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Original research

Placental mesenchymal stem cells boost M2 alveolar over M1 bone marrow macrophages via IL-1 β in *Klebsiella*-mediated acute respiratory distress syndrome

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ABSTRACT

Rationale Acute respiratory distress syndrome (ARDS) is a lethal complication of severe bacterial pneumonia due to the inability to dampen overexuberant immune responses without compromising pathogen clearance. Both of these processes involve tissue-resident and bone marrow (BM)-recruited macrophage (M Φ) populations which can be polarised to have divergent functions. Surprisingly, despite the known immunomodulatory properties of mesenchymal stem cells (MSCs), simultaneous interactions with tissue-resident and recruited BMM Φ populations are largely unexplored.

Objectives We assessed the therapeutic use of human placental MSCs (PMSCs) in severe bacterial pneumonia with elucidation of the roles of resident alveolar M Φ s (AM Φ s) and BMM Φ s.

Methods We developed a lethal, murine pneumonia model using intratracheal infection of a clinically relevant *Klebsiella pneumoniae* (KP) strain with subsequent intravenous human PMSC treatment. Pulmonary AM Φ and recruited BMM Φ analyses, histological evaluation, bacterial clearance and mice survival were assessed. To elucidate the role of resident AM Φ s in improving outcome, we performed AM Φ depletion in the KP-pneumonia model with intratracheal clodronate pretreatment.

Measurements and main results Human PMSC treatment decreased tissue injury and improved survival of severe KP-pneumonia mice by decreasing the presence and function of recruited M1 BMM Φ while preserving M2 AM Φ s and enhancing their antibacterial functions. Interestingly, PMSC therapy failed to rescue AM Φ -depleted mice with KP pneumonia, and PMSC-secreted IL-1 β was identified as critical in increasing AM Φ antibacterial activities to significantly improve pathogen clearance—especially bacteraemia—and survival.

Conclusions Human PMSC treatment preferentially rescued resident M2 AM Φ s over recruited M1 BMM Φ s with overall M2 polarisation to improve KP-related ARDS survival.

INTRODUCTION

Acute respiratory distress syndrome (ARDS) is still a lethal complication of severe bacterial pneumonia, with *Klebsiella pneumoniae* (KP) among the

Key messages

What is already known on this topic

- ⇒ Severe bacterial pneumonia often results in acute respiratory distress syndrome (ARDS), a lethal complication due to overexuberant immune responses which cause tissue injury without effective pathogen clearance.
- ⇒ These processes involve tissue-resident alveolar macrophages (AM Φ) as well as recruited bone marrow (BM) M Φ s, both of which can be further polarised to have divergent functions.
- ⇒ However, no previous reports have studied human mesenchymal stem cell (MSC) interactions with these two different M Φ populations simultaneously in a clinically relevant model.

What this study adds

- ⇒ We studied the possible therapeutic role of human placental MSCs (PMSCs) in a murine severe bacterial pneumonia/ARDS model using a clinically derived *Klebsiella pneumoniae* (KP) strain, and found that human PMSC treatment preferentially rescued resident M2 AM Φ s over recruited M1 BMM Φ s with overall M2 polarisation of all M Φ s to improve severe KP pneumonia/ARDS survival.

How this study might affect research, practice and/or policy

- ⇒ Our findings demonstrate the distinct roles of different sources of M Φ populations and their polarisation in bacterial infection and recovery, as well as strongly implicate a potential therapeutic role for PMSCs in severe bacterial pneumonia/ARDS.

most common causative agents.¹ A Gram-negative bacterium with high multidrug resistance and over 80 serotypes, drug design and vaccine development has been difficult for KP.^{2,3} Among virulent KP members, the K2 serotype is the most studied since it can cause highly lethal bacterial pneumonia clinically.⁴ While previous reports demonstrated that augmenting the innate immune response is



effective,^{5,6} both bacteraemia and overexuberant inflammation damaged multiple organs/tissues.⁷ Blockage of acute infection mediators including tumour necrosis factor (TNF)- α muted organ/tissue damage but did not improve survival,⁸ showing that careful balancing of the bactericidal immune response against excessive inflammation is critical for disease resolution. Recent clinical trials using corticosteroids—arguably the most potent anti-inflammatory agent—to mitigate excessive host responses have not consistently reduced mortality,⁹ necessitating better strategies to combat severe bacterial pneumonia or acute respiratory distress syndrome (ARDS).

Multilineage mesenchymal stem cells (MSCs) have been isolated from diverse post-natal sources including the placenta, a temporary organ with fetal contributions which is ethically compliant and easily accessible.¹⁰ MSC sources including placental MSCs (PMSCs) not only have regenerative properties but also strong immunomodulation with therapeutic value.^{11–13} Surprisingly, despite well-established interactions of MSCs with adaptive and innate immune cells like T lymphocytes and natural killer cells, respectively,¹⁴ reports on MSC-macrophage (M Φ) interactions are relatively scarce. Moreover, there are almost no MSC studies in which different populations of M Φ s are simultaneously evaluated, despite the clear understanding currently that tissue-resident M Φ s—important in maintaining tissue/organ homeostasis—are functionally and developmentally distinct from bone marrow (BM)-derived M Φ s,¹⁵ which are among the most potent effector cells in host responses against pathogens. The lungs, being in constant interactions with environmental pathogens and toxins, has a resident M Φ population to maintain homeostasis: the alveolar M Φ (AM Φ), derived from the yolk sac.^{16,17} When local infection occurs, AM Φ s are the first in line to mount an immune response and recruit effector BMM Φ s for effective elimination of respiratory pathogens.¹⁵ Both pathogen clearance and tissue damage in bacterial ARDS have been linked to M Φ activity, but the existence of two populations of M Φ s during pulmonary infection—resident AM Φ s and recruited BMM Φ s—makes it difficult to resolve which population is responsible for tissue injury versus disease resolution. Furthermore, all M Φ s can undergo functional polarisation into classically activated or M1 M Φ s, crucial in fighting off invading pathogens, or alternatively activated or M2 M Φ s which are important in tissue repair.^{18,19} An imbalance in M Φ polarisation can influence disease outcome, ranging from successful resolution to persistent infection with tissue injury.¹⁷ Given the strong immunomodulatory properties of MSCs and the protean impact of M Φ populations in health and disease, we studied the possible therapeutic role of MSCs for severe bacterial pneumonia/ARDS in a murine disease model and the mechanisms involved using a clinically relevant and virulent KP strain.

METHODS

A detailed description of the methods is provided in the online supplemental information.

Cell culture

Human PMSCs were isolated from term placental tissue obtained after institutional board approval as previously reported, and cultured as previously reported.^{10,20}

Mouse model of KP-induced bacterial pneumonia and PMSC injection

A clinical KP strain of serotype 2 was isolated from patient sputum¹ for intratracheally inoculation into C57BL/6 mice at

a sublethal or lethal dose (5×10^6 or 5×10^7 CFUs, respectively) according to previous report.⁵ PMSCs (3×10^5 cells) or phosphate buffered saline (PBS) were intravenously injected 2 hours after KP injection. Mice were euthanised with CO₂ at 4 hours, with peripheral blood collected for evaluation of bacterial load and lungs collected for evaluation of bacterial load, M Φ analyses and histological evaluation.

