Molecular Therapy
Original Article

The ARDS microenvironment enhances MSC-induced repair via VEGF in experimental acute lung inflammation

Courteney Tunstead,[1,2] Evelina Volkova,[1,2] Hazel Dunbar,[1,2] Ian J. Hawthorne,[1,2] Alison Bell,[3,4] Louise Crowe,[2,5] Joanne C. Masterson,[2,5] Claudia C. Dos Santos,[6] Bairbre McNicholas,[3,4] John G. Laffey,[3,4] and Karen English[1,2]

[1]Cellular Immunology Lab, Department of Biology, Maynooth University, Maynooth, Co. Kildare, Ireland; [2]Kathleen Lonsdale Institute for Human Health Research, Maynooth University, Maynooth, Co. Kildare, Ireland; [3]Anesthesia and Intensive Care Medicine, School of Medicine, College of Medicine Nursing and Health Sciences, University of Galway, Galway, Ireland; [4]Anesthesia and Intensive Care Medicine, Galway University Hospitals, Saolta University Hospitals Groups, Galway, Ireland; [5]Allergy, Inflammation & Remodelling Research Lab, Department of Biology, Maynooth University, Maynooth, Co. Kildare, Ireland; [6]Keenan Research Centre for Biomedical Research, St. Michael's Hospital, Toronto, ON, Canada

Clinical trials investigating the potential of mesenchymal stromal cells (MSCs) for the treatment of inflammatory diseases, such as acute respiratory distress syndrome (ARDS), have been disappointing, with less than 50% of patients responding to treatment. Licensed MSCs show enhanced therapeutic efficacy in response to cytokine-mediated activation signals. There are two distinct sub-phenotypes of ARDS: hypo- and hyper-inflammatory. We hypothesized that pre-licensing MSCs in a hyper-inflammatory ARDS environment would enhance their therapeutic efficacy in acute lung inflammation (ALI). Serum samples from patients with ARDS were segregated into hypo- and hyper-inflammatory categories based on interleukin (IL)-6 levels. MSCs were licensed with pooled serum from patients with hypo- or hyper-inflammatory ARDS or healthy serum controls. Our findings show that hyper-inflammatory ARDS pre-licensed MSC conditioned medium (MSC-CM_{Hyper}) led to a significant enrichment in tight junction expression and enhanced barrier integrity in lung epithelial cells *in vitro* and *in vivo* in a vascular endothelial growth factor (VEGF)-dependent manner. Importantly, while both MSC-CM_{Hypo} and MSC-CM_{Hyper} significantly reduced IL-6 and tumor necrosis factor alpha (TNF-α) levels in the bronchoalveolar lavage fluid (BALF) of lipopolysaccharide (LPS)-induced ALI mice, only MSC-CM_{Hyper} significantly reduced lung permeability and overall clinical outcomes including weight loss and clinical score. Thus, the hypo- and hyper-inflammatory ARDS environments may differentially influence MSC cytoprotective and immunomodulatory functions.

INTRODUCTION

Acute respiratory distress syndrome (ARDS) is an inflammatory disease with a mortality rate of ∼60% in patients with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-induced ARDS.[1,2] ARDS is largely characterized by low blood oxygen levels, excessive alveolar damage, and acute respiratory failure.[3–5] One of the primary treatments for ARDS is mechanical ventilation, with ∼70% of patients in need of ventilation. Unfortunately, ventilation can further exacerbate the disease state of the patient and contribute to disease pathology through ventilator-induced lung injury.[2,6] The Berlin definition of ARDS states ventilator support as a key diagnostic criterion, but the more recent global definition suggests that this is no longer a critical diagnostic component.[3,7] Due to the lack of available treatments, cell therapies have long been investigated as a potential treatment for ARDS. Mesenchymal stromal cell (MSC) therapies have been investigated in the treatment of ARDS due to their ability to modulate over-zealous inflammatory responses; however, clinical trials have generated disappointing results, with only ∼30%–50% of patients responding to treatment as of the latest clinical trials.[8–10]

MSCs are primarily known for their immunomodulatory properties and reparative capacity. This makes them an excellent candidate in the treatment of inflammatory diseases, such as ARDS. MSCs are known to show enhanced efficacy when exposed to cytokine-mediated activation signals, often referred to as "MSC licensing."[11–14] Although ARDS is traditionally characterized as an inflammatory disease containing the necessary levels of inflammation to effectively license the MSCs *in vivo*, sub-phenotypic stratification of patients suggests that there are two distinct sub-groups: hypo- and hyper-inflammatory ARDS.[15] Hyper-inflammatory ARDS is largely characterized by high levels of pro-inflammatory cytokines, such as interleukin (IL)-6 and IL-8, while hypo-inflammatory has significantly less of an inflammatory signature.[16] Since MSC licensing largely impacts their functional capacity *in vivo*, this would suggest that MSCs would be

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Correspondence: Karen English, Cellular Immunology Lab, Department of Biology, Maynooth University, Maynooth, Co. Kildare, Ireland.
E-mail: karen.english@mu.ie

more efficacious in the treatment of hyper-inflammatory ARDS. Unlicensed MSCs, or MSCs that are placed into an environment lacking a threshold level of inflammation, are less likely to exert beneficial effects.[17–19] Therefore, understanding the MSC response to the ARDS patient microenvironment is a crucial, but often overlooked, point of interest prior to clinical trials.

The acute lung inflammation (ALI) and cellular infiltration seen in ARDS is known to lead to disruptions in the lung epithelium, thus leading to pulmonary edema and, ultimately, death of the patient.[20–22] Tsikis et al. recently highlighted that endotoxin-induced ALI in mice led to a significant reduction in vascular endothelial growth factor (VEGF) and its associated receptor, VEGF receptor 2 (VEGFR2).[23] MSCs are already known to secrete VEGF, and many studies have already investigated the role of MSC-derived VEGF in the context of cytoprotection, wound healing, and permeability.[24–26] Other studies have highlighted the enrichment of tight junction expression and the capacity for MSCs and MSC-derived extracellular vesicles to reduce epithelial barrier permeability.[27–33] However, this study is the first to investigate the impact of the hypo- and hyper-inflammatory patient microenvironments on MSC functionality, using human bone marrow-derived MSCs (hBM-MSCs), clinically relevant ARDS patient samples, and a pre-clinical model of ALI.

