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Healthy versus Inflamed Lung Environments Differentially Effect MSC Functions

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A short running head: ARDS BALF effects on hMSCs

ABSTRACT

Background: Despite increased interest in MSC-based cell therapies for the acute respiratory distress syndrome (ARDS), clinical investigations have not yet been successful and understanding of the potential *in vivo* mechanisms of MSC actions in ARDS remain limited. ARDS is driven by an acute severe innate immune dysregulation, often characterized by inflammation, coagulation, and cell injury. How this inflammatory microenvironment influences MSC functions remains to be determined.

Aim: To comparatively assess how the inflammatory environment present in ARDS lungs vs. the lung environment present in healthy control subjects alters MSC behaviors.

Methods: Clinical grade human bone marrow-derived MSCs (hMSCs) were exposed to bronchoalveolar lavage fluid (BALF) samples obtained from ARDS patients or from healthy control subjects. Following exposure, hMSCs and their conditioned media were evaluated for a broad panel of relevant properties including viability, levels of expression of inflammatory cytokines, gene expression, cell surface HLA expression, and activation of coagulation and complement pathways.

Results: Pro-inflammatory, pro-coagulant, and major histocompatibility complex (self recognition) related gene and protein expression was markedly up-regulated in hMSCs exposed *ex vivo* to BALF obtained from healthy control subjects. In contrast, these changes were less apparent and often opposite in hMSCs exposed to ARDS BALF samples.

Conclusion: These data provide new insights into how hMSCs behave in healthy vs. inflamed lung environments strongly suggesting that the inflamed environment in ARDS induces hMSC responses potentially benefical for cell survival and actions. This further highlights the need to understand how different disease environments affect hMSC functions.

Key words: Cell Therapy, Inflammation, Acute Respiratory Distress Syndrome, Mesenchymal Stromal Cells, Bronchoalveolar Lavage Fluid

Word count: 246 (max 250)

INTRODUCTION

Mesenchymal stromal cells (MSCs) are being increasingly investigated as a cell-based therapy to suppress excessive inflammation in the acute respiratory distress syndrome (ARDS) (1, 2). However, results of clinical investigations of MSCs in ARDS, while uniformly demonstrating safety, have not as yet demonstrated efficacy (3-5). While a number of factors may be responsible for the lack of improved outcome, including tissue of MSC origin (bone marrow vs adipose tissue vs umbilical cord vs other), dose, dosing, route of administration, use of freshly thawed vs continuously cultured cells, and other considerations, there remains a fundamental lack of knowledge as to the fate and actions of the administered MSCs *in vivo* in the diseased human lung microenvironment (reviewed in (6)). This raises the possibility that the inflammatory environment encountered may significantly alter potential MSC efficacy and potency.

A growing number of studies, including our own, have found that MSC functions, and thus potential therapeutic actions, differ depending on the inflammatory environment encountered (7-13). MSCs express a range of damage- and pathogen-associated molecular pattern receptors on their cell surface, including the toll-like receptors (TLRs), and differential activation of these receptors results in different patterns of MSC actions (14-16). Earlier work has suggested pro- and anti-inflammatory MSC phenotypes based, in part, on different patterns of TLR activation (17, 18). This is highly relevant for ARDS as different inflammatory stimuli in the ARDS lung can activate a range of TLRs. Further, other inflammatory stimuli may influence MSC functions through mechanisms in parallel with TLR activation.

We and others have found that *ex vivo* exposure to bronchoalveolar lavage fluid (BALF) or serum samples from ARDS patients has significant impact on MSC functions including profile of secreted mediators and downstream effects on macrophage functions, often enhancing anti-inflammatory actions (8-12). In one example, BALF from cystic fibrosis patients who have pulmonary *Aspergillus* infection is rapidly toxic to MSCs, in part related to the fungal product gliotoxin (13). This raises the possibility that certain inflammatory lung environments may have deleterious effects on MSCs with according implications for potential therapeutic use. Moreover, contrary to previously held beliefs, systemically administered allogeneic MSCs rapidly undergo apoptosis, autophagy, efferocytosis, and possibly other means of clearance and/or inactivation (19-22). In part, this may be related to a phenomenon known as the instant blood mediated inflammatory reaction (IBMIR), an immediate inflammatory response to systemically administered allogeneic MSCs that includes activation of the complement and coagulation cascades and includes stimulation of tissue factor release by the MSCs (23-25).

Thus, to investigate the effects of the ARDS inflammatory lung environment on hMSC viability and function, clinical grade human bone marrow-derived MSCs obtained from healthy volunteers (hMSCs) were exposed *ex vivo* to individual BALF samples obtained from ARDS patients and from healthy volunteers for comparison. Cell viability, mitochondrial function, pro- and anti-inflammatory cytokine gene and protein expression, cell surface HLA expression, and activation of coagulation and complement pathways were assessed following exposure. Unexpectedly, hMSCs exposed to healthy control BALF developed an inflammatory response and also increased gene and protein expression associated with self- vs non-self recognition, notably increased Class II HLA expression, and increased complement expression. These results suggest that an otherwise

non-inflamed normal lung environment stimulates mechanisms for clearance of allogeneic hMSCs. In contrast, these changes were either mitigated, absent, or opposite in hMSCs exposed to BALF from ARDS patients. These findings provide evidence of the plasticity of hMSC responses in different clinically relevant lung environments and shed new light into the potential mechanisms of action of MSC-based cell therapy for ARDS.

RESULTS

ARDS BALF contains elevated inflammatory mediators compared to HC BALF

To initially determine if the BALF samples differed between the HC and ARDS patient lungs, inflammatory mediators in clinical BALF samples were assessed. The BALF samples utilized for each assay in the overall study are depicted in **Table 1**. Although there were variations between the different clinical isolates, the levels of total protein, dsDNA, and a range of inflammatory mediators were significantly elevated in ARDS compared to HC BALF samples (**Table 2**). There were no significant differences between ARDS and HC BALF in the levels of anti-inflammatory and Th2 mediators such as IL-10, IL-4, and IL-13, also depicted in **Table 2**.

BALF from both ARDS healthy control patients is non-toxic to hMSCs

To determine if BALF samples from ARDS and HC lungs were associated with increased cell death, hMSCs were exposed *ex vivo* for 24 hours to individual clinical BALF samples. There was no significant difference in toxicity between hMSCs exposed to ARDS or HC BALF samples as determined by light microscopy (**Figure 1A**) and by LDH release, although there was one outlier in the ARDS BALF-exposure group (**Figure 1B**). To further determine toxicity, mitochondrial respiration in hMSCs exposed to different BALFs was assessed. Neither HC or ARDS BALF significantly affected altered hMSC basal respiration rate (**Figure 1C**), maximal respiration rate (**Figure 1D**), or spare respiratory capacity (**Figure 1E**) compared to PBS-exposed hMSCs. Interestingly, a significant reduction in spare respiratory capacity was similarly observed in ARDS and HC BALF-exposed hMSCs compared to control hMSCs (serum free media only) ($p=0.030$ and $p=0.034$, respectively, **Figure 1E**). However, this reduction was also observed in PBS exposed hMSCs compared to unstimulated control hMSCs ($p=0.059$, **Figure 1E**). No differences were

observed in mitochondrial proton leak, ATP-production, coupling efficiency, or non-mithochondrial oxygen consumption between hMSCs exposed to HC vs ARDS BALF (**Supplemental Figure 1**). The lack of visible cytotoxicity, LDH release into conditioned media, and mitochondrial functional abnormality (**Supplemental Figure 1**) suggested that, at least in the first 24 hours, the BALF samples used in this study were not toxic to the hMSCs.