Assessment of in vivo M Φ function

Single-cell suspensions derived from lung lobes were stained with antibodies recognising CD45, CD11b, F4/80, CD206, inducible nitric oxide synthase (iNOS), arginase-1 (Arg1), TNF- α (all from eBioscience, San Diego, California, USA), or 2',7'-dichlorofluorescein diacetate (DCFDA, Sigma-Aldrich, Missouri, USA) for 30 min according to manufacturer's instructions, then assessed by flow cytometry or analysed with t-distributed stochastic neighbor embedding (t-SNE)-based algorithm.^{20,21}

In vivo depletion of AM Φ s

C57BL/6J mice were intratracheally injected with 100 μ L of PBS or 15 mM clodronate (Cayman Chemical, Ann Arbor, Michigan, USA).²² After 48 hours, the frequency and absolute numbers of CD11b^{low}F4/80⁺ AM Φ s and CD11b^{high}F4/80⁺ BMM Φ s in lung tissues were assessed by flow cytometry.

Statistics

For comparisons between two groups, non-parametric Mann-Whitney test was used, while for comparisons between multiple groups, non-parametric analysis of variance was used followed by Dunn's test. Comparisons of survival curves were made using Kaplan-Meier method with log-rank tests. A value of $p < 0.05$ was considered statistically significant. Analyses were performed using GraphPad Prism software (California, USA), and data are shown as mean \pm SD.

RESULTS

Pulmonary KP infection dramatically decreases resident AM Φ s while increasing recruited BMM Φ s in the lungs

To assess whether M Φ s are critically involved during K2 KP pneumonia, we first performed bioinformatics analysis which demonstrated broad transcriptomic changes in lung tissue from control versus KP-infected mice (figure 1A), and found M Φ -related pathways to be highly enriched (figure 1B). To further delineate immune responses at the cellular level, we performed analyses on immune cells at 2 (h2) and 4 hours (h4) in lungs infected with an LD₅₀ of a clinically isolated serotype K2 KP, revealing a dramatic shift in the pattern of lung-isolated M Φ s at h2 versus h4 post-infection in comparison to control/PBS-injected mice by t-SNE (figure 1C), with significant increases at h4 post-infection in a population designated G1 which is identified as CD11b^{high}M Φ s (figure 1D–F). A population designated G2 and identified as CD11b^{low}M Φ s was significantly decreased at h4 post-infection (figure 1E,F). These M Φ populations can be further differentiated on CD11b^{high} (G1) and CD11b^{low} (G2) expression as BMM Φ s and AM Φ s, respectively.¹⁶ Flow cytometric analyses confirmed these findings, showing an increase in the frequency as well as absolute number of BMM Φ s over AM Φ s at h4 (figure 1G–I). These results demonstrate that K2 KP infection rapidly decreased resident AM Φ s while gradually increasing recruited BMM Φ s to cause an imbalance in pulmonary M Φ populations, which possibly contribute to subsequent ARDS.

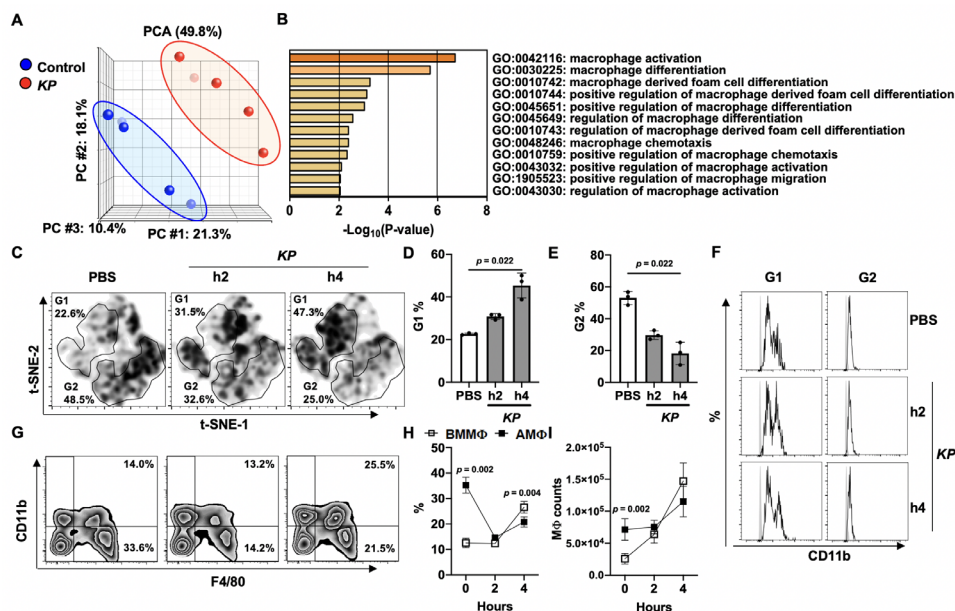


Figure 1 Pulmonary infection of a clinically isolated *Klebsiella pneumonia* (KP)-serotype K2 dramatically decreases resident alveolar macrophages (AMΦs) while increasing recruited bone marrow (BM) MΦs in the lungs. (A) Principal component analysis (PCA) of transcriptomic profiles (National Center for Biotechnology Information-Gene Expression Omnibus or NCBI-GEO database: GSE121970) of KP-infected (n=5) compared to uninfected (n=5) murine lung tissues. The first principal component (PC1) accounts for the largest data variance at 21.3%, while data variance was 18.1% for PC2, and 10.4% for PC3. (B) Metascape pathway analysis for MΦ-related pathways using transcriptomic data of KP-infected murine lung tissue compared with uninfected control, with pathways coloured according to p values. (C) Representative data of t-distributed stochastic neighbor embedding (t-SNE) plots for analyses of changes in MΦs harvested from lung tissues of C57BL/6J mice after intratracheal injection of phosphate buffered saline (PBS; no infection), or infection with sublethal dose (5×10^6 CFUs) of K2 KP for 2 or 4 hours (h2 or h4). Single-cell suspensions derived from lung lobes were stained with anti-CD45, anti-CD11b and anti-F4/80 for flow cytometric analysis, with gating on CD45⁺F4/80⁺ cells for analysis of MΦ populations using tSNE-based algorithm. G1 and G2 were then manually gated on the t-SNE plot by signal profiles. (D, E) Pooled data for G1 and G2 as defined in (C) (n=3 for each group). (F) Histogram graph for CD11b expression levels in G1 representing CD11b^{high} MΦs or and G2 representing CD11b^{low} MΦs as defined in (C). (G) Representative data for frequency analysis of recruited BMMΦs and resident AMΦs in lung lobes taken from uninfected (PBS) and sublethally KP-infected mice sacrificed at h2 and h4 as assessed by flow cytometry. Immune cells harvested from lung tissues were stained with anti-CD45, anti-CD11b and anti-F4/80. Gating for CD45⁺ cells was first performed, with subsequent frequency analysis for CD11b^{high}F4/80⁺ and CD11b^{low}F4/80⁺ to identify for BMMΦs and AMΦs, respectively. (H, I) Pooled data for frequency and absolute numbers, respectively, of BMMΦs (□) and AMΦs (■) in lung lobes as assessed by flow cytometry, with gating for CD45⁺ cells first and then analysis for cell numbers of CD11b^{high}F4/80⁺ and CD11b^{low}F4/80⁺ identified as BMMΦs and AMΦs, respectively (n=6 for each group). Data are shown as mean±SD.

