sun, X.F. (2009). Survivin expression quantified by Image Pro-Plus compared with visual assessment. Appl. Immunohistochem. Mol. Morphol. *17*, 530–535.