ARDS and HC BALF activate hMSCs to release a spectrum of pro-inflammatory and some anti-inflammatory mediators

Level of IL-6 (p=0.0034 and p=0.0257, respectively) and other pro-inflammatory mediators such as IL-8 (p=0.0008 and p=0.0084, respectively) and IL-18 (p=0.0591 and p=0.0157, respectively) were increased in hMSC-conditioned media following exposure to either HC or ARDS BALF samples compared to PBS-exposed hMSCs (**Figure 2A-C**, **Table 3**). Moreover, significantly increased levels of CD44 (p=0.0041 and p=0.0342), respectively) and SP-D (p=0.0162 and p=0.0055, respectively) were comparably observed in hMSCs exposed to both ARDS and HC BALFs compared to PBS-exposed controls (**Figure 2D-E**, **Table 3**). Interestingly, hMSCs exposed to ARDS but not HC BALF samples induced significantly higher levels of HGF (p=0.0180) and in particular MMP-3 (p=0.0041) compared to controls (**Figure 2F-G**). In contrast, hMSCs exposed to HC but not ARDS BALF induced significantly higher CCL2 levels (p=0.0208) compared to controls (**Figure 2H**). Taken together, these data suggest that hMSCs exposed to both HC or ARDS BALF samples are activated to release a spectrum of mostly pro- but also anti-inflammatory cytokines with both overlap and differences with respect to specific mediators. These data suggest that hMSCs can acquire both pro- and anti-inflammatory phenotype in response to specific mediators present or absent in the BALF.

BALF IL-1β predicts hMSC cytokine secretion

We thus next determined whether specific BALF cytokines correlated with hMSC inflammatory mediator production. Notably, IL-1β in both ARDS or HC BALF samples was predictive of the presence of several inflammatory and apoptosis-inducing mediators in hMSC-conditioned media including IL-6 (p=0.0173), IL-36 (p=0.0334), IL-2 (p=0.0340), MMP-3 (p=0.0034), FAS (p=0.0427), and IL-8 (p=0.0346) (**Figure 3A, Table 4,** and **Supplementary Figure 2**). As shown in **Figure 3B**, expression of IL-1β was not correlated with other cytokines measured in BALF samples. However, expression of cytokines detected in conditioned media were frequently correlated with each other. For example, expression of IL-6 was demonstrated to be correlated with CCL2, CCL3, IL-8, HGF, IL-1β, IL-2, and IL-36β (**Figure 3C**). Interestingly, none of the cytokines measured in conditioned media were co-expressed with FAS (**Figure 3C**). These data suggest that the presence of higher concentrations of IL-1β in BALF samples may be used to predict presence a number of pro-inflammatory mediators and also FAS in hMSC-conditioned media. Similar observations were made with several other BALF cytokines, including IL-10 and IL12p70, as predictive of specific patterns of mediator production by the exposed hMSCs (**Supplementary Figure 2**). Taken together, these data suggest that presence of specific mediators and/or combined mediator patterns in BALF can predict hMSC behaviors.

HC BALF-exposed hMSCs demonstrate increased overall gene expression compared to ARDS BALF- and PBS-exposed hMSCs

To further probe BALF exposure effects on hMSC functions, HC and ARDS BALF-exposed hMSCs were analyzed by RNA sequencing and compared to PBS-exposed hMSCs. A heat map demonstrates the cytokine profiles of the individual BALF samples (5 each of HC or ARDS BALF

samples) utilized for hMSC exposures prior to RNA sequencing analyses (**Figure 4A).** These data demonstrate that both the HC and ARDS BALF samples utilized were representative of the full set of BALF samples analyzed (**Table 2**) and that the ARDS BALF samples contained higher concentrations of pro- and anti-inflammatory mediators compared to HC BALF samples. However, despite high levels of inflammatory mediators in ARDS BALF samples, RNA sequencing analysis demonstrated that HC BALF samples were more potent overall in inducing increased hMSC gene expression whereas ARDS BALF decreased gene expression compared to that induced by control PBS exposure (**Figure 4B**). Notably increased expression of many genes observed with HC BALF exposures were decreased with ARDS BALF exposure (**Figure 4B**). These data suggest that a normal non-inflamed lung environment induces expression of a range of hMSC genes while in contrast an inflammatory lung environment does not significantly induce but rather has an overall inhibitory effect on hMSC gene expression. Interestingly, the COVID-19 binding and entry receptors ACE2 and TMPRSS2 were expressed in the hMSCs, however only at minimal levels with no differences observed between the BALF exposure groups (**Supplementary Table 1**).

Exposure to HC but not ARDS BALF increases pro-inflammatory cytokine gene expression

hMSCs exposed to HC BALF samples demonstrated an overall increased expression of genes involved in multiple immune-regulatory pathways as compared to PBS-exposed (control) hMSCs, including TNF, ICAM-1, CXCL10, CCL2, CCL8, and IFN-β1 (**Figure 5A**). In striking contrast ARDS BALF-exposed hMSCs demonstrated expression of the majority of those genes at levels similar to those observed in PBS-exposed hMSCs. However, ARDS BALF-exposed hMSCs

demonstrated an increased expression of cytokines known to be involved in neutrophil trafficking including CXCL1, CXCL2 (MIP-2α), CXCL3, CXCL8/IL-8, and IL-6 (**Figure 5A**). However, these genes were expressed at levels lower than those observed following exposure to HC BALF. Further evaluations were undertaken to assess BALF exposure effect on hMSC expression of other inter-related genes associated with selected prominent inflammatory and other mediators for which both gene (**Figures 3B, F**) and protein levels (**Figure 2A** and **Supplementary Figure 3**) were increased following BALF exposures, specifically IL-6, IL-8, and FAS. Notably, HC BALF exposures significantly induced a range of IL-6 interacting genes whereas in contrast ARDS BALF exposures resulted in similar expression as observed in PBS-exposed hMSCs with only a significant increase in IL-6 gene expression itself. Interestingly, both HC and ARDS BALF exposure had an inhibitory effect on secretion of the anti-inflammatory cytokine IL-27 compare to PBS-exposed cells (**Figure 5B**). Similarly, a marked induction of a wide range of FAS interactive genes was only observed in hMSCs exposed to HC BALF samples (**Figure 5C**).

A similar pattern was observed for the pro-inflammatory cytokine IL-8 in which hMSC gene but not protein expression was increased following BALF exposures. Increase in expression of a range of IL-8 interacting genes was increased following HC BALF exposure with ARDS BALF exposure increasing expression of only a few genes (**Figure 5D**). Taken together, these data suggest that HC BALF exposure induces increased expression of genes involved in multiple immune-regulatory pathways. However, this is not seen in ARDS BALF-exposed hMSCs except for a few genes, some of which are involved in neutrophil trafficking.

Exposure to HC but not ARDS BALF increases complement gene and protein expression but not tissue factor or other coagulation cascade gene expression

hMSCs exposed to HC BALF samples demonstrated increased gene expression of complements C3b and C4a as well as the C3A complement receptor (C3AR) compared to PBS-exposed hMSCs (**Figure 6A**). In contrast, ARDS BALF-exposed hMSCs demonstrated only increased C3b expression with expression of other complement cascade genes at levels similar to those observed in PBS-exposed hMSCs (**Figure 6A**). Direct comparison of ARDS vs. HC BALF-exposed hMSCs demonstrated respective decrease in C4a and C3AR as well as C2a and BFb gene expression. Assessment of the conditioned media from BALF and PBS exposed to hMSCs obtained from two different donors demonstrated no detectable complement (C3) production by either HC BALF or PBS-exposed hMSCs (**Figure 6B-C**). However, low but detectable levels of complement were seen in some of the conditioned media from hMSCs exposed to ARDS BALF (**Figure 6B-C**).

Parallel assessment of coagulation-associated gene expression demonstrated no changes in tissue factor (TFP1) expression in hMSCs exposed to either HC or ARDS BALF as compared to PBS-exposed cells (**Figure 6D**). Isolated increase in gene expression of KNG1a, part of the intrinsic coagulation cascade was observed in HC-exposed hMSCs whereas isolated increase in SERPIN A1 (plasminogen activator-1) gene expression was observed in ARDS BALF-exposed hMSCs (**Figure 6D**). These results suggest that although complement-related gene and protein expression is increased following BALF exposure, unlike IBMIR, there is no increase tissue factor and only isolated changes in other coagulation cascade gene expression.