Human PMSCs reduce recruitment of BMMΦs while preserving AMΦs in KP-infected lungs

To elucidate the mechanism underlying the interaction of recruited BMMΦs and resident AMΦs in KP pneumonia, we first used the Molecular Activation Prediction (MAP) tool from Ingenuity Pathway Analysis (IPA) which revealed that during pulmonary KP infection, for the process of BMMΦ migration, many inflammatory cytokines including CXCL3, CCL2 and TNF- α were involved, while for the process of AMΦ quantity, other cytokines including CSF1, CXCL17 and interleukin (IL)-36 γ were highly activated (figure 2A). Among these predicted cytokines, TNF- α has been reported as critical in causing pneumonia exacerbation by several infectious agents including KP.^{23–25} We therefore performed transcriptomic analyses in TNF- α -treated BMMΦs and found that multiple inflammatory pathways involving either innate or adaptive immunity were activated (figure 2B). To validate the strong presence of TNF- α during in vivo KP pneumonia and assess whether PMSC therapy could limit the highly inflammatory process represented by this cytokine, we detected TNF- α secretion in harvested lung immune cells from uninfected or KP-infected mice 2 hours post-infection which were then co-cultured ex vivo with PMSCs for another 2 hours (figure 2C). We found that PMSCs significantly decreased levels of KP-induced

TNF- α even at this early time point post-infection; interestingly, this early post-infection time point is prior to recruited BMMΦs arriving in the lungs (figure 1H,I). We then further assessed PMSC interactions with both AMΦ and BMMΦ populations (figure 2D), and found both percentages (figure 2E) and absolute counts (figure 2F) of BMMΦs were significantly increased whether treated with PBS or PMSCs after KP infection, while a slight but non-significant decrease in absolute BMMΦ numbers after PMSC treatment compared with after PBS treatment in infected mice was seen. Moreover, while BMMΦ expression of CD11b—a critical adhesion molecule involved in recruited BMMΦ tissue migration²⁶—was significantly increased after KP infection, PMSC but not PBS treatment resulted in a significant decrease back to nearly baseline levels (figure 2G,H). Furthermore, after KP infection, AMΦ percentages (figure 2I) were significantly decreased when only PBS was given; PMSC treatment, on the other hand, significantly rescued percentages (figure 2J). The supportive effects of PMSCs on AMΦs during KP infection were also seen when absolute cell counts were assessed (figure 2J), in which PMSC treatment nearly doubled the average number of AMΦs compared with PBS treatment. Responses of BMMΦ and AMΦ absolute numbers were further confirmed in CD11c⁺SIGLEC-F[−] and CD11c⁺SIGLEC-F⁺ cells

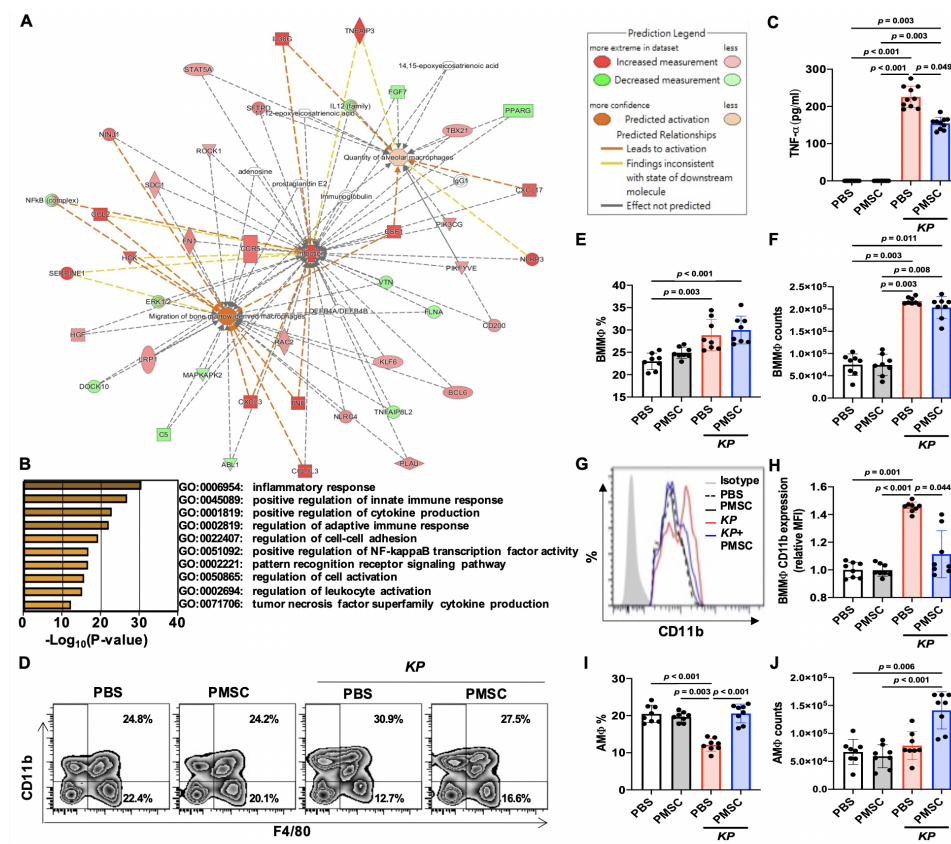


Figure 2 Human placenta mesenchymal stem cells (PMSCs) prevent bone marrow MΦ (BMMΦ) recruitment but preserve alveolar MΦ (AMΦ) population in *Klebsiella pneumoniae* (KP)-infected lungs. (A) Pathway analyses of transcriptomic profiles obtained from KP-infected versus uninfected lung lobes (NCBI-GEO database: GSE121970) as performed with Molecular Activation Prediction (MAP) tool in Ingenuity Pathway Analysis (IPA), for prediction of mechanisms involved in migration of recruited BMMΦs or quantity of resident AMΦs during pulmonary KP infection. IPA showed regulatory relationships between downregulated (green) and upregulated (red) proteins; the MAP tool showed that 'migration of BMMΦs' and 'quantity of AMΦs' are upregulated (orange) through positively regulated downstream molecules (orange lines) or inconsistent findings on some mediators (yellow lines) after occurrence of KP pneumonia (red). (B) Enrichment of immune-related pathways as assessed in transcriptomic data of tumour necrosis factor (TNF)-α-treated BMMΦs versus control BMMΦs (NCBI-GEO database: GSE160163) using Metascape analysis and coloured by p values. (C) Assessment of ex vivo lung inflammation with detection of TNF-α in non-infected and KP-infected lung tissues after phosphate buffered saline (PBS) and PMSC treatment. Mice were intratracheally infected with 5×10^6 CFUs of K2 KP for 2 hours, and then sacrificed for extraction of lung immune cells which were ex vivo co-cultured with human PMSCs at the ratio of 10:1 for 2 hours further and supernatants collected for TNF-α detection (n=10 for each group). (D) Representative data for frequency analysis of recruited BMMΦs and resident AMΦs in lung lobes as assessed with flow cytometry. C57BL/6J mice were intratracheally injected with 5×10^6 CFUs of K2 KP followed by intravenous administration of PBS or 3×10^5 PMSCs 2 hours later. Single-cell suspensions harvested from lung tissues of non-infected mice as well as infected mice injected with PBS or PMSCs were stained with anti-CD45, anti-CD11b and anti-F4/80 for flow cytometric analysis. Gating for CD45⁺ cells was first performed, with subsequent frequency analysis for CD11b^{high}F4/80⁺ BMMΦs and CD11b^{low}F4/80⁺ AMΦs. (E, F) Pooled data for analyses of frequency and absolute counts of recruited BMMΦs in lung lobes of each experimental group, respectively (n=8 for each group). (G, representative data; H, pooled data) Relative CD11b expression levels of recruited BMMΦs in lung lobes of each experimental group by flow cytometric analysis (n=8 for each group). (I, J) Pooled data for analyses of frequency and absolute number of resident AMΦs in lung lobes of each experimental group, respectively (n=8 for each group). Data are shown as mean±SD.

of CD11b^{high}F4/80⁺ and CD11b^{low}F4/80⁺ MΦs, respectively (online supplemental figure 1). These results suggest that PMSC administration can prevent recruitment and function of BMMΦs—likely through downregulation of KP-induced TNF-α—while significantly preserving resident AMΦs.