RESULTS

Segregation of ARDS patient serum samples

ARDS patient serum samples were stratified based on their IL-6 level upon arrival to the intensive care unit. ARDS patient demographics, including age, sex, World Health Organization score[34], and O_2 requirement, are included in Table S1 and Figures 1A–1D. For this study, patients with IL-6 levels <50 pg/mL were considered hypo-inflammatory, and IL-6 levels >50 pg/mL were considered hyper-inflammatory (Figures 1E and 1F). For comparison, serum samples collected from healthy donors were included. Upon pooling the samples, we confirmed that the hyper-inflammatory pool contained significantly higher levels of IL-6 and IL-8, both known to be of higher levels in the hyper-inflammatory ARDS sub-phenotype (Figures 1G and 1H). In addition, the hyper-inflammatory pool contained significantly higher levels of migration inhibitory factor (MIF) and tumor necrosis factor alpha (TNF-α) (Figures 1I and 1J). All subsequent experiments were carried out using these distinct pools.

Hyper-, but not hypo-, inflammatory ARDS serum licenses MSCs and enhances their secretion of VEGF

MSC conditioned medium (MSC-CM) from MSCs that had been exposed to healthy or hypo-inflammatory serum showed no significant increase in cytokines or growth factors in comparison to naive MSC-CM. There was no difference in IL-6 secretion between MSC-CM groups (Figure 2B). Hyper-inflammatory ARDS serum, however, led to a significant increase in MSC secretion of IL-8, MIF, and VEGF (Figures 2C–2E). Gene expression studies also highlighted that *vegf-a* and *kdr*, the genes that encode for VEGF-A and VEGFR2, respectively, were also significantly increased after exposure to hyper-inflammatory serum (Figures 2F and 2G). There were no significant differences in expression of *il-8*, *mif*, or any of their corresponding receptors (*cxcr1*, *cxcr2*, *cxcr4*, *cd74*) (Figures S1A–S1F). For this reason, we chose to further investigate the role of VEGF.

MSC-CM enhances tight junction expression in CALU-3 lung epithelial cells in a VEGF-dependent manner

CALU-3 lung epithelial cells exposed to endotoxin showed a significant reduction in *tjp1*, the gene that encodes for zonula occludens-1 (Figures 3A and 3B). There was no significant difference in *ocln* or *cld4*, the genes that encode for occludin and claudin-4, respectively, in response to endotoxin (Figures 3C and 3D). However, upon exposure to 2 mL of naive MSC-CM, containing a high concentration of ∼1,000 pg/mL VEGF, correlating with the VEGF concentration seen in MSC-CM_{Hyper} (Figure 2E), there was a significant enrichment of *tjp1*, *ocln*, and *cldn4* expression. Pre-treatment of CALU-3 lung epithelial cells with SU-5416, a VEGFR2 inhibitor, abrogated this effect (Figures 3B–3D), demonstrating that VEGF derived from MSC-CM was required for the enhancement of tight junction gene expression in response to endotoxin challenge.

MSC-CM_{Hyper} reduces lung permeability *in vitro*

Transepithelial electric resistance (TEER) measurements were performed in CALU-3 cells in submerged cultures on days 1, 3, and 5 and in subsequent air-liquid interface cultures from day 8. This exposure to air allows for a more respiratory-like cell stratification, as lung epithelial cells are associated with exposure to both air and liquid in the body. Upon exposure to 2 μg/mL of endotoxin on day 15, TEER measurements (ohm cm^2) demonstrate a significant decrease in epithelial membrane integrity by day 17 (Figures 4A and 4B). The PBS control remained steady throughout. Subsequently, when exposed to 500 μL of MSC-$CM_{Healthy}$ (containing ∼500 pg/mL VEGF) or MSC-CM_{Hyper} (containing ∼1,000 pg/mL VEGF) on day 17 (Figures 4B and 4C), there was a significant improvement in barrier integrity on day 19. This was not seen in MSC-CM_{Hypo} (∼250 pg/mL VEGF). Neutralizing VEGF with a bevacizumab biosimilar led to the abrogation of this effect (Figures 4B and 4C), confirming the importance of VEGF in MSC therapeutic efficacy. Gene expression studies on the cells from this assay also showed significant enrichment of *tjp1*, *ocln*, and *cldn-4* in response to MSC-CM_{Hyper} (Figures 4D–4F).

MSC-CM_{Hyper} reduces overall disease severity in a pre-clinical model of ALI

MSC-$CM_{Healthy}$, MSC-CM_{Hypo}, and MSC-CM_{Hyper} all had the ability to reduce the inflammatory profile seen in our model of ALI, primarily in the context of IL-6 and TNF-α (Figures 5A and 5B). In the context of clinical parameters in the endotoxin-induced lung inflammation model, both MSC-CM_{Hyper} ($p = 0.0041$) and MSC-$CM_{Healthy}$ ($p = 0.0297$) significantly reduced the clinical score when compared to lipopolysaccharide (LPS)-treated mice (Figure 5C). In contrast, only MSC-CM_{Hyper} had the ability to significantly reduce the percentage of weight loss ($p = 0.0071$) when compared to LPS-treated mice (Figure 5D). MSC-CM_{Hypo} did not significantly reduce the clinical score ($p = 0.2728$) or the percentage of weight loss ($p = 0.0994$); however,