Exposure to HC but not ARDS BALF increases hMSC HLA gene and cell surface protein expression

We next assessed BALF exposure effects on expression of genes and proteins that might result in recognition of the MSCs by the host immune system, notably HLA expression. HC BALF exposure resulted in significantly increased expression of a number of HLA Class I and II genes and pseudogenes when compared to PBS-exposed hMSCs. These included classical (A, B, C) and non-classical (E, F, G) HLA Class I and also HLA Class II including DRA and DRB1, DMA, DMB, and DPA1 (major Class I and II genes depicted in **Figure 7A** with all HLA genes evaluated depicted in **Supplementary Figure 4)**. In contrast, exposure to ARDS BALF resulted in significant increase only in C and F and significant decrease in G, with no changes in A, B, or E compared to PBS-exposed hMSCs (**Figure 7A** and **Supplementary Figure 4**). Comparably, exposure to ARDS BALF resulted in significant increase only in DMA and significant decrease in DRA, with no changes in any of the other Class II genes. Strikingly, a significant decrease in expression of genes encoding for HLA class I genes A, B, and G, and HLA class II DRB1 was observed when comparing ARDS BALF to HC BALF-exposed hMSCs (**Figure 7A**).

To further validate these observations, cell surface hMSC HLA-ABC and DR protein expression was evaluated by flow cytometry following 24 hours exposure to HC vs. ARDS BALF using two different hMSC donors. These results demonstrated an upregulation of HLA-DR expression in HC BALF-exposed hMSCs (**Figure 7C, E**). In contrast, exposure to ARDS BALF or to PBS resulted in no upregulation of HLA-DR surface marker expression (**Figure 7C, E**). No change in HLA-ABC expression compared to control was observed after exposure to either BALF samples or PBS (**Figure 7B, D**). Scattergrams from these studies are shown in **Supplementary Figure 4**. These

results demonstrate that exposure of allogeneic hMSCs to a normal lung environment up-regulates expression of a non-self antigen recognized by the host immune system that will provoke clearance of the hMSCs. In contrast, hMSCs exposed to inflammatory ARDS BALF appear to be protected in this regard.

DISCUSSION

There remains a fundamental lack of knowledge as to the fate and actions of MSCs in clinical lung disease inflammatory environments. Here we found that hMSCs exposed to BALF collected from patients with ARDS behaved quite differently from the same hMSCs exposed to BALF from healthy volunteers. Not only was the pro-inflammatory response observed with healthy control BALF exposure blunted with ARDS BALF exposure but also the increased expression of genes and proteins associated with self- vs non-self recognition following healthy control BALF exposure, notably HLA-DR, that signal the host immune system to recognize the hMSCs as foreign and presumably initiate various clearance mechanisms. At least one pro-inflammatory cytokine in the BALF, IL-1β, was found to be predictive of hMSC behaviors. These are novel observations that provide insight into the potential mechanisms of action of MSC-based cell therapies in ARDS.

Our data support the tantalizing hypothesis that a mechanism similar to IBMIR may be responsible for the aggressive removal of hMSCs in healthy individual lungs. Similar to the IBMIR literature centered on blood exposure, when stimulated with BALF from healthy individuals, hMSCs responded by engaging in behaviors that will provoke an acute innate immune response including marked increase in pro-inflammatory, complement, and Class II HLA gene and protein expression (24, 25). In striking contrast, stimulation of the same hMSCs with BALF from ARDS patients either failed or comparatively resulted in decreased expression of these self antigen-encoding genes and proteins. However, in contrast to IBMIR, exposure of hMSCs to either HC or ARDS BALF did not increase tissue factor gene expression, an important observation supporting the safety of these cells in clinical practice. Notably, a recent study found that allogeneic MSC exposure to blood of trauma patients was less potent in inducing tissue factor expression than was exposure to healthy volunteer blood (26). This suggests that IBMIR can be affected by the patient's

inflammatory status but there is little other data in this regards. hMSCs are not well recognized as producing complement factors although they do produce anti-bacterial peptides such as LL-37 (27). Paradoxically, some of the ARDS but not healthy control BALF samples stimulated detectable levels of complement (C3a) in hMSC-conditioned media. At present, the significane of these findings remains unknown.

Human MSCs generally express low levels of HLA class I molecules A, B, and C and no constitutive expression of Class II DR molecules (23, 28, 29), attributes that have long been thought to minimize recognition of systemically administered allogeneic hMSCs by host immune surveillance mechanisms (30). In particular, HLA class II molecule expression is generally limited to antigen-presenting cells. However, both mouse and human MSC HLA-ABC and HLA-DR expression can be induced by exposure to several factors, including IFN-γ, which contributes to increased immune recognition and clearance of hMSCs (31, 32). Thus, the observed results demonstrating increased gene and cell surface protein expression of several Class II molecules following exposure to HC but not ARDS BALF further suggests that an intact normal allogeneic lung environment can activate hMSCs to participate in immune surveillance activities that may result in their own inactivation and clearance. Importantly, similar results were observed using hMSCs from two different donors, which strengthens the hypothesis that this is a general hMSC response. These observations parallel data from the literature showing hMSCs may persist for longer time in inflamed vs non-inflamed lungs presumably allowing for more opportunity to exert effects on inflammatory pathways (26, 33, 34). However, although IFN-γ was detected at low levels in both HC and ARDS BALF, there was no significant difference between groups except a non-significant trend towards increased IFN-γ in the ARDS BALF samples. As such, even though

exposure to BALF from HC patients triggers increased hMSC Class II HLA gene expression, this does not appear to be correlated with BALF IFN-γ. We acknowledge that HLA expression was assessed at only a single time point and that the kinetics of IFN-γ exposure and potential changes in related HLA gene and protein expression in response to BALF exposure remains unknown. An additional possible explanation is that low doses of IFN-γ have been demonstrated to have less effects on MSC immunosuppressive potency compared to higher doses of IFN-γ (35). An increased expression of the IFN-γ-dependent HLA class II transactivator C2Ta (also known as CIITA) was observed (data not shown), however the significane of this finding remains unknown (36).

Attempting to identify specific BALF components responsible for the observed effects yielded further novel observations. Notably, BALF IL-1β was significantly predictive of hMSC production of a range of important pro-inflammatory mediators including IL-2, IL-6, IL-8, IL-36, and MMP-3 as well as FAS. This suggests that IL-1β, commonly elevated in ARDS lungs, can drive hMSC behaviors and that blocking IL-1β may potentially alter MSC actions. However, the overall picture is complex, as demonstrated in **Figure 3** and **Supplemental Figure 2**, and a number of both pro- and anti-inflammatory cytokines in BALF correlated with hMSC production of both pro- and anti-inflammatory cytokines. These are somewhat surprising findings as, while inflammatory ARDS BALF is expected to influence hMSC activities, presumably non-inflamed HC BALF would be expected to do this to a lesser degree or not at all. This suggests that healthy lung environments, are capable of provoking inflammatory behaviors in allogeneic bone marrow-derived hMSCs. These results are also potentially consistent with IBMIR and that heatlhy non-inflamed lung

environments can both increase immune recognition of allogeneic MSCs as well as to activate them to produce a range of inflammatory mediators.

This study has several strengths. First, the hMSCs utilized are clinically relevant, having also been utilized in the recent START trial of systemic hMSC administration in ARDS patients (3, 4). Second, the BALF samples were assessed individually rather than as pooled samples in order to provide a more comprehensive understanding of the range of potential actions on the hMSCs. Importantly, the current findings are robust and reproducible across multiple individual healthy or ARDS BALF samples. Further, the effects on complement and HLA protein expression were observed with two different bone marrow-deerived MSC isolates obtained from different healthy volunteers. However, one caveat is that the BALF samples utilized came from a range of participating institutions and were obtained by different operators. As such, it is not possible to demonstrate uniformity in either bronchoscopy procedures utilized or in any potential sample dilutions. In addition, the ARDS and HC samples were stored differently, -20°C (ARDS) and -70°C (HC) respectively, however cytokine levels were higher in the ARDS samples which indicates that the proteins were intact and had not degraded. Further, given HIPAA limitations, no clinical data, including microbiologic data, on the underlying etiology of the ARDS patients is available except that for the ARDSNET clinical study under which these samples were obtained, patients with sepsis/septic shock were excluded (37). This will be important information for future studies given increasing understanding of the heterogeneity of the inflammatory environment in different ARDS patients (34). Similarly, limited clinical information is available for the HC patients. Nonetheless, the overall patterns observed within and between different ARDS and HC patients were robust. One additional significant caveat to this study is that the BALF samples

utilized were cell-free supernatants. As such, we speculate that the absence of direct toxicity following overnight hMSC exposure to the BALF samples (**Figure 1**) likely reflects the absence of immune effector cells that would then clear the hMSCs through efferocytosis and possibly other means of clearance and/or inactivation. Further investigations, for example with mixed lymphocyte studies utilizing BALF-exposed hMSCs or otherwise adding immune effector cells to the BALF samples, will further provide important information on the lung inflammatory environment effects on hMSC behaviors.