Human PMSCs decrease pulmonary inflammation and tissue injury, as well as significantly increase survival of mice with KP pneumonia

To determine PMSC therapeutic effects on bacterial clearance and tissue injury in KP pneumonia, we first assessed bacterial load and found that PMSC treatment significantly reduced bacterial numbers in infected lungs and decreased incidence

of bacteraemia in LD₅₀-infected mice at 4 hours (figure 3A,B). PMSCs also significantly downregulated levels of KP-induced TNF-α within lung tissues (online supplemental figure 2), suggesting TNF-α-mediated immunity was not critical for bacterial clearance after PMSC administrated. We then assessed lung tissues of uninfected and infected mice 4 hours after LD₅₀ infection histologically (figure 3C). H&E staining of lung tissue sections showed that KP infection increased immune cell infiltration (figure 3C-c), oedematous changes (figure 3C-g) and bronchial epithelium hyperplasia (figure 3C-k), which collectively indicated significant tissue injury similar to ARDS (figure 3D). These changes were reduced (figure 3C-d,h,l) after PMSC treatment. To confirm the degree of inflammatory infiltrates, we also

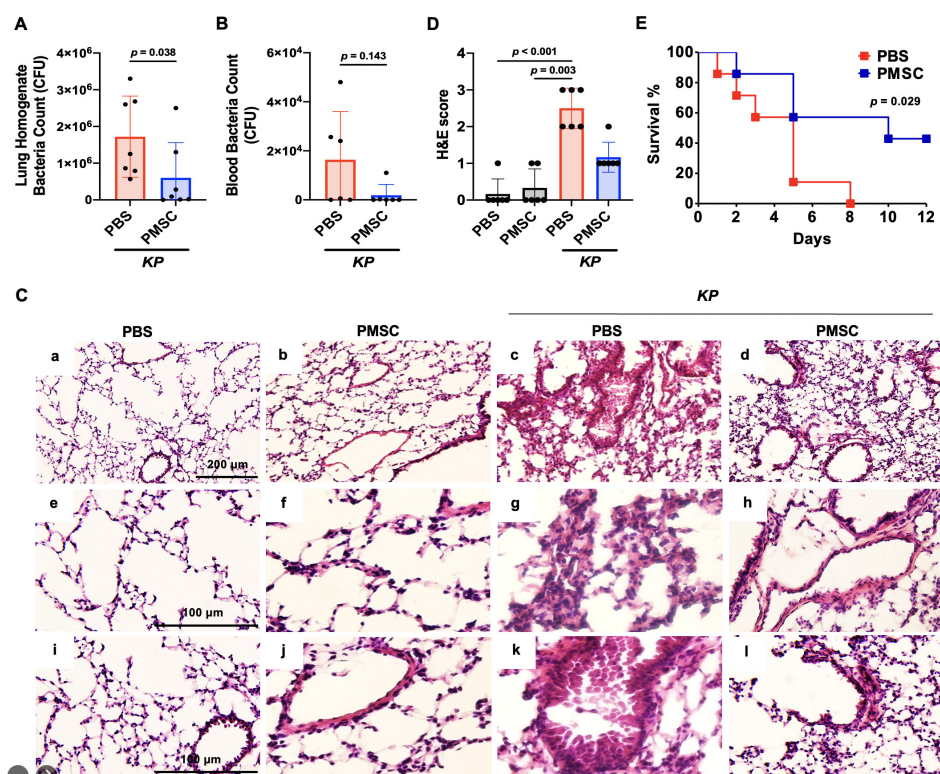


Figure 3 Human placental mesenchymal stem cells (PMSCs) decrease pulmonary inflammation and tissue injury, as well as significantly increase survival of mice with *Klebsiella pneumoniae* (KP) pneumonia. (A, B) Assessment of bacterial load within lung homogenate (A; n=7 for each group) and peripheral blood (B; n=6 for each group). Mice were intratracheally injected with a 5×10^6 CFUs of K2 KP followed by intravenous administration of phosphate buffered saline (PBS) or 3×10^5 PMSCs 2 hours later, with lung lobes excised and peripheral blood collected for CFU calculation. (C, representative data; D, pooled data) H&E staining of histological sections of lung tissues from KP-infected mice after PBS or PMSC treatment (n=6 for each group). Upper panel (a–d): scale bar, 200 μ m. Middle (e–h) and bottom panels (i–l), magnifications of lung parenchyma and airways, respectively: scale bar, 100 μ m. (E) Kaplan-Meier survival analysis of infected mice treated with or without PMSC treatment. Wild-type C57BL/6J mice were intratracheally injected with a lethal dose (5×10^7 CFUs) of K2 KP followed by intravenous administration of PBS or 3×10^5 PMSCs 2 hours later. Survival was observed for 12 days (n=7 for each group). Data are shown as mean \pm SD.

collected whole lungs of non-infected and infected mice after PBS or PMSC treatment for analysis of white blood cell (WBC) frequency and cell numbers at 4 hours post-infection. Pulmonary WBC frequency was relatively unchanged 4 hours post-infection (online supplemental figure 3A,B), but absolute numbers were significantly increased, which after PMSC administration was partially reversed (online supplemental figure 3C). To assess whether PMSCs are therapeutic for KP-induced pneumonia/ARDS, intravenously PBS or PMSCs was injected 2 hours after LD₁₀₀ KP infection, and we found that PMSC administration significantly improved KP-infected mice survival rates from 0% to 42.86% (figure 3E). Collectively, these findings demonstrate that PMSC intravenous administration decreased bacterial load, local inflammation and lung tissue injury, to result in significantly improved overall survival of mice with K2 KP pneumonia.

Human PMSCs preserve resident AMΦs over BMMΦs and drive the overall milieu to an M2 immunomodulatory phenotype in mice with KP pneumonia

To assess whether PMSCs can affect polarisation of BMMΦs and/or AMΦs, we first analysed the functional M1 and M2 polarisation markers iNOS and Arg1, respectively. After KP infection, iNOS expression (figure 4A,B) was significantly increased but Arg1 expression (figure 4C,D) was significantly decreased in BMMΦs with PBS treatment when compared with uninfected

groups; interestingly, expression of both markers was significantly reversed with PMSC treatment. For AMΦs, iNOS expression levels were similar in both uninfected and infected mice (figure 4E,F); however, Arg1 expression was slightly decreased in infected mice with PBS treatment when compared with uninfected groups although non-significantly, but could be significantly reversed with PMSC treatment (figure 4G,H).