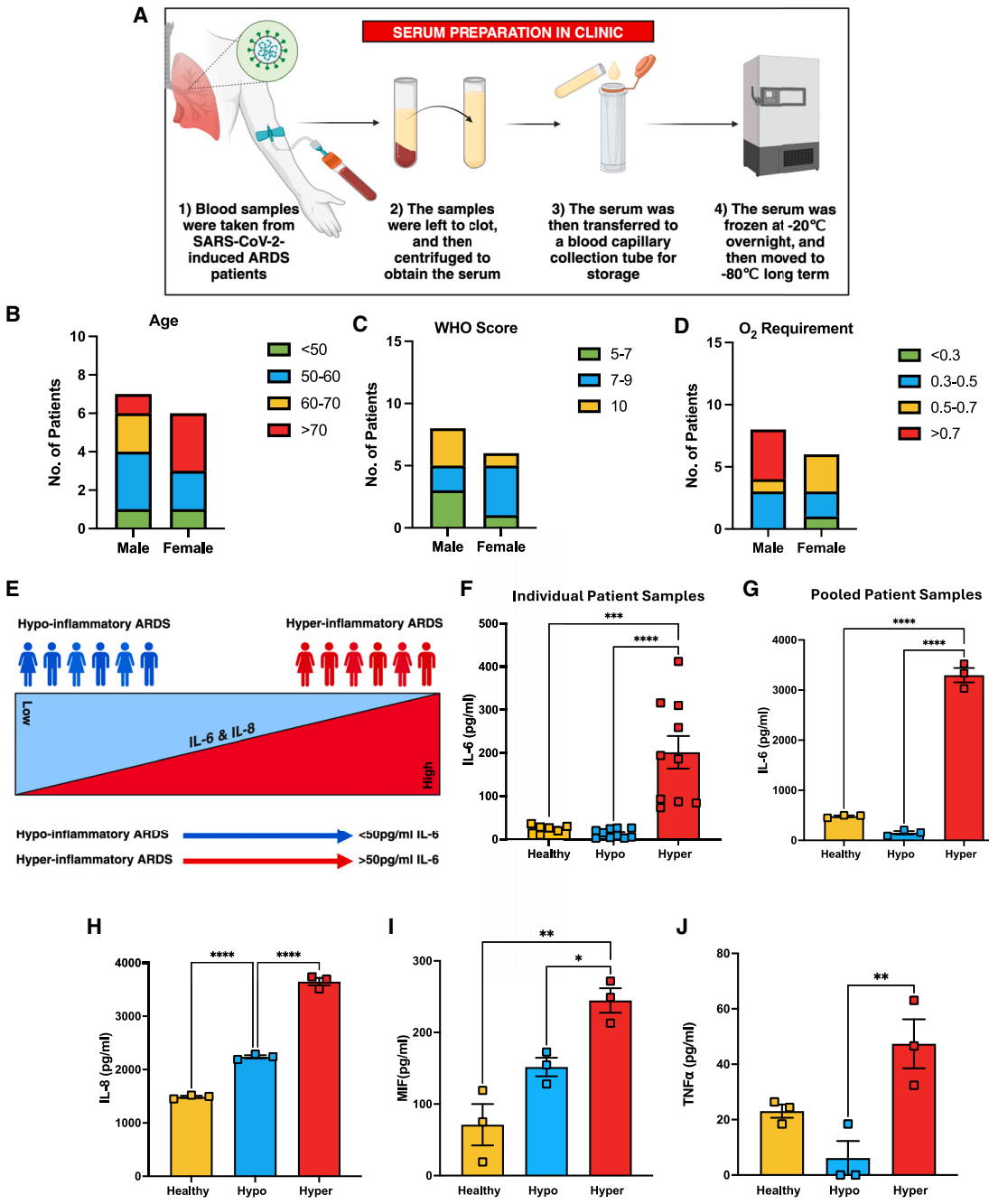

Figure 1. ARDS patient sub-phenotype stratification

(A) Schematic created using Biorender.com. ARDS patient serum samples were obtained from patients of (B–D) differing backgrounds (age, World Health Organization [WHO] score, O_2 level) and (E and F) stratified into a hypo- or hyper-inflammatory sub-phenotype based on their IL-6 levels at the time of admission ($n = 7$). Patients with <50 pg/mL IL-6 were considered hypo-inflammatory, and patients with >50 pg/mL IL-6 were considered hyper-inflammatory. The samples were then pooled, and the (G) IL-6 (BioLegend), (H) IL-8 (R&D), (I) MIF (R&D), and (J) TNF-α (BioLegend) levels were observed in each pool. Serum pooled from 6 healthy age-matched patients was used as a control. Data are presented as mean ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

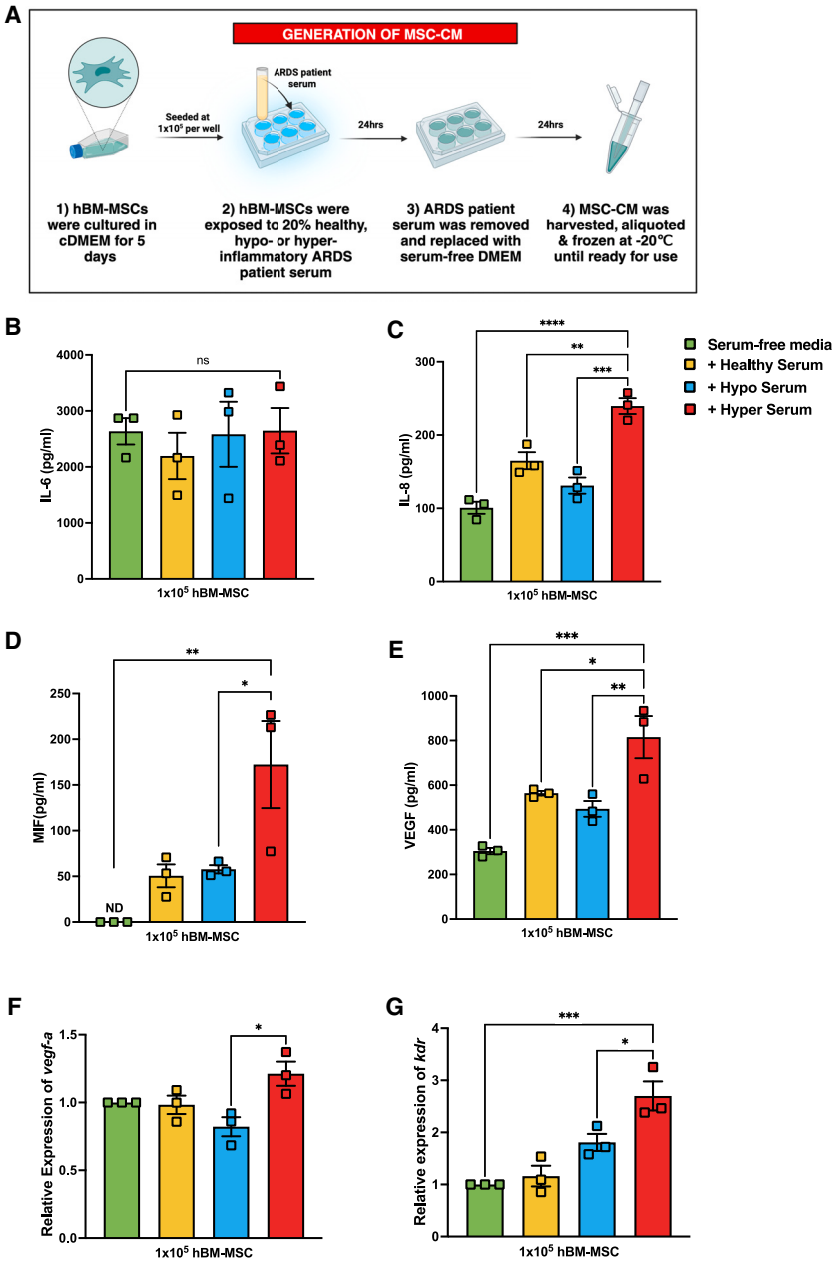

Figure 2. The MSC secretome in response to healthy or hypo- or hyper-inflammatory ARDS serum

(A) Schematic created using Biorender.com. hBM-MSCs ($n = 3$ donors) were seeded at a density of 1×10^5 in a 6-well plate, left to attach, and exposed to 20% ARDS patient serum or healthy serum control. The serum was removed after 24 h, the cells were washed with PBS, and serum-free DMEM was added for a further 24 h to generate MSC-CM. The (B) IL-6 (BioLegend), (C) IL-8, (D) MIF, and (E) VEGF (R&D) levels were then analyzed via ELISA. RT-PCR was also carried out on (F) *vegf-a* and (G) *kdr*. Data are presented as mean ± SEM; $n = 3$ per group; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

there was no significant difference observed between MSC-CM_{Hyper} and MSC-CM_{Hypo} ($p = 0.0690$).