In summary, hMSC exposure to healthy lung environments induces expression of a range of genes encoding for inflammation and also for recognition as foreign to the host immune system. These changes are not observed or in some cases are opposite following hMSC exposure to inflammatory ARDS lung environments. Nonetheless, both environments provoke often comparable production and/or release of both pro- and anti-inflammatory-related mediators by the hMSCs. Further, selected components in the BALF are correlated with and in some cases predictive of hMSC mediator release. These observations provide a growing understanding of the complex interplay of inflammatory and other pathways involved in hMSC actions in the lung and provide important information towards developing more effective MSC-based cell therapies for ARDS and other lung diseases.

METHODS

BALF samples

BALF samples from ARDS patients without sepsis, with an APACHE II score >25, were collected prospectively as part of an unrelated clinical investigation conducted by the National Heart Lung Blood Institute (NHLBI) ARDSNET (ClinicalTrials.gov NCT0011216) (37). ARDS BALF samples were collected approximately 48 hours after enrollment under appropriate approved IRB protocols (UCSF) at the different participating institutions. For one of the complement ELISA and one of the flow cytometry measurements, ARDS BALF obtained on study day 3 were used, for all other studies ARDS BALF obtained on study day 1 was utilized. These samples were subsequently made available for the analyses in the current studies. Control BALF samples were obtained from healthy volunteers at Dartmouth under appropriate institutional IRB protocols. The BALF samples were centrifuged to pellet cells and debris and cell-free supernatants were stored at -20°C or -70°C prior to use in the current studies. All BALF samples were de-identified and numerically coded. Only limited clinical information is available on the patient samples.

BALF Assessments

Total protein was measured using Pierce BSA Protein Assay Kit (Thermo Scientific) (ARDS: n=6, healthy control: n=15). Total dsDNA content was measured using a Qubit Fluorometer dsDNA HS (10pg/ul-100ng/ul) assay (ARDS: n= 9, Healthy controls: n=15). Levels of pro- and anti-inflammatory cytokines were assessed using a Luminex assay as further described below. Some of the data from healthy control samples have also been presented in a previous publication and are used here for comparison purposes (13). IFN-γ was measured using ELISA MAX TM as further described below.

Ex vivo exposure of hMSCs

Human MSCs (hMSCs) were obtained from the NHLBI's Production Assistance for Cellular Therapies (PACT) program (University of Minnesota) and routinely cultured in MEM/EBSS media (Hyclone) supplemented with 20% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin (Lonza) in standard tissue culture incubators. The hMSCs utilized were obtained from a single volunteer (except for the complement ELISA and HLA the flow cytometry measurements in which bone marrow-derived hMSCs from a second donor were also utilized) and were the same as those utilized in the recent START trial of systemic MSC administration in ARDS patients (3, 4). Cells at passage 3-5 were used for experiments. For exposures, hMSCs were seeded (200,000 cells/6-well) in normal growth media and allowed to attach overnight. Following adherence, cells were washed and synchronized for 24 hours in serum free media (MEM/EBSS). After synchronization, media was replaced with individual ARDS or healthy control BALF samples diluted into serum free media (20% BALF final concentration as delineated in prior studies (11, 13). Serum free media with 20% PBS was added to unstimulated hMSCs, and serum free media only was added to control hMSCs. After 24 hours incubation (37°C), media was aspirated, cells washed with PBS, 1 ml serum free media added, and cells incubated for an additional 24 hours (37°C). Conditioned media was then collected for different analyses and cells lysed with TRIzol Reagent for RNA sequencing and other analyses.

Cytotoxicity

Following BALF exposure, hMSC viability was assessed using Pierce LDH Cytotoxicity Assay Kit (Thermo Scientific) according to manufacturer's instructions. Briefly, conditioned media samples were analyzed for LDH activity and absorbance was measured at 490 nm and 680 nm

using a spectrophotometer plate reader. hMSCs exposed to serum free media only served as negative control, Triton X-100-exposed hMSCs served as positive control, and serum free media served as background control. An AMG EVOS Cell Imaging System was utilized to obtain photomicrographs of cells following exposures to assess qualitative appearance. All samples and controls were analyzed in duplicate.

Mitochondrial Respiration

For real-time analysis of the oxygen consumption rate (OCR), hMSCs pre-exposed (24 hours) to BALF samples, PBS, or serum free media were analyzed using an XF-96^{e} Extracellular Flux Analyzer (Seahorse/Agilent, Bioscience). Briefly, hMSCs were seeded into XF-96^{e} Seahorse cell culture microplates (5 x 10^4 cells/well in 100 ul normal hMSC growth media) and allowed to attach overnight. After attachment, cells were either stimulated for 24 hours with individual ARDS (n=9) or healthy control (n=8) BALF samples (20% in serum free media), 20% PBS in serum free media (n=3), or serum free media (n=3). Prior to analyses, cells were equilibrated in a non-CO_2 incubator with XF running buffer (non-buffered DMEM supplemented with 5mM glucose and 2mM L-glutamine) and XF-96^{e} probe plates were hydrated and prepared according to manufacturer's instructions. OCR rates were measured under basal conditions and in response to 1µM Oligomycin, 1.5µM fluoro-carbonyl cyanide phenylhydrazone (FCCP), and 100nM Rotenone-Antimycin A. For the data acquisition protocol, three baseline reads were recorded, followed by injection of Oligomycin treatment, followed by the second injection of FCCP, followed by Rotenone/Antimycin-A, followed by 27 final reads. Data were analyzed using Wave software 2.6.1 (Agilent) and all samples were analyzed in 3-4 technical replicates.

Cytokine Analyses

Conditioned media and BALF samples were analyzed using a human magnetic Luminex Assay kit (R&D Systems). All samples were diluted 1:2 and analyzed using Bio-Rad Bioplex Analyzer in duplicate according to manufacturer's instructions. Analytes included ADAMTS13, CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CXCL8/IL-8, CD44, FAS, FAS Ligand, G-CSF, GM-CSF, HGF, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-13, IL-18, IL-36β/IL-1F8, Leptin, MIF, MMP-3, Osteopontin, SP-D, and TNF-α as previously described (13). Samples included BALF samples (ARDS patients (n=15) and healthy controls (n=15)) and conditioned media from ARDS BALF-exposed hMSCs (n=7), healthy control BALF-exposed hMSCs (n=10), and PBS-exposed hMSCs (n=7). Extrapolated values are presented as mean with SD and values out of range below were set to 1.0. The cytokine data from BALF samples of 7 of the healthy control subjects have been used as a control group in a previous publication (13). Cytokine values detected in conditioned media were log2 normalized in order to fit on the same scale.

IFN-γ was measured using ELISA MAX TM (BioLegend) on ARDS (n=15) and healthy control (n=14) BALF samples and on conditioned media from hMSCs exposed to ARDS BALF (n=3) and healthy control BALF (n=5) BALF or PBS (n=4) according to manufacturer's instructions.

RNA Isolation and Sequencing Analysis

Total RNA was extracted from either naïve (serum free media), PBS-exposed, or BALF-exposed hMSCs using standard Trizol extraction. In brief, cells were lysed with TRIzol Reagent (1ml per 6-well), samples were phase separated using chloroform, and RNA was isolated using 100% isopropanol followed by ethanol wash. RNA pellets were allowed to air dry and RNA were re-

suspended in RNase free waster prior to an additional cleaning step using RNeasy spin columns (Qiagen) according to manufacturer's instructions. RNA was quality assessed on a Fragment Analyzer instrument (Agilent) and quantified on a qubit fluorometer by the Genomics Shared Resource at the Geisel School of Medicine. RNA sequencing analyses were performed on RNA extracted from hMSCs exposed to PBS (n=4), ARDS BALF samples (n=5), and healthy control samples (n=5) and were aligned to human genes using salmon (38). Transcript level information from salmon was imported into R using tximport(39), normalized in edgeR based on library size to create counts per million (CPM) for each gene and differential gene expression was assessed. Pathway analysis genes that differed significantly from unstimulated control samples were identified using IPA (www.ingenuity.com).