To further delineate whether PMSCs can modulate the polarisation of both BMMΦs and AMΦs simultaneously, we first performed t-SNE analyses to visualise the complex changes. Concatenation of all experimental groups demonstrated that the majority of recruited BMMΦs are of the M1 phenotype (identified as CD206^{low}), whereas the majority of resident AMΦs are of the M2 phenotype (identified as CD206^{high}) (figure 5A). Visualisation of individual experimental groups shows that after KP infection, M1 BMMΦs were more prominent, whereas M2 AMΦs were less prominent, with PMSC treatment after KP infection rescuing M2 AMΦ presence (figure 5B). Confirmation with flow cytometric analyses (figure 5C, representative data) demonstrated that both percentages (figure 5D) and counts (figure 5E) of M1 BMMΦs treated with PBS were significantly increased from uninfected levels which were reversed by PMSC treatment. However, when compared with uninfected groups, M2 BMMΦs percentages (figure 5F) were significantly decreased to similar levels in KP-infected mice whether treated with PBS or

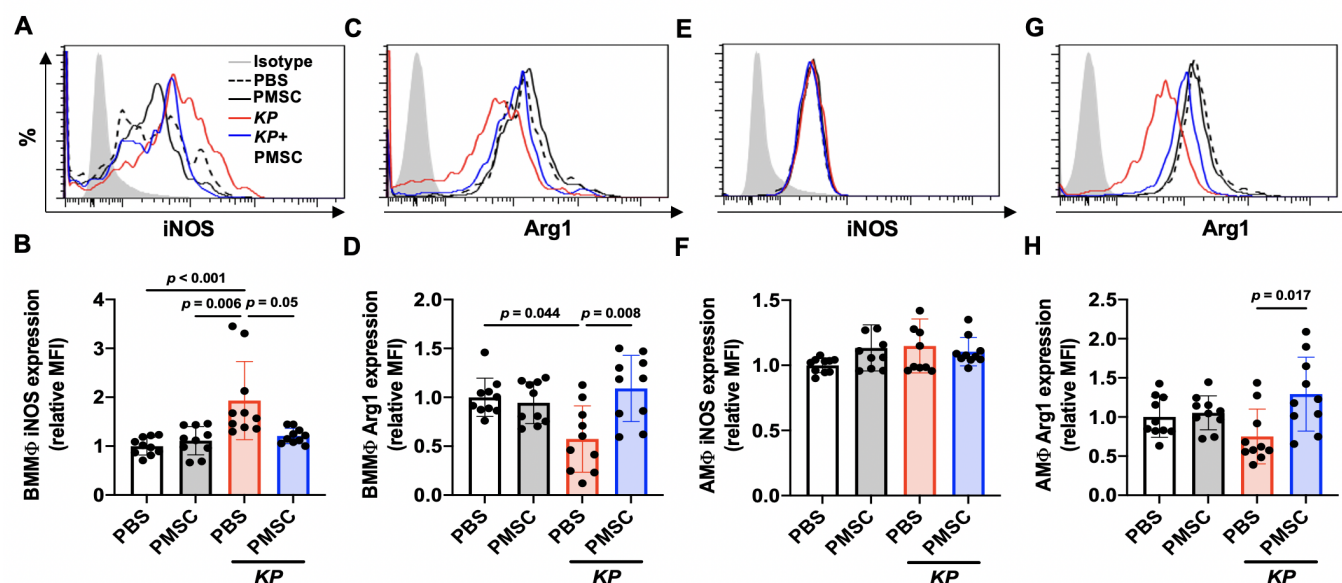


Figure 4 Human PMSCs modulate both bone marrow MΦs (BMMΦs) and alveolar MΦs (AMΦs) to M2 polarisation in mice with *Klebsiella pneumoniae* (KP) pneumonia. (A, representative data; B, pooled data) Relative inducible nitric oxide synthetase (iNOS) expression levels of CD11b^{high}F4/80⁺ BMMΦs in lung lobes of each experimental group as assessed by flow cytometry (n=10 for each group). (C, representative data; D, pooled data) Relative arginase-1 (Arg1) expression levels of CD11b^{high}F4/80⁺ BMMΦs in lung lobes of each experimental group as assessed by flow cytometry (n=10 for each group). (E, representative data; F, pooled data) Relative iNOS expression levels of CD11b^{low}F4/80⁺ AMΦs in lung lobes of each experimental group as assessed by flow cytometry (n=10 for each group). (G, representative data; H, pooled data) Relative Arg1 expression levels of CD11b^{low}F4/80⁺ AMΦs in lung lobes of each experimental group as assessed by flow cytometry (n=10 for each group). Data are shown as mean±SD.

PMSCs, while absolute counts (figure 5G) in infected mice were significantly increased only with treatment of PMSCs but not PBS. When we turned to evaluate polarised subpopulations of AMΦs, we found that both percentages (figure 5H) and absolute counts (figure 5I) of M1 AMΦs were significantly increased from uninfected levels whether treated with PBS or PMSCs. However, PBS treatment significantly decreased M2 AMΦs percentages (figure 5J), while absolute counts (figure 5K) were similar to those in uninfected mice; PMSC treatment in infected mice, on the other hand, not only significantly reversed M2 AMΦ percentages as well as increased absolute counts compared with levels in uninfected mice. Since M1 MΦs are also an important source of TNF-α, we next assessed whether PMSCs can modulate TNF-α expression levels in M1 subpopulation of both BMMΦs and AMΦs. We found that in uninfected mice, treatment with PBS or PMSCs did not change TNF-α levels significantly in M1 BMMΦs, while in infected mice, TNF-α levels in M1 BMMΦs was increased with PBS treatment but significantly reversed with PMSC treatment (figure 5L,M). Interestingly, there were no significant changes in TNF-α levels of M1 AMΦs between uninfected and infected mice regardless of PBS or PMSC treatment (figure 5N,O). Collectively, PMSC treatment can modulate both BMMΦs and AMΦs towards a more M2, tolerogenic response in pulmonary KP infection with particular preservation of M2 AMΦs.

Human PMSCs enhance multiple antibacterial functions in AMΦs but not BMMΦs to significantly improve bacterial clearance and survival in mice with KP pneumonia which were abrogated after AMΦ depletion

To investigate how the prominent immunomodulation of BMMΦs and AMΦs by PMSCs impact disease outcome, we first assessed two classical MΦ antibacterial functions in both

MΦ populations: bacterial phagocytosis and reactive oxygen species (ROS) production. In BMMΦs, co-culture with PMSCs surprisingly decreased in vitro bacterial phagocytosis capacity significantly (figure 6A,B), while in AMΦs, PMSC co-culture significantly increased this capacity (figure 6C,D). To assess whether PMSCs can modulate the production of antibacterial effector molecules from the two MΦ populations in vivo, we measured ROS levels in AMΦs and BMMΦs harvested from lung tissues of uninfected and KP-infected mice. ROS production by BMMΦs treated with PBS or PMSCs was similar in uninfected mice, with production rising significantly after KP infection with PBS treatment which could be partially reversed when PMSCs were administered (figure 6E,F). For AMΦs, ROS production was also similar in uninfected mice treated with PBS or PMSCs, with levels significantly increased in PBS-treated infected mice, which were further increased in PMSC-treated infected mice compared with those in uninfected groups (figure 6G,H). Moreover, we concurrently assessed phagocytosis and ROS production in both M1 and M2 BMMΦs and AMΦs, and found that PMSCs suppressed both antibacterial functions in M1 BMMΦs while boosting these functions in M2 AMΦs (online supplemental figure 4). We also excluded that PMSCs have direct antibacterial properties, finding that PMSCs does not affect KP growth in vitro or virulence gene expression in vivo (online supplemental figure 5). These findings collectively demonstrate that during an active bacterial infection, PMSCs modulate BMMΦs and AMΦs in divergent directions: decreasing effector antibacterial functions in BMMΦs while increasing similar functions in AMΦs.