MSC-CM_{Hyper} reduces lung permeability in a pre-clinical model of ALI

Evans Blue dye was used as a measure of lung permeability *in vivo* (Figure 6A). Lungs with a visible blue coloration are considered permeable, or "leaky," as the Evans Blue dye leaks to the exterior of the lung. MSC-$CM_{Healthy}$, MSC-CM_{Hypo}, or MSC-CM_{Hyper} was concentrated using centrifugal filters, and VEGF levels were measured following the addition of a bevacizumab biosimilar (to neutralize VEGF) or an immunoglobulin G (IgG) control (Figures 6B and 6C). The lungs from mice exposed to endotoxin followed by MSC-CM_{Hyper} showed a significant visual improvement in barrier integrity (Figure 6D). This effect was abrogated when VEGF was neutralized. Mice that were treated with MSC-CM_{Hyper} also showed a significant reduction in total Evans Blue in the lung extract (normalized to the serum Evans Blue and divided by the lung wet weight). This effect was abrogated when VEGF was neutralized (Figure 6E). There was also a significant reduction in the wet:dry weight ratio of the lungs of mice that received MSC-CM_{Hyper}, which, again, was lost when VEGF was neutralized (Figure 6F).

DISCUSSION

The patient microenvironment is thought to have an impact on MSC therapy, with MSCs typically exerting their beneficial effects in response to an inflammatory stimulus. MSCs that have not been subjected to a threshold level of inflammation may lack therapeutic efficacy and may further contribute to disease pathology.[11–14] Other studies have shown that MSC-CMs generated from MSCs exposed to ARDS patient bronchoalveolar lavage fluid (BALF) have had increased levels of cytokines in comparison to MSCs exposed to a healthy BALF control.[35] However, this study lacked patient stratification. Our research illustrates that MSCs are more responsive to the hyper-, but not hypo-, inflammatory ARDS patient microenvironment in the context of MSC cytoprotective effects. This is outlined in Figure 2, where we show that MSC-CM_{Hyper}, but not MSC-CM_{Hypo}, had increased levels of IL-8, MIF, and VEGF. MSC-CM_{Hypo} had a comparable level of protein secretion to that of MSC-$CM_{Healthy}$. Gene expression studies called further attention to VEGF, as *vegf-a* and *kdr* were significantly increased in the hyper-, but not hypo-, pre-licensed MSCs.

Based on previous literature, MSCs have already been shown to enhance epithelial tight junction gene expression in a VEGF-dependent

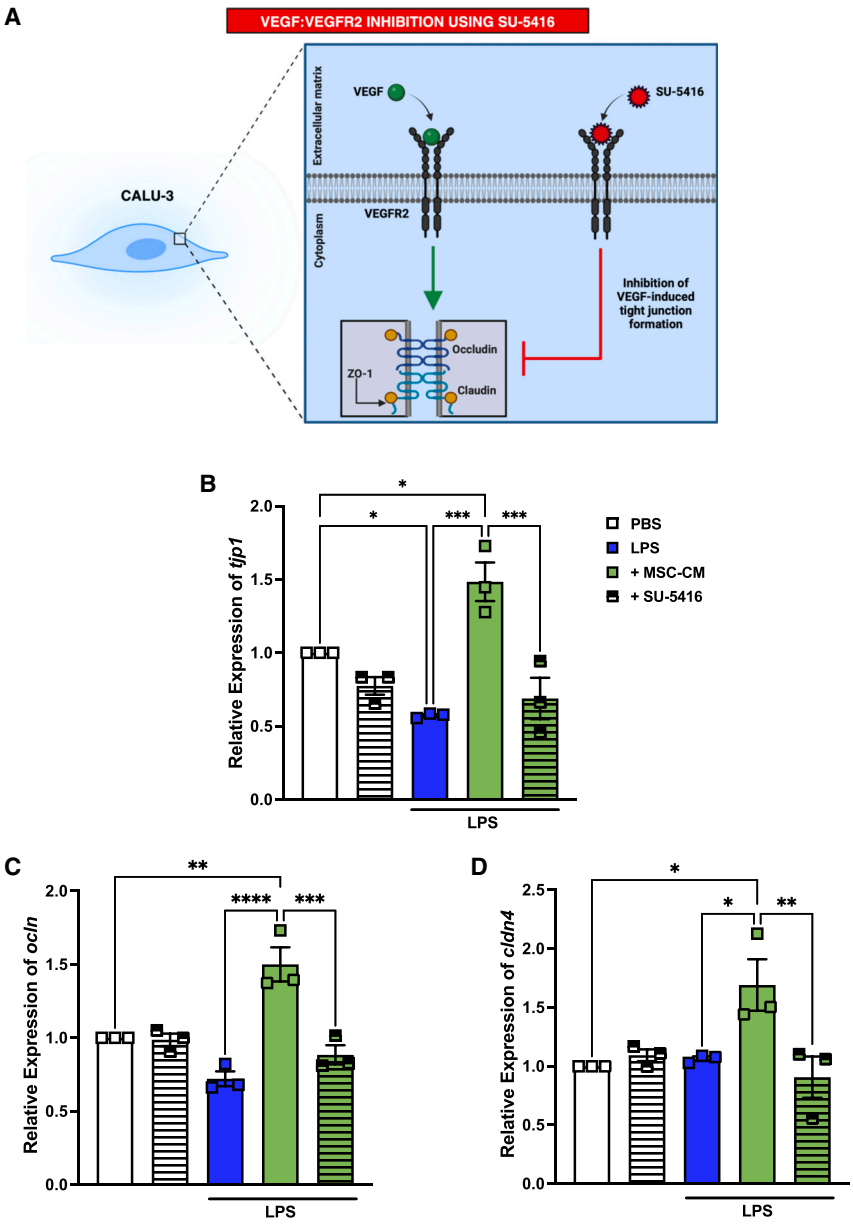

Figure 3. The MSC-CM enhancement of tight junction expression

(A) Schematic created using Biorender.com. CALU-3 cells (passages 10–12) were seeded at a density of 5×10^5 in a 6-well plate, left to attach, and exposed to 10 μM SU-5416, a VEGFR2 inhibitor, for 4 h before being stimulating with 2 μg/mL endotoxin for 48 h. Some groups were subsequently exposed to 2 mL of MSC-CM containing ~1,000 pg/mL VEGF for 24 h. The cells were then harvested for gene expression studies of tight junction genes: (B) *tjp1*, (C) *ocln*, and (D) *cldn4*. Data are presented as mean ± SEM; $n = 3$ per group; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