Complement Assays

The complement cascade activation product c3a des (Arg) was measured in conditioned media collected from hMSCs exposed for 24 hrs to BALF samples or PBS 24 hours using an ELISA assay (Enzo LifeSciences; ADI-900-058) following the manufacturers' instructions.

Flow Cytometry

hMSCs were harvested after exposure to BALF samples (HC and ARDS) or PBS 24 hours and used for flow-cytometry analysis. The cells were fixed with 4% PFA solution and stained for DAPI (1:2500 dilution from 5mg/ml stock solution), HLA-ABC antibody conjugated to FITC (Biolegend; #311404), and HLA-DR antibody conjugated to AlexaFluor 647 (Biolegend; #307622) at the concentration recommended by manufacturers' protocol. The samples were run on BDLSRII Flow Cytometer and analyzed in FlowJo.

Statistical Analyses

Shapiro-Wilk test was used for normality test and data was presented as mean ± SD (normal distribution) or media ± IQR (non-normal distribution). Non-parametric Mann-Whitney test was used to assess differences between two groups. Kruskal-Wallis tests (Dunn's post hoc test) or one-way ANOVA (Dunnett's post hoc test) were used to assess differences between three or more groups. Statistical analyses were performed using GraphPad Prism software. P-values ≤ 0.05 were considered as significant, except in the case of RNA sequencing data analyzed in edgeR, where a multiple hypothesis corrected false discovery rates (FDR) less than 0.05 were considered significant. Spearman correlations were calculated in base R, using the t distribution to calculate p-values in those cases that included ties in rank.

AUTHOR CONTRIBUTIONS

S.R.E designed and performed experiments, analyzed and interpreted result, prepared figures and wrote the manuscript. T.H.H performed the statistical analysis, designed and analyzed the RNA sequencing experiment, prepared figures and reviewed the manuscript. J.B performed experiments, analyzed results, and reviewed the manuscript. D.H.M and A.A prepared and provided patient samples and cells, and reviewed the manuscript. E.A designed and interpreted results the extracellular flux analysis and reviewed the manuscript. C.d.S, K.D.L, B.A.S, A.D.K, A.D.K, K.E, M.A.M, and P.R.M.R interpreted results, reviewed the manuscript, provided comments and conducted discussions. D.J.W conceived strategies and designed experiments, interpreted results, and wrote the manuscript.

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REFERENCES

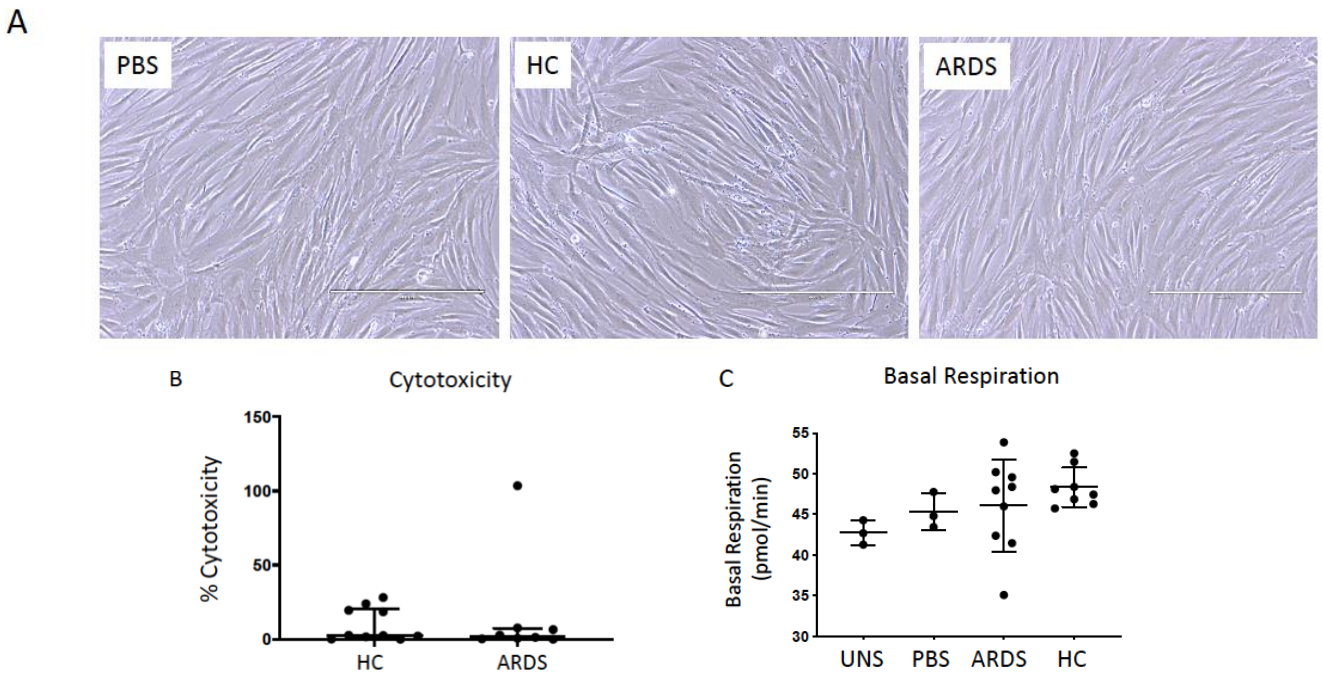
1. Matthay MA, Zemans RL, Zimmerman GA, Arabi YM, Beitler JR, Mercat A, et al. Acute respiratory distress syndrome. *Nat Rev Dis Primers.* 2019;5(1):18.
2. Khoury M, Cuenca J, Cruz FF, Figueroa FE, Rocco PRM, and Weiss DJ. Current Status of Cell-Based Therapies for Respiratory Virus Infections: Applicability to COVID-19. *Eur Respir J.* 2020.
3. Wilson JG, Liu KD, Zhuo H, Caballero L, McMillan M, Fang X, et al. Mesenchymal stem (stromal) cells for treatment of ARDS: a phase 1 clinical trial. *Lancet Respir Med.* 2015;3(1):24-32.
4. Matthay MA, Calfee CS, Zhuo H, Thompson BT, Wilson JG, Levitt JE, et al. Treatment with allogeneic mesenchymal stromal cells for moderate to severe acute respiratory distress syndrome (START study): a randomised phase 2a safety trial. *Lancet Respir Med.* 2019;7(2):154-62.
5. Simonson OE, Mougiakakos D, Heldring N, Bassi G, Johansson HJ, Dalen M, et al. In Vivo Effects of Mesenchymal Stromal Cells in Two Patients With Severe Acute Respiratory Distress Syndrome. *Stem Cells Transl Med.* 2015;4(10):1199-213.
6. Sara Rolandsson Enes GW-T. In: Janette K. Burgess IHH ed. *Stem Cell-Based Therapy for Lung Disease*. Springer; 2019.
7. Kusuma GD, Carthew J, Lim R, and Frith JE. Effect of the Microenvironment on Mesenchymal Stem Cell Paracrine Signaling: Opportunities to Engineer the Therapeutic Effect. *Stem Cells Dev.* 2017;26(9):617-31.
8. Xu AL, Rodriguez LA, 2nd, Walker KP, 3rd, Mohammadipoor A, Kamucheka RM, Cancio LC, et al. Mesenchymal Stem Cells Reconditioned in Their Own Serum Exhibit Augmented Therapeutic Properties in the Setting of Acute Respiratory Distress Syndrome. *Stem Cells Transl Med.* 2019;8(10):1092-106.
9. Islam D, Huang Y, Fanelli V, Delsedime L, Wu S, Khang J, et al. Identification and Modulation of Microenvironment Is Crucial for Effective Mesenchymal Stromal Cell Therapy in Acute Lung Injury. *Am J Respir Crit Care Med.* 2019;199(10):1214-24.
10. Bustos ML, Huleihel L, Meyer EM, Donnenberg AD, Donnenberg VS, Sciurba JD, et al. Activation of human mesenchymal stem cells impacts their therapeutic abilities in lung injury by increasing interleukin (IL)-10 and IL-1RN levels. *Stem Cells Transl Med.* 2013;2(11):884-95.
11. Abreu SC, Enes SR, Dearborn J, Goodwin M, Coffey A, Borg ZD, et al. Lung Inflammatory Environments Differentially Alter Mesenchymal Stromal Cell Behavior. *Am J Physiol Lung Cell Mol Physiol.* 2019.
12. Abreu SC, Xisto DG, de Oliveira TB, Blanco NG, de Castro LL, Kitoko JZ, et al. Serum from Asthmatic Mice Potentiates the Therapeutic Effects of