To assess whether PMSC preferential enhancement of AMΦ antibacterial functions was critical to improving disease outcome, we depleted AMΦs but not BMMΦs prior to KP infection using intratracheal injection of clodronate (figure 6I). In uninfected mice, intratracheal clodronate did

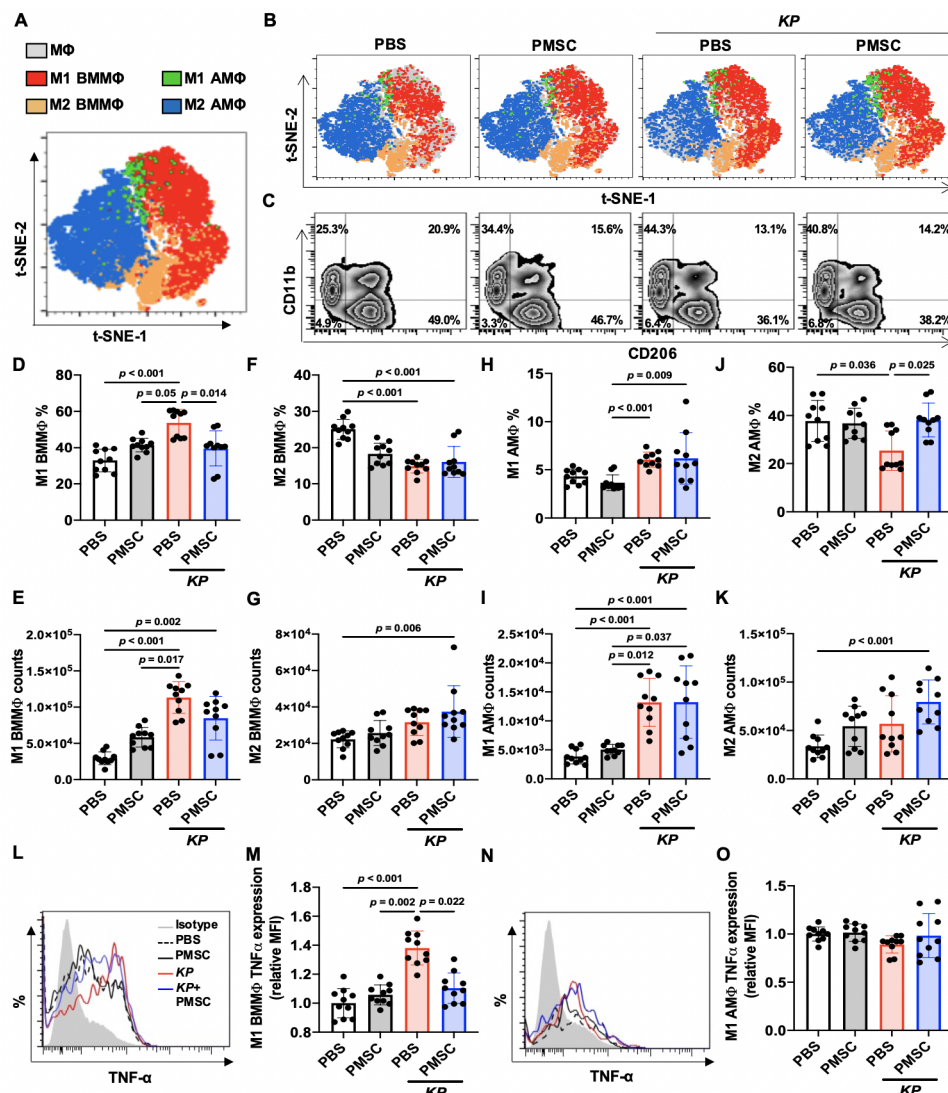


Figure 5 Human placental mesenchymal stem cells (PMSCs) reduce the population and function of M1 bone marrow MΦs (BMMΦs) while preserving M2 alveolar MΦs (AMΦ) population in mice with *Klebsiella pneumoniae* (KP) pneumonia. (A, pooled data; B, representative data) Population analyses for M1 and M2 BMMΦs as well as M1 and M2 AMΦs with PhenoGraft on the MΦ-based t-distributed stochastic neighbor embedding (t-SNE) map. Single-cell suspensions harvested from lung tissues of non-infected mice as well as infected mice injected with phosphate buffered saline (PBS) or PMSCs were stained with anti-CD45, anti-CD11b, anti-F4/80 and anti-CD206, assessed by flow cytometry and analysed with t-SNE-based algorithm. Gating for CD45⁺ cells was first performed, and then CD206⁺CD11b^{high}F4/80⁺ M1 BMMΦs and CD206⁺CD11b^{high}F4/80⁺ M2 BMMΦs as well as CD206⁺CD11b^{low}F4/80⁺ M1 AMΦs and CD206⁺CD11b^{low}F4/80⁺ M2 AMΦs were gated on the two t-SNE dimensions. (C) Representative data for frequency analyses of M1 and M2 populations in BMMΦs or AMΦs from lung lobes as assessed with flow cytometry. Gating for CD45⁺F4/80⁺ cells was first performed, with subsequent analyses for CD11b and CD206 signals. (D, E) Pooled data for analyses of frequency and absolute number, respectively, of CD206⁺CD11b^{high}F4/80⁺ M1 BMMΦs (n=10 for each group). (F, G) Pooled data for analyses of frequency and absolute number, respectively, of CD206⁺CD11b^{high}F4/80⁺ M2 BMMΦs (n=10 for each group). (H, I) Pooled data for analyses of frequency and absolute number, respectively, of CD206⁺CD11b^{low}F4/80⁺ M1 AMΦs (n=10 for each group). (J, K) Pooled data for analyses of frequency and absolute number, respectively, of CD206⁺CD11b^{low}F4/80⁺ M2 AMΦs (n=10 for each group). (L, representative data; M, pooled data) Relative TNF-α expression levels of M1 BMMΦs as assessed in CD45⁺CD11b^{high}F4/80⁺CD206⁺. Single-cell suspensions harvested from lung tissues, followed by intracellular staining of tumour necrosis factor (TNF)-α (n=10 for each group). (N, representative data; O, pooled data) Relative TNF-α expression levels of M1 AMΦs as assessed in CD45⁺CD11b^{low}F4/80⁺CD206⁺ cells harvested from lung tissues, followed by intracellular staining of TNF-α (n=10 for each group). Data are shown as mean±SD.

not alter BMMΦ percentages or absolute counts (figure 6J,K), whereas AMΦ percentages and counts were sharply decreased (figure 6L,M), demonstrating a fivefold decrease specifically of AMΦs without affecting BMMΦs. Using this model, we then infected mice with LD₅₀ of KP with subsequent PBS or PMSC treatment. We found that in KP-infected mice after AMΦ depletion, PMSC treatment did not significantly

improve pulmonary bacterial clearance compared with PBS treatment (figure 6N). More importantly, PMSC treatment in AMΦ-depleted mice infected with sublethal KP dose not only failed to rescue mice but actually worsened the survival rate (figure 6O). These findings demonstrate that the therapeutic effects of PMSCs in severe KP pneumonia were dependent on AMΦs and their antibacterial capacities.