manner.[26–33] Yang et al. highlighted that MSCs could stabilize endothelial lung permeability in a model of ALI through the enrichment of VEGF in the MSC-CM.[26] We wanted to further investigate the impact of MSC-CM on tight junction enhancement as a mechanism for repairing the epithelial barrier. To do this, we exposed CALU-3 lung epithelial cells to endotoxin to interrupt the epithelial barrier and then exposed them to MSC-CM (containing ~1,000 pg/mL of VEGF), a comparable concentration to that of hyper-licensed MSC-CM, from three independent, non-treated MSC donors (Figure 2E). We also used a VEGFR2 inhibitor, SU-5416, to block VEGFR2 on the CALU-3 cells to confirm VEGF dependence (Figures 3B and 3C). Our study confirmed that MSC-CM could enhance tight junction expression of *ocln*, *cldn4* and *tjp1* in a VEGF-dependent manner in CALU-3 lung epithelial cells, a clinically relevant cell type for lung barrier studies due to their ability to generate tight junctions.

Furthermore, we investigated the relevance of this at a functional level, *in vitro*, by performing a TEER assay in CALU-3 cells. Endotoxin significantly decreased the barrier integrity, and MSC-CM_{Hyper}, but not MSC-CM_{Hypo}, had the ability to significantly enhance barrier integrity in response to endotoxin (Figures 4B and 4C). MSC-$CM_{Healthy}$ could also significantly enhance barrier integrity but not to the same extent as MSC-CM_{Hyper}, which had the ability to fully restore the barrier. Using an anti-VEGF bevacizumab biosimilar monoclonal antibody or an IgG isotype control, we investigated the mechanistic impact of VEGF. This antibody functionally inhibits VEGF, and neutralizing VEGF in this manner significantly abrogated the increased barrier integrity mediated by the MSC-CM_{Hyper} group. Gene expression studies further highlighted that hyper-licensed MSCs had the ability to enhance the expression of *tjp1*, *cldn4*, and *ocln* in CALU-3 cells cultured at the air-liquid interface in a VEGF-dependent manner. There was no significant increase in these genes in the healthy-licensed MSCs. This is in line with studies in rat MSCs, whereby lentiviral VEGF knockdown rat MSCs failed to enhance epithelial barrier integrity when compared to control MSCs in a model of endotoxin-induced ALI.[32]

To investigate the functional relevance *in vivo*, we exposed C57BL6/J mice to endotoxin-induced ALI, which shares some aspects of human ARDS pathology, including the permeable, or leaky, lung. We then exposed the mice to each of our treatment groups before injecting Evans Blue dye to assess the lung permeability.[36] MSCs and MSC-CM have been shown to reduce pro-inflammatory cytokine levels and reduce pathology and clinical score in endotoxin-induced ALI models.[37,38] Upon analyzing the cytokine profile of the BALF of the