Mesenchymal Stromal Cells in Experimental Allergic Asthma. *Stem Cells Transl Med.* 2019;8(3):301-12.
13. Soraia C. Abreu THH, Evan Hoffman, Jacob Dearborn, Alix Ashare, Karatatiwant Singh Sidhu, Dwight E. Matthews, David H. McKenna, Eyal Amiel, Jayita Barua, Anna Krasnodembskaya, Karen English, Bernard Mahon, Claudia dos Santos, Fernanda F. Cruz, Daniel C. Chambers, Kathleen D. Liu, Michael A. Matthay, Robert A. Cramer, Bruce A. Stanton, Patricia R. M. Rocco, Matthew J. Wargo, Daniel J. Weiss, Sara Rolandsson Enes. Differential effects of the cystic fibrosis lung inflammatory environment on mesenchymal stromal cells. *American Journal of Physiology-Lung Cellular and Molecular Physiology.* 2020;In Press.
14. Pevsner-Fischer M, Morad V, Cohen-Sfady M, Rousso-Noori L, Zanin-Zhorov A, Cohen S, et al. Toll-like receptors and their ligands control mesenchymal stem cell functions. *Blood.* 2007;109(4):1422-32.
15. Tomchuck SL, Zwezdaryk KJ, Coffelt SB, Waterman RS, Danka ES, and Scandurro AB. Toll-like receptors on human mesenchymal stem cells drive their migration and immunomodulating responses. *Stem Cells.* 2008;26(1):99-107.
16. Liotta F, Angeli R, Cosmi L, Fili L, Manuelli C, Frosali F, et al. Toll-like receptors 3 and 4 are expressed by human bone marrow-derived mesenchymal stem cells and can inhibit their T-cell modulatory activity by impairing Notch signaling. *Stem Cells.* 2008;26(1):279-89.
17. Waterman RS, Tomchuck SL, Henkle SL, and Betancourt AM. A new mesenchymal stem cell (MSC) paradigm: polarization into a pro-inflammatory MSC1 or an Immunosuppressive MSC2 phenotype. *PLoS One.* 2010;5(4):e10088.
18. Romieu-Mourez R, Francois M, Boivin MN, Bouchentouf M, Spaner DE, and Galipeau J. Cytokine modulation of TLR expression and activation in mesenchymal stromal cells leads to a proinflammatory phenotype. *J Immunol.* 2009;182(12):7963-73.
19. Galleu A, Riffo-Vasquez Y, Trento C, Lomas C, Dolcetti L, Cheung TS, et al. Apoptosis in mesenchymal stromal cells induces in vivo recipient-mediated immunomodulation. *Sci Transl Med.* 2017;9(416).
20. Weiss DJ, English K, Krasnodembskaya A, Isaza-Correa JM, Hawthorne IJ, and Mahon BP. The Necrobiology of Mesenchymal Stromal Cells Affects Therapeutic Efficacy. *Front Immunol.* 2019;10:1228.
21. Weiss ARR, and Dahlke MH. Immunomodulation by Mesenchymal Stem Cells (MSCs): Mechanisms of Action of Living, Apoptotic, and Dead MSCs. *Front Immunol.* 2019;10:1191.
22. Galipeau J, and Sensebe L. Mesenchymal Stromal Cells: Clinical Challenges and Therapeutic Opportunities. *Cell Stem Cell.* 2018;22(6):824-33.

23. Moll G, Hult A, von Bahr L, Alm JJ, Heldring N, Hamad OA, et al. Do ABO blood group antigens hamper the therapeutic efficacy of mesenchymal stromal cells? *PLoS One.* 2014;9(1):e85040.
24. Hoogduijn MJ, Roemeling-van Rhijn M, Engela AU, Korevaar SS, Mensah FK, Franquesa M, et al. Mesenchymal stem cells induce an inflammatory response after intravenous infusion. *Stem Cells Dev.* 2013;22(21):2825-35.
25. Moll G, Jitschin R, von Bahr L, Rasmusson-Duprez I, Sundberg B, Lonnies L, et al. Mesenchymal stromal cells engage complement and complement receptor bearing innate effector cells to modulate immune responses. *PLoS One.* 2011;6(7):e21703.
26. George MJ, Prabhakara K, Toledano-Furman NE, Gill BS, Wade CE, Cotton BA, et al. Procoagulant in vitro effects of clinical cellular therapeutics in a severely injured trauma population. *Stem Cells Transl Med.* 2020;9(4):491-8.
27. Krasnodembskaya A, Song Y, Fang X, Gupta N, Serikov V, Lee JW, et al. Antibacterial effect of human mesenchymal stem cells is mediated in part from secretion of the antimicrobial peptide LL-37. *Stem Cells.* 2010;28(12):2229-38.
28. Wang Y, Huang J, Gong L, Yu D, An C, Bunpetch V, et al. The Plasticity of Mesenchymal Stem Cells in Regulating Surface HLA-I. *iScience.* 2019;15:66-78.
29. Kot M, Baj-Krzyworzeka M, Szatanek R, Musial-Wysocka A, Suda-Szczurek M, and Majka M. The Importance of HLA Assessment in "Off-the-Shelf" Allogeneic Mesenchymal Stem Cells Based-Therapies. *Int J Mol Sci.* 2019;20(22).
30. Ryan JM, Barry FP, Murphy JM, and Mahon BP. Mesenchymal stem cells avoid allogeneic rejection. *J Inflamm (Lond).* 2005;2:8.
31. Chinnadurai R, Copland IB, Garcia MA, Petersen CT, Lewis CN, Waller EK, et al. Cryopreserved Mesenchymal Stromal Cells Are Susceptible to T-Cell Mediated Apoptosis Which Is Partly Rescued by IFNgamma Licensing. *Stem Cells.* 2016;34(9):2429-42.
32. Stagg J, Pommey S, Eliopoulos N, and Galipeau J. Interferon-gamma-stimulated marrow stromal cells: a new type of nonhematopoietic antigen-presenting cell. *Blood.* 2006;107(6):2570-7.
33. Moll G, Drzeniek N, Kamhieh-Milz J, Geissler S, Volk HD, and Reinke P. MSC Therapies for COVID-19: Importance of Patient Coagulopathy, Thromboprophylaxis, Cell Product Quality and Mode of Delivery for Treatment Safety and Efficacy. *Front Immunol.* 2020;11:1091.
34. Reilly JP, Calfee CS, and Christie JD. Acute Respiratory Distress Syndrome Phenotypes. *Semin Respir Crit Care Med.* 2019;40(1):19-30.
35. Romieu-Mourez R, Francois M, Boivin MN, Stagg J, and Galipeau J. Regulation of MHC class II expression and antigen processing in murine

and human mesenchymal stromal cells by IFN-gamma, TGF-beta, and cell density. *J Immunol.* 2007;179(3):1549-58.
36. Tang KC, Trzaska KA, Smirnov SV, Kotenko SV, Schwander SK, Ellner JJ, et al. Down-regulation of MHC II in mesenchymal stem cells at high IFN-gamma can be partly explained by cytoplasmic retention of CIITA. *J Immunol.* 2008;180(3):1826-33.
37. Liu KD, Levitt J, Zhuo H, Kallet RH, Brady S, Steingrub J, et al. Randomized clinical trial of activated protein C for the treatment of acute lung injury. *Am J Respir Crit Care Med.* 2008;178(6):618-23.
38. Patro R, Duggal G, Love MI, Irizarry RA, and Kingsford C. Salmon provides fast and bias-aware quantification of transcript expression. *Nat Methods.* 2017;14(4):417-9.
39. Soneson C, Love MI, and Robinson MD. Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences. *F1000Res.* 2015;4:1521.

Figure 1.