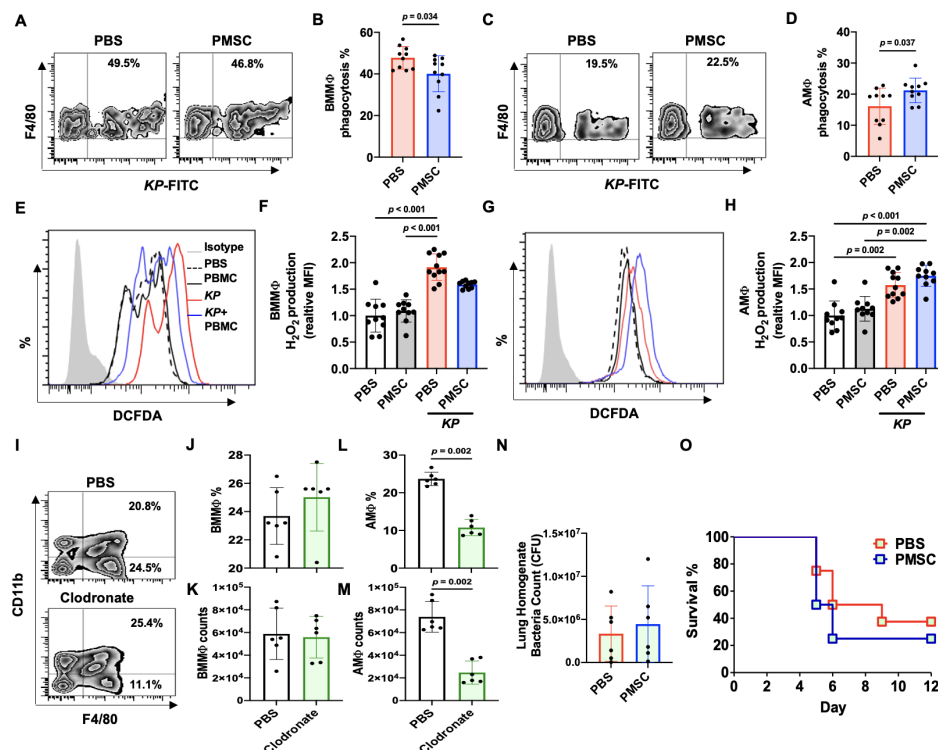
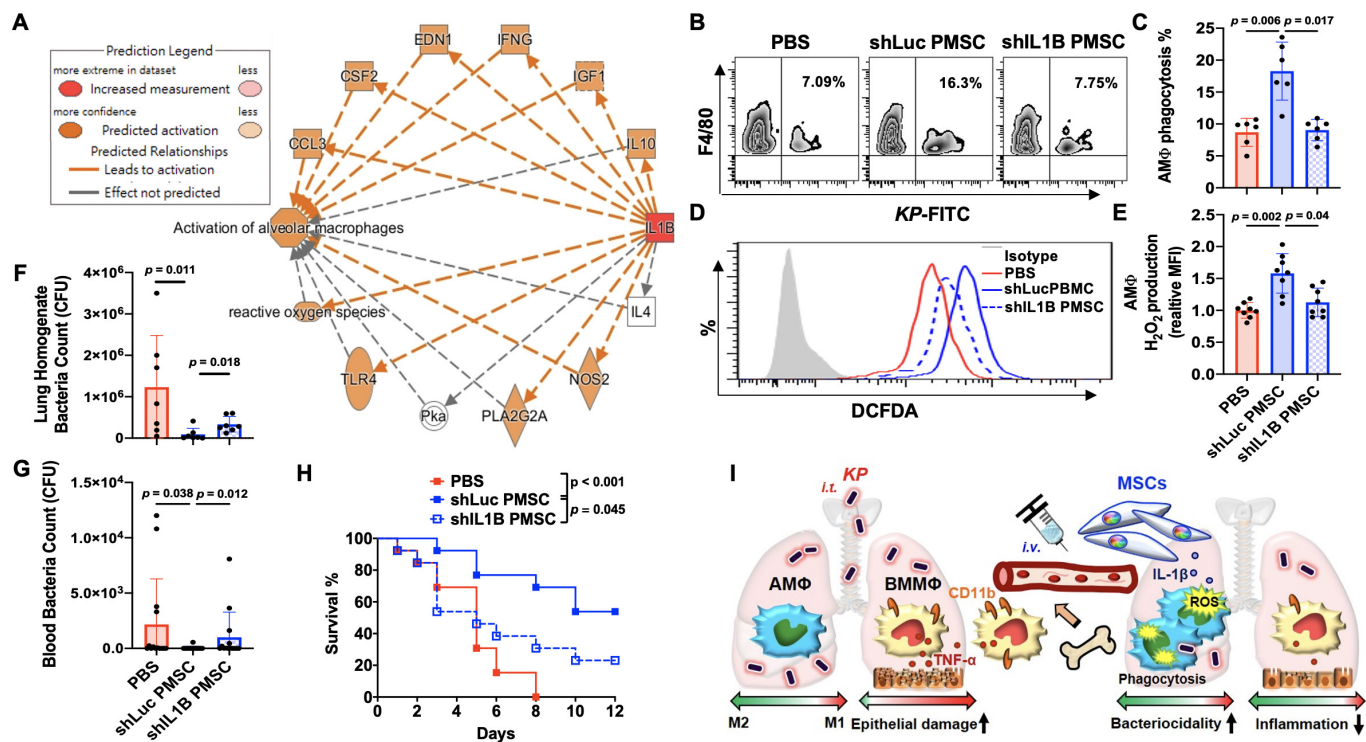