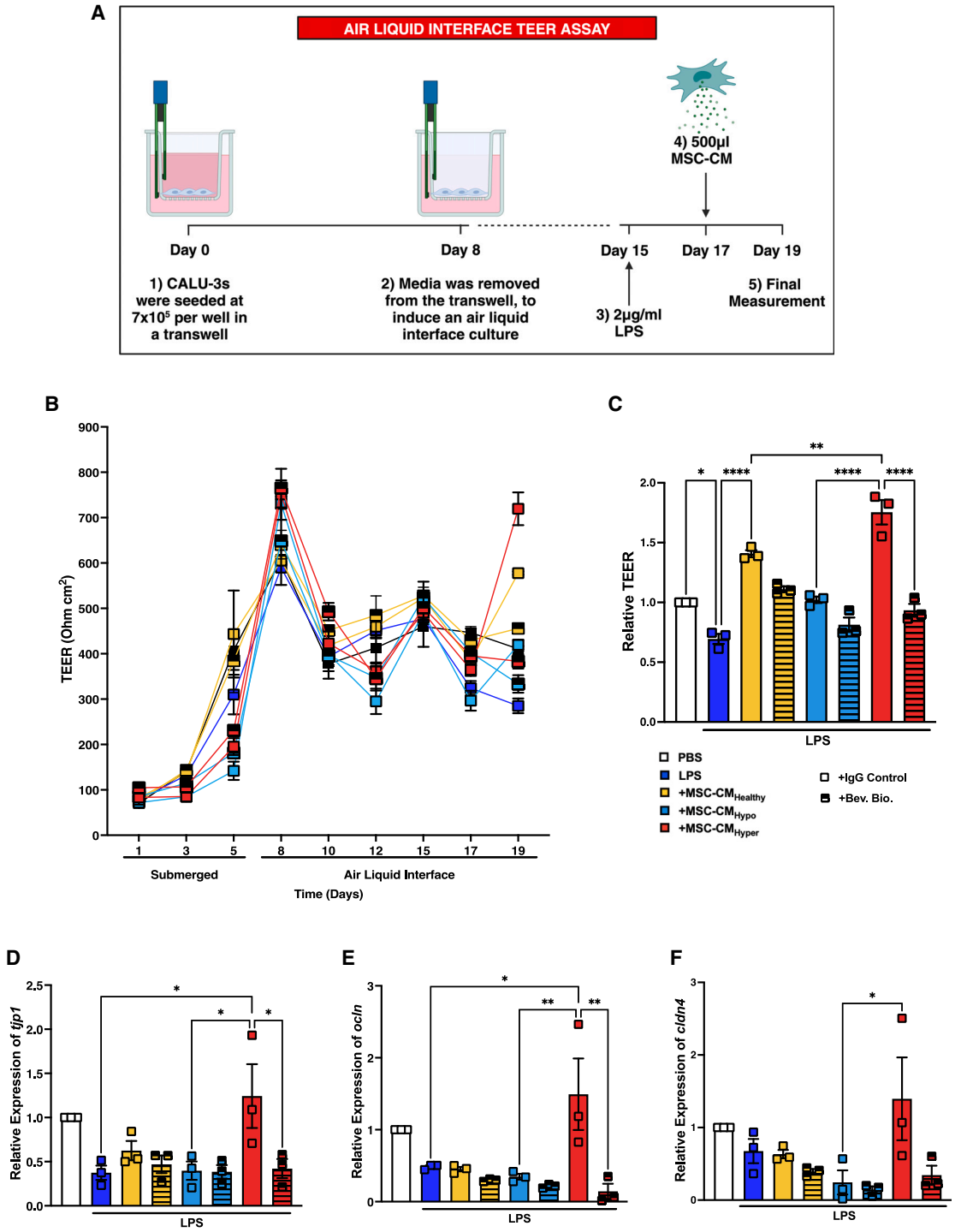

Figure 4. Barrier integrity of CALU-3 cells in response to MSC-CM

(A) Schematic created using Biorender.com. CALU-3s were seeded at a density of 7 × 10^5 in a Transwell (0.4 µm) and grown under air-liquid interface conditions from day 8 of culture. On day 15, the cells were stimulated with 2 µg/mL of endotoxin, and on day 17, they were exposed to 500 µL of MSC-CM from the groups depicted in Figure 2E (± bevacizumab biosimilar or the appropriate IgG isotype control). (B) TEER measurements were taken 3 times a week for 3 weeks, and (C) final measurements were taken on day 19. (D–F) Gene expression studies highlighted differences in tight junction expression. Data are presented as mean ± SEM; $n = 3$ per group; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

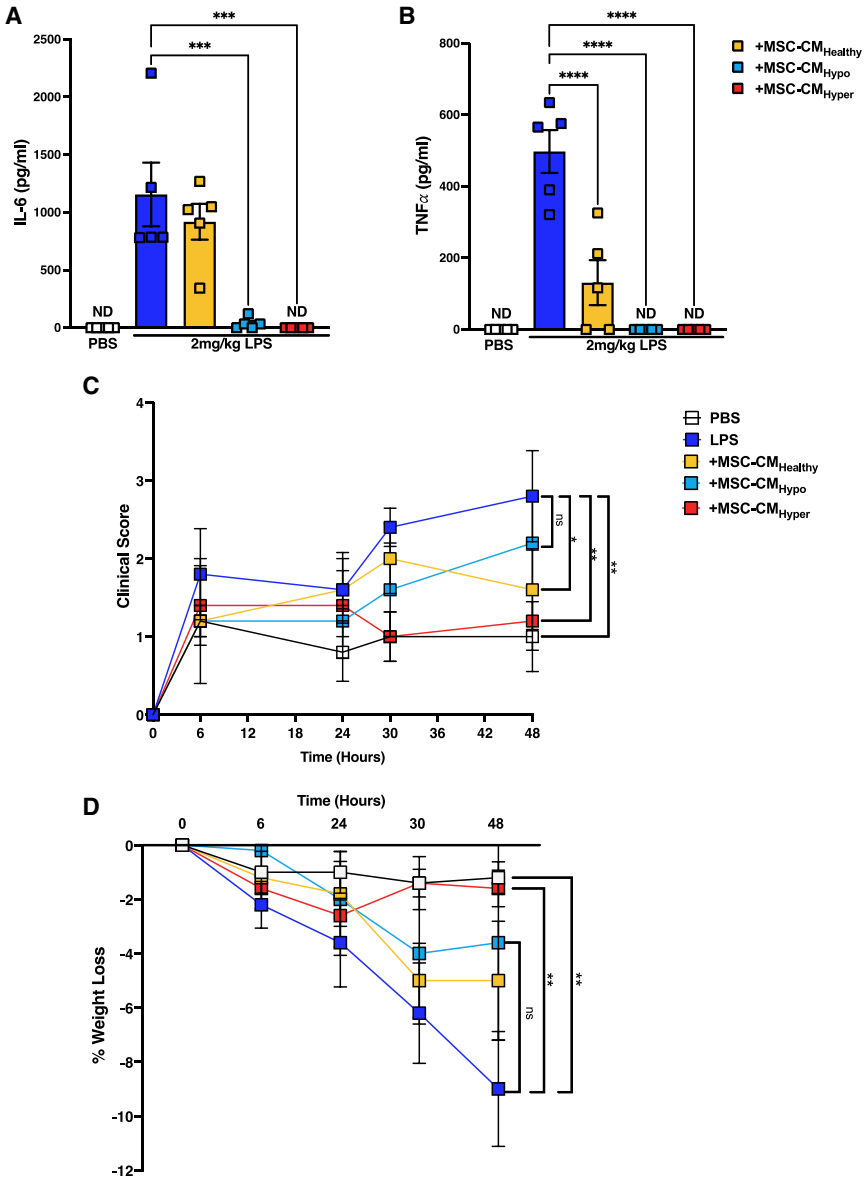

Figure 5. Cytokine levels in BALF and clinical scoring from ALI mouse model

BALF was harvested from C57BL6/J mice (n = 5) exposed to our ALI model and (A) IL-6 and (B) TNF-α were quantified by ELISA. (C) Clinical score and (D) percentage of weight loss were also assessed. Data are presented as mean ± SEM; n = 5 per group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

mice subjected to each treatment, we detected significantly less cytokines, in particular IL-6, in mice that received any form of MSC-CM, and this aligns with previous studies.[39–41] The overall clinical parameters highlighted that both MSC-$CM_{Healthy}$ and MSC-CM_{Hyper} had the ability to significantly reduce clinical score, with p values suggesting that MSC-CM_{Hyper} reduced this to a greater extent (Figure 5C). MSC-CM_{Hyper} also showed a significant reduction in the percentage of weight loss, something we did not see in our MSC-CM_{Hypo} group (Figure 5D). Importantly, only MSC-CM_{Hyper} significantly reduced lung permeability, and this effect was VEGF dependent. This data suggests that MSC cytoprotective and immunomodulatory effects may be mediated by different factors present in the secretome. Our study focused on the cytoprotective effects mediated by VEGF and demonstrated that pre-licensing MSCs with high-IL-6 ARDS patient serum (denoted as hyper-inflammatory ARDS) had a significant impact in reducing lung permeability in comparison to healthy or hypo-inflammatory pre-licensed MSC-CM. However, in the same model, all MSC-CMs had the ability to reduce the pro-inflammatory cytokines IL-6 and TNF-α in the BALF. These findings confirm that MSCs of any form may be beneficial in the treatment of inflammatory disease, but only MSC-CM_{Hyper} appeared to be efficacious across all parameters, further highlighting the impact of the patient microenvironment on MSC functionality. Indeed, a recent study by Faircloth et al. confirms this hypothesis, showing that VEGF secretion and immunosuppression by MSCs are two distinct potency mechanisms.[42]

This study provides novel insight into the impact of the patient microenvironment in dictating response to MSC therapy, offering a solution to the urgent unmet need for patient stratification prior to further MSC studies and clinical trials in the treatment of ARDS.