A
PBS
HC
ARDS
B
Cytotoxicity
% Cytotoxicity
150
100
50
0
HC
ARDS
C
Basal Respiration
Basal Respiration (pmol/min)
55
50
45
40
35
30
UNS
PBS
ARDS
HC

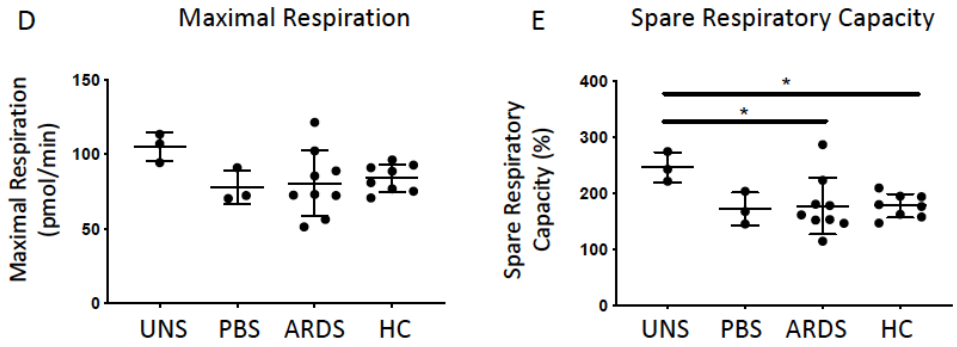


Figure 1. Exposure to BALF from ARDS patients and healthy controls are non-toxic to hMSCs. Representative phase contrast photomicrographs (10X) of hMSCs exposed for 24 hours to 20% BALF samples obtained from ARDS patients or from healthy control subjects (HC). PBS-exposed (20%) hMSCs were used as controls. Scale bar represents 400μm and photomicrographs have been brightness/contrast adjusted (**A**). Cytotoxicity was evaluated in conditioned medium utilizing a standard LDH assay following 24 hours exposure (ARDS: n= 8 and HC: n=10). Data are presented as median with interquartile range of % cytotoxicity (**B**). To assess the impact of ARDS (n=9) and healthy control BALF (n=8) samples on hMSC mitochondrial function, basal respiration (**C**), maximal respiration (**D**), and spare respiration capacity (**E**) was measured in hMSCs pre-exposed (24 hours) utilizing XF-96e Extracellular Flux Analyzer and compared to PBS-exposed (n=3) and unstimulated (serum free media only, n=3). Data are presented as means $\pm$ with SD and statistical analysis was performed by Shapiro-Wilk test, followed by a one-way ANOVA with Dunnett's post hoc test. Abbreviations: HC, healthy control; ARDS, acute respiratory distress syndrome; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline; uns, unstimulated (serum free media only); *, $p \leq 0.05$.

Figure 2.

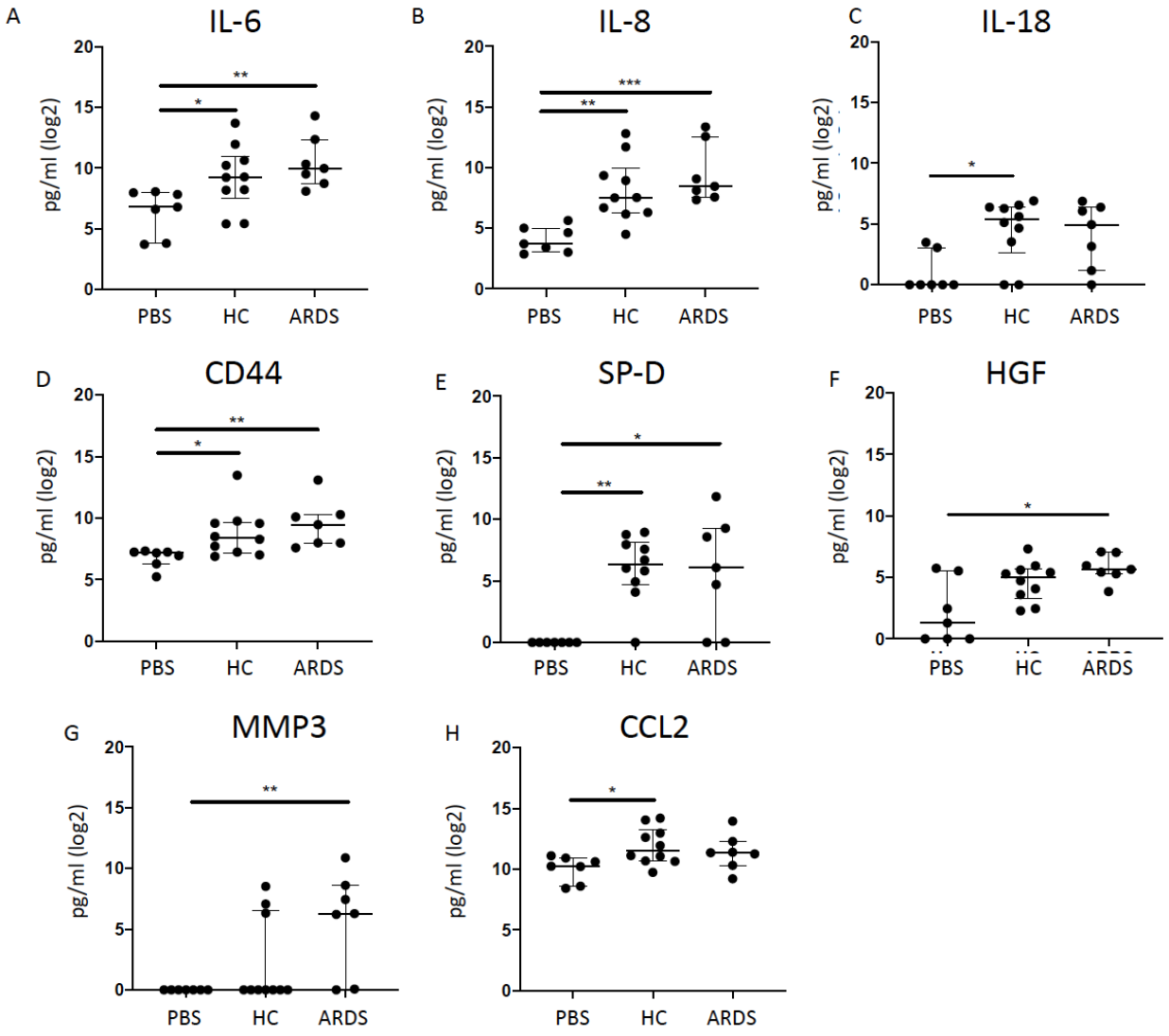


Figure 2. ARDS BALF as well as healthy control BALF exposure activates hMSCs to release a spectrum of mostly pro- but some anti-inflammatory mediators. To assess if the secretome profiles of hMSCs exposed to ARDS BALF (n=7) samples differed from healthy control BALF-exposed hMSCs (n=10) and PBS-exposed hMSCs (n=7), conditioned media after BALF or PBS exposure was assessed for a range of inflammatory and other mediators including IL-6 (**A**), IL-8 (**B**), IL-18 (**C**), CD44 (**D**), SP-D (**E**), HGF (**F**), MMP3 (**G**), and CCL2 (**H**). Data are presented as median with interquartile range of log2 normalized values, and statistical analysis was performed by Shapiro-Wilk test, followed by Kruskal-Wallis followed by Dunn's post hoc test by comparing to the unstimulated control group. Abbreviations: HC, healthy control; ARDS, acute respiratory distress syndrome; PBS, phosphate-buffered saline; IL-6, Interleukin 6; IL-8, Interleukin 8; IL-18, Interleukin 18; CD44, CD44 Molecule/Hyaluronate Receptor; SP-D, Surfactant protein D; HGF, Hepatocyte growth factor; MMP3, matrix metalloproteinase-3; CCL2, chemokine (C-C motif) ligand 2/ monocyte chemoattractant protein 1; *, $p \leq 0.05$; **, $p \leq 0.01$.

Figure 3.