Figure 6 Human placental mesenchymal stem cells (PMSCs) enhanced multiple antibacterial functions in alveolar MΦs (AMΦs) but not bone marrow MΦs (BMMΦs) to significantly improve bacterial clearance and survival in mice with *Klebsiella pneumoniae* (KP) pneumonia which were abrogated after AMΦ depletion. (A, representative data; B, pooled data) The capability of KP phagocytosis in BMMΦs harvested from lung tissues co-cultured in phosphate buffered saline (PBS) or with PMSCs (n=10 for each group) for 2 hours as assessed with subsequent addition of FITC-labelled KP for 30 min at 37°C. Phagocytic capability of BMMΦs was measured by detecting for the frequency of FITC⁺ cells in CD11b^{high}F4/80⁺. (C, representative data; D, pooled data) The capability of KP phagocytosis in AMΦs harvested from lung tissues co-cultured in PBS or with PMSCs (n=10 for each group) for 2 hours as assessed with subsequent addition of FITC-labelled KP for 30 min at 37°C. Phagocytic capability of AMΦs was measured by detecting for the frequency of FITC⁺ cells in CD11b^{low}F4/80⁺. (E, representative data; F, pooled data) Assessment of BMMΦ respiratory burst as measured by reactive oxygen species (ROS) production of H₂O₂ in each experimental group. Isolated cells from lung tissues were incubated with 10 μM of 2',7'-dichlorofluorescein diacetate (DCFDA) for 30 min, and then analysed by flow cytometry to assess ROS levels in CD45⁺CD11b^{high}F4/80⁺ BMMΦs (n=8 for each group). (G, representative data; H, pooled data) Assessment of AMΦ respiratory burst as measured by ROS production of H₂O₂ in each experimental group. Isolated cells from lung tissues were incubated with 10 μM DCFDA for 30 min, and then analysed by flow cytometry to assess ROS levels in CD45⁺CD11b^{low}F4/80⁺ AMΦs (n=8 for each group). (I) Representative data for frequency analyses of CD11b^{high}F4/80⁺ BMMΦs and CD11b^{low}F4/80⁺ AMΦs in lung lobes treated with PBS or clodronate as assessed with flow cytometry. C57BL/6J mice were intratracheally injected with 100 μL of PBS or 15 mM clodronate solution. After 2 days, single-cell suspensions harvested from lung tissues were stained with anti-CD45, anti-CD11b and anti-F4/80 for flow cytometric analyses. Gating for CD45⁺ cells was first performed, with subsequent frequency analysis for CD11b^{high}F4/80⁺ BMMΦs and CD11b^{low}F4/80⁺ AMΦs. (J, K) Pooled data for analyses of frequency and absolute number of CD11b^{high}F4/80⁺ BMMΦs, respectively (n=6 for each group). (L, M) Pooled data for analyses of frequency and absolute number of CD11b^{low}F4/80⁺ AMΦs, respectively (n=6 for each group). (N) Assessment of bacterial load within lung homogenate in clodronate-pretreated mice infected with 5 × 10⁶ CFU of K2 KP. Clodronate-pretreated mice were then intratracheally infected with sublethal dose (5 × 10⁶ CFU) of K2 KP followed by intravenous administration of PBS or 3 × 10⁵ PMSCs 2 hours later. Lung lobes were collected for detection of bacterial burden (n=6 for each group). (O) Kaplan-Meier survival analysis of clodronate-pretreated/AMΦ-depleted mice with K2 KP infection (5 × 10⁶ CFU) after PBS or PMSC treatment. Survival was observed for 12 days (n=8 for each group). Data are shown as mean ± SD.

Human PMSC-secreted IL-1β prominently increase AMΦ antibacterial functions to significantly improve KP pneumonia disease outcome

To identify which factor may be involved in the potent capacity of PMSCs to modulate and significantly increase multiple AMΦ antibacterial functions, we performed transcriptome profiling of PMSCs and MAP analysis, finding IL1B to be the most potential candidate (figure 7A).²⁰ To ascertain whether IL-1β is responsible for PMSC-mediated enhancement of AMΦ antibacterial functions, we assessed both in vitro KP phagocytosis and in vivo ROS production of AMΦs under KP infection with subsequent treatment of PMSCs after specific knockdown of IL1B (shIL1B-PMSCs) or non-target knockdown (shLuc-PMSCs). Compared

with PBS group, co-culture with shLuc-PMSCs significantly increased AMΦ in vitro bacterial phagocytic capacity while co-culture with shIL1B-PMSCs reversed the capacity back to baseline (figure 7B–C). ROS production in AMΦs of KP-infected mice was significantly increased with shLuc-PMSC treatment, but not with shIL1B-PMSC treatment which resulted in levels close to PBS-treated levels (figure 7D,E). To determine whether PMSC-expressed IL-1β was critical to improving disease outcome, we first assessed bacterial clearance in KP-infected mice 4 hours post-infection. We found that the local pulmonary bacteria load in KP-infected mice after PBS treatment was significantly increased than that in infected mice treated with shLuc-PMSCs (figure 7F). However, in KP-infected mice treated with



shIL1B-PMSCs, a significantly higher bacterial load was seen when compared with levels in shLuc-PMSC-injected infected mice. The importance of PMSC-secreted IL-1β was even more prominent in reducing systemic bacterial load (figure 7G). Most importantly, treatment of shLuc-PMSCs but not shIL1B-PMSCs significantly improved survival of LD₁₀₀-infected mice (figure 7H). These findings demonstrate the importance of IL-1β in PMSC modulation of AMΦs to improve disease outcomes of severe bacterial pneumonia/ARDS.

DISCUSSION

The lungs are in constant contact with the environment, thus requiring resident AMΦs to maintain immune homeostasis and tissue repair,¹⁷ and during infections, recruited BMMΦs to rapidly produce effector responses.¹⁵ The careful orchestration in the actions of these two MΦ populations during severe bacterial infections is crucial not only for pathogen clearance but also limiting the concomitant, potentially lethal tissue injury. Thus, it is surprising that no previous reports have evaluated MSC interactions towards AMΦs and BMMΦs simultaneously, especially since MSC therapy appear particularly suited for pulmonary diseases given the tremendous first-pass effect with intravenous delivery,^{27–29} effects which are further increased

with inflammation.^{30–31} Surprisingly, many studies of MSCs for bacterial pulmonary infections have used less clinically relevant models such as applying lipopolysaccharide (LPS) only or *Escherichia coli*, given MSCs intratracheally, or not evaluated survival.^{32–35} Two reports have used more clinically relevant bacteria, such as *Streptococcus pneumoniae* but not evaluating MΦs in vivo,³⁶ and another used KP but treated with murine BMMSCs intratracheally³⁷; neither reports studied resident AMΦs. Two other reports evaluated MSC-pulmonary MΦ interactions using *E. coli*, and found that MSCs reduced overall pulmonary MΦ inflammatory responses including enhancing bacterial phagocytosis.^{38–39} However, the study did not ascertain whether the isolated pulmonary MΦs from infected lungs were truly AMΦs. MSC-CD11b^{low} AMΦ interactions have been evaluated in one early study, finding that MSC-derived medium can modulate these MΦs to an M2 phenotype after in vitro LPS stimulation.⁴⁰ Despite considerable differences in methodology and endpoints, findings collectively from these earlier studies are surprisingly similar to our more detailed investigation of MΦ subpopulations and specific roles: during pulmonary inflammation/infection, MSCs modulate MΦ populations towards an immunomodulatory M2 phenotype to decrease overexuberant inflammatory responses while enhancing antibacterial functions

to significantly improve tissue repair and disease outcome. Our report particularly highlights the importance of AMΦs—and PMSC treatment in supporting these MΦs quantitatively and qualitatively—in combating severe bacterial pulmonary infection and ARDS.

We demonstrate that PMSC-secreted IL-1β, the endogenous pyrogen highly produced by innate immune cells in response to infection, is critical in mediating survival benefits against severe KP pneumonia and complications. Generally considered to be immunomodulatory rather than inflammatory, PMSC production of IL-1β is unexpected, but may make sense teleologically. The placenta has few effector-phenotype immune cell populations, and it is plausible that non-immune cells such as PMSCs ‘take over’ these functions, although at a much less vigorous level, as the amount of IL-1β expressed by PMSCs is very low compared with levels in bacterial infection (data not shown). Further research is warranted to explore whether PMSCs have source-specific immunomodulatory properties.

Overall, we demonstrate that human PMSCs differentially modulate resident AMΦs and recruit BMMΦ in severe KP-pneumonia to minimise tissue injury and improve disease outcome, with the overall immune milieu shifted towards an M2, immunomodulatory phenotype. Specifically, PMSC-secreted IL-1β increases AMΦ antibacterial functions and is critical in improving pathogen clearance especially systemic bacteraemia, and survival (figure 7I). These findings demonstrate the distinct roles of MΦ populations in infection and recovery, as well as strongly implicate a potential therapeutic role for PMSCs in severe bacterial pneumonia/ARDS.

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idea, oversaw the research, revised the manuscript and provided funding. MLY is the guarantor for this work.

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