MATERIALS AND METHODS

Study cohorts and ethical approval

Full ethical approval was obtained from both the Galway University Medical Ethics Committee and the Maynooth University Ethics Committee. A cohort of healthy adult donors was recruited from St Vincent's Healthcare Group. All participants provided full consent.

Ethical approval and HPRA compliance

Ethical approval was granted by the ethics committee of Maynooth University (BRESC-2022-2453953) and project authorization from the HPRA (AE19124/P031), in accordance with the Animal Research: Reporting of In Vivo Experiments criteria.

ARDS serum extraction

Blood samples were taken from patients with SARS-CoV-2-induced ARDS, fractionated to obtain the serum, and placed in 5 mL serum collection tubes. The samples were frozen at −20°C overnight before

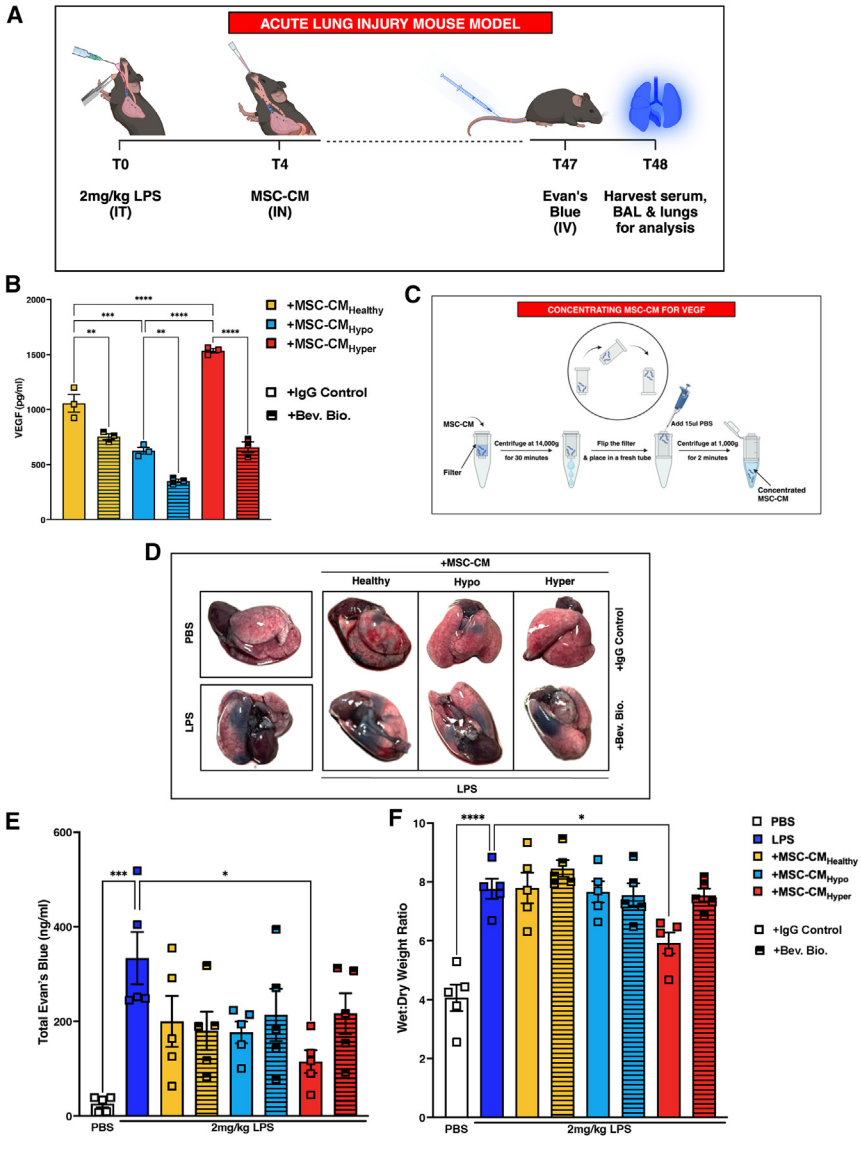

Figure 6. The impact of MSC-CM on lung permeability *in vivo*

(A) Schematic created using Biorender.com. C57BL6/J mice (n = 5) were exposed to 2 mg/kg of endotoxin intratracheally at T0. (B and C) At T4, 500 μL of MSC-CM that had been concentrated down to 30 μL (± bevacizumab biosimilar or an appropriate IgG isotype control) was administered intranasally. 1 h before harvesting (T47), a 10% solution of Evans Blue dye was administered intravenously, and mice were sacrificed at T48. (D and E) Evans Blue dye and (F) wet:dry weight ratio were used as indicators of permeability. Data are presented as mean ± SEM; n = 5 per group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

being transferred to −80°C. Samples were derived from both male and female patients in the age range of 40–80 years old. Healthy serum samples were sourced from St Vincent's University Hospital as a control. These samples were processed in the same manner and age matched accordingly.

ARDS patient sample pool generation

IL-6 levels were measured to stratify patient samples into either the hypo- or hyper-inflammatory ARDS sub-phenotype. Patient samples with an IL-6 level <50 pg/mL were pooled and used as a representative of hypo-inflammatory ARDS (n = 7). Patient samples with an IL-6 level > 50 pg/mL were pooled and used as a representative of hyper-inflammatory ARDS (n = 7). Once pooled, the IL-6, TNF-α (BioLegend), IL-8, and macrophage MIF (R&D) levels were analyzed via enzyme-linked immunosorbent assay (ELISA) to ensure that our hyper-inflammatory cohort has significantly more of both.

ELISA

96-well half-area ELISA plates (COSTAR) were coated as per manufacturer guidelines (human IL-6 [BioLegend], IL-8, MIF, VEGF [R&D]). The plate was then blocked with reagent diluent (1% BSA in PBS, sterile filtered) for 1 h before adding samples in appropriate dilutions for 2 h. The detection antibody was then added as per manufacturer guidelines and left for a further 1 (BioLegend) to 2 h (R&D). Streptavidin-HRP was added for 30 min, followed by a TMB substrate. The reaction was stopped using a 2N stop solution made up of $H2SO_4$ and distilled H_2O when color appeared in a gradient in the standard. The plate was washed in an automated washer (ELx50 Biotek) three times between each step and measured at absorbances of 450 and 570 nm using a CLARIOstar microplate reader.