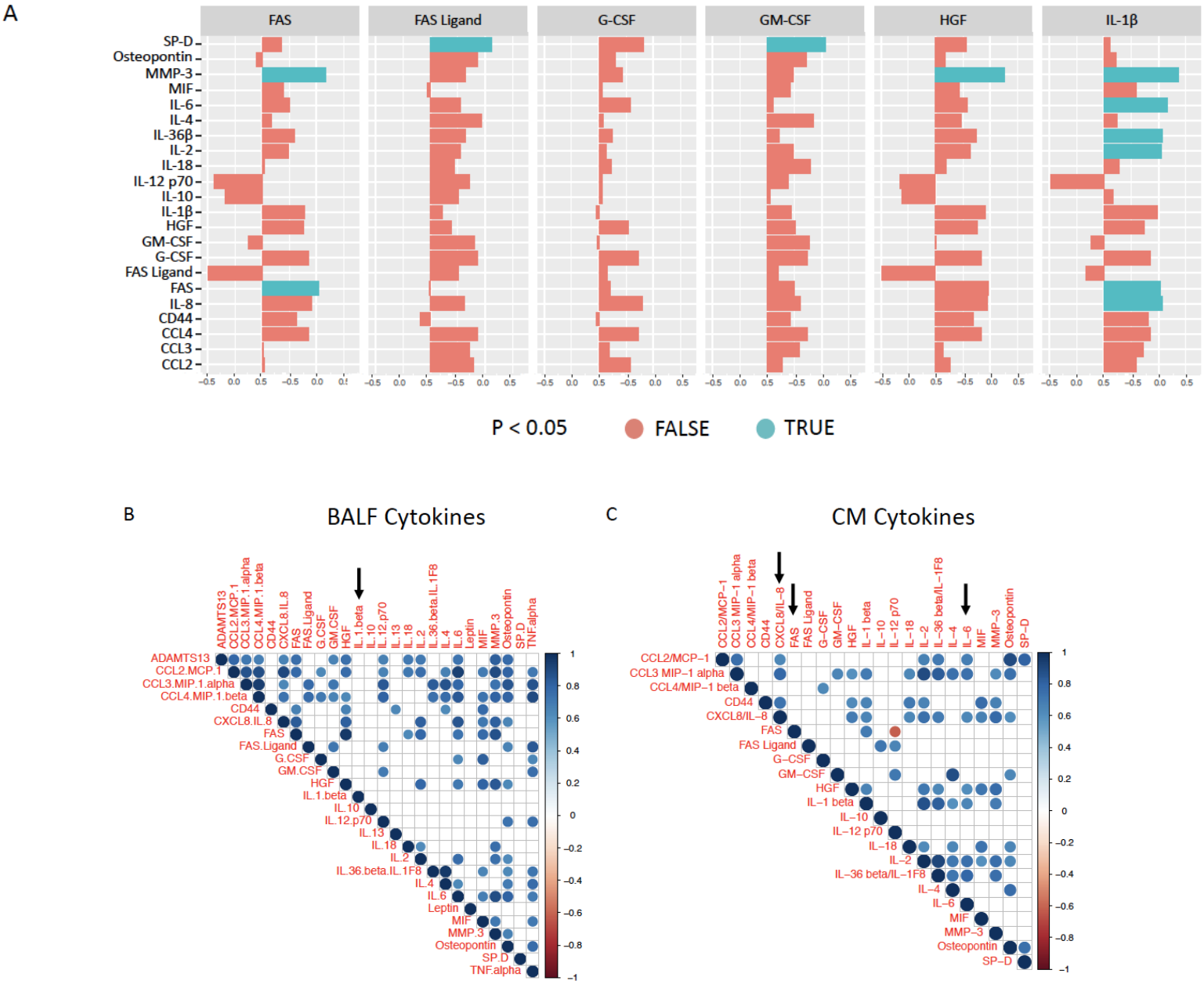


Figure 3. BALF IL-1β predicts hMSC pro-inflammatory cytokine secretion. Cytokines measured in BALF samples (top) were correlated with cytokines detected in conditioned media (left) from ARDS (n=6) and HC (n=10) BALF-exposed hMSC cultures (**A**). Red color indicates no significant difference ($p<0.05$) and blue color indicates significant difference ($p<0.05$). Cytokines measured in BALF samples were correlated with each other (**B**) and cytokines measured in conditioned media from BALF-exposed hMSC cultures was correlated with each other (**C**). Red color indicates significantly decreased expression ($p<0.01$) and blue color indicates significantly increased expression ($p<0.01$). Arrows indicate cytokines of specific interest (IL-1β , IL-6, and FAS). Spearman correlation was calculated in R. P-values are estimated and not exact because there were ties in the data.

Figure 4.

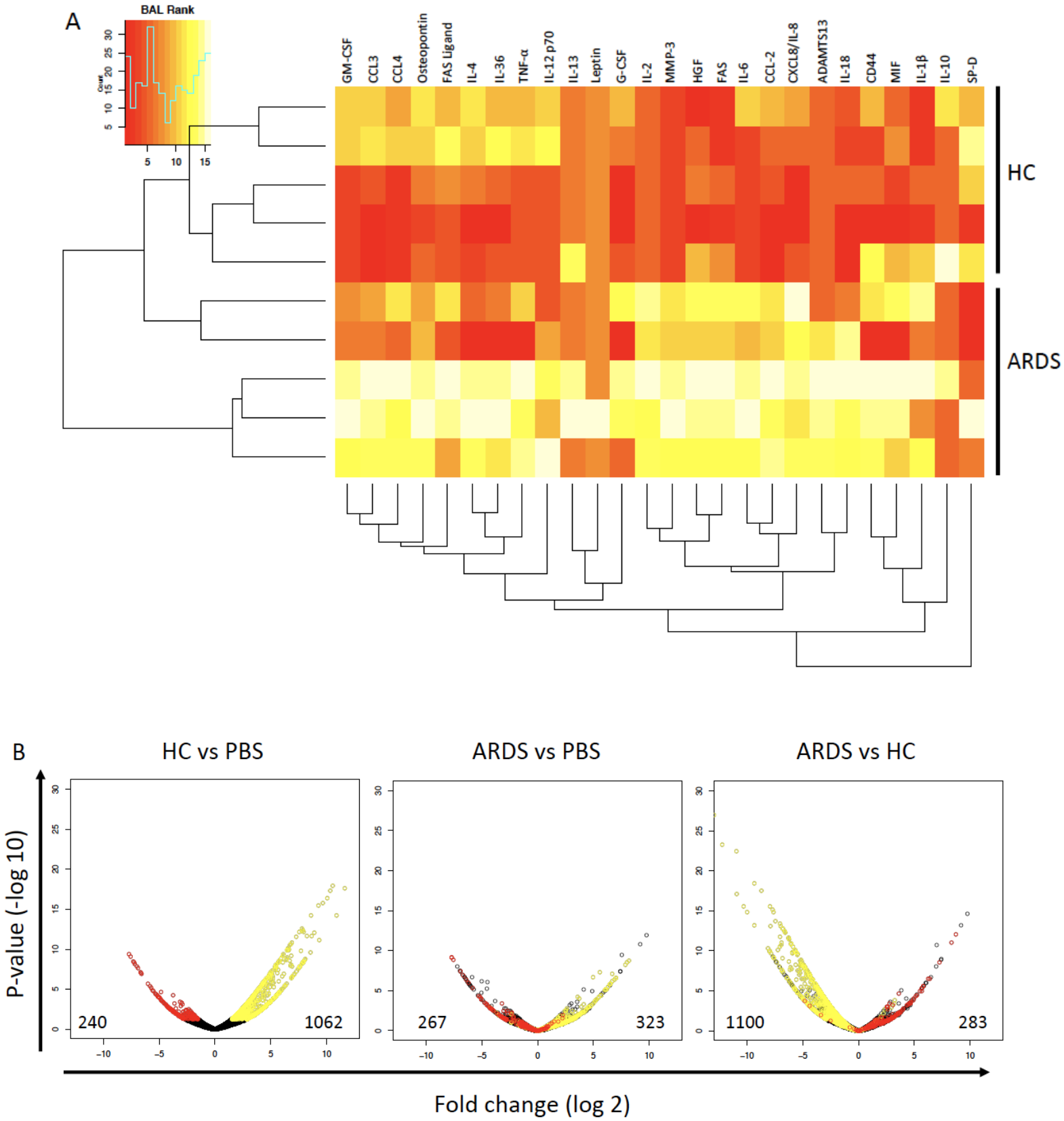