Cell culture of hBM-MSCs

hBM-MSCs from three different donors were obtained from RoosterBio and expanded as per the manufacturer's guidelines using Rooster Nourish expansion media (Rooster Basal + Rooster Booster). Cells were further cultured in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum and 1% *Penicillin streptomycin* (cDMEM), for 5 days at 37°C + 5% CO_2. All experiments were a representation of three independent MSC donors.

Generation of MSC-CM

hBM-MSCs (passage 2–4) were seeded at a density of 1×10^5 per well in a 6-well plate in 1 mL of cDMEM and left overnight to attach. Once attached, the medium was removed, and 1 mL of medium containing 20% healthy or hypo- or hyper-inflammatory ARDS serum was added to each corresponding well and left for 24 h. This medium was then removed, the cells were washed with PBS to remove any leftover serum, and serum-free DMEM was added for a further 24 h to allow

for the generation of MSC-CM. Supernatants were harvested, centrifuged at 300*g* for 5 min to remove debris, aliquoted, and stored at −20°C for future experiments.

Concentration of MSC-CM using Amicon Ultra Centrifugal Filters

For use in *in vivo* studies, the MSC-CM was concentrated into a smaller volume of liquid for administration to the mice. MSC-CM was placed in an Amicon filter of appropriate size for VEGF purification (50 kDa) and centrifuged at 14,000*g* for 30 min. The filter was then inverted, placed in a clean Eppendorf, and centrifuged at 1,000*g* for 2 min to elute the concentrated MSC-CM. This concentrate was reconstituted in the appropriate amount of PBS, and 30 μL was given, intranasally, per mouse.

Cell culture of CALU-3s

Human CALU-3 lung epithelial cells obtained from Dr. Mark Robinson (MU) were cultured in cDMEM at 37°C + 5% CO_2. The cells were split at a 1:2 ratio every 3–4 days and monitored closely.

CALU-3 stimulation

CALU-3s were seeded at a density of 5×10^5 per well in a 6-well plate and stimulated with 2 μg/mL of LPS/endotoxin or a PBS control. 24 h post-endotoxin stimulation, a 10 μM solution of SU-5416, a VEGFR2-inhibitor, or an appropriate DMSO vehicle control was added for 4 h. The cells were then washed with PBS, and the endotoxin (or PBS control) was re-added, along with 2 mL of MSC-CM (containing ∼1,000 pg/mL of VEGF) for a further 24 h. Cells were harvested in TRIzol for gene expression analysis.

Gene expression

RNA was extracted from hBM-MSCs that had been exposed to 20% healthy or hypo- or hyper-inflammatory ARDS serum. The RNA was nano-dropped to assess concentration and purity. The RNA was then normalized to 100 ng/μL, and cDNA was made using the QuantBio cDNA Synthesis kit (as per the manufacturer's instructions). Real-time PCR (RT-PCR) was then carried out using PerfeCta SYBR Green FastMix (QuantBio) and the required primers (Table S1). Expression was quantified in relation to the housekeeper gene HPRT, and the relative fold change was measured by calculating the $2^{-\Delta\Delta CT}$ values.

TEER assay

CALU-3 cells were seeded at a density of 7×10^5 per well in a 6-well Transwell insert that had been coated with 1% fibronectin for 1 h. For the first week of culture, the cells were grown in submerged conditions (cDMEM in the lower well and Transwell). For the second week, the cells were exposed to air-liquid interface culture conditions (cDMEM in the lower well only). On day 15, the cells were stimulated with 2 μg/mL of endotoxin (Serotype: 0111:B4 [Sigma]). On day 17, the medium was supplemented with 1 mL of MSC-CM. Measurements were taken using the EVOM on days 1, 3, 5, 8, 10, 12, 15, 17, and 19. The medium was changed 1 h prior to measurement. Cells were harvested in TRIzol for gene expression analysis. To analyze the TEER, a blank value (from a Transwell containing no cells) was subtracted from each value, and the values were then multiplied by the area of the well (4.67 cm^2). The relative TEER was then generated by dividing each replicate's final TEER value on day 19 by the corresponding PBS control well on day 19.

ALI mouse model

Male and female C57BL6/J mice (Charles River), aged 12–16 weeks, were given 2 mg/kg of endotoxin, or a PBS control, intratracheally and monitored closely for 48 h. MSC-CM was concentrated using Amicon Ultra Centrifugal Filters and incubated with either an anti-VEGF monoclonal antibody (bevacizumab biosimilar [InVivoSIM]) to inhibit VEGF functionality or an IgG isotype control (InVivoMAb). The MSC-CM was administered, intranasally, 4 h post-endotoxin administration.

Evans Blue dye

A 10% solution of Evans Blue tetrasodium salt (Tocris) was made up in PBS. The solution was filter sterilized, and 200 μL was injected intravenously into the tail vein of the mice. 1 h post-injection, the mice were sacrificed using an intraperitoneal injection of pentobarbital, and the blood, BALF, and lungs were harvested for analysis. The BALF was used for cytokine analysis by ELISA, and all animal scoring was graphed (weight/temperature/clinical score). To analyze this, absorbance readings were taken to assess the total Evans Blue in the lung and serum (620 and 740 nm) and compared to a serial-diluted standard of Evans Blue dye. The total lung Evans Blue was then divided by the serum Evans Blue values to account for tail vein injection error, and this was further divided by the lung wet weight to get the absolute Evans Blue concentration.[36]

Statistical analysis of animal studies

Power calculations were performed to guide sample size, and data were analyzed using GraphPad Prism 10 software. One-way ANOVA, followed by the post hoc Tukey's multiple comparison test, was used to assess significance except in Figures 6D and 6E, where a two-way ANOVA was used. All data are presented as mean ± SEM, with $n = 5$ per group.

DATA AND CODE AVAILABILITY

The data supporting the findings of this study are available upon request from the corresponding author.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.ymthe.2024.08.003.

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AUTHOR CONTRIBUTIONS

C.T. performed research, data analysis, and study design and wrote the manuscript. E.V., H.D., and I.J.H. performed research and data analysis. A.B. and B.McN. provided patient samples and data for the study. L.C. and J.C.M. provided reagents and contributed to study design and data analysis. C.C.D.S. and J.G.L. contributed to study design. K.E. designed and supervised the study and wrote the manuscript. All authors approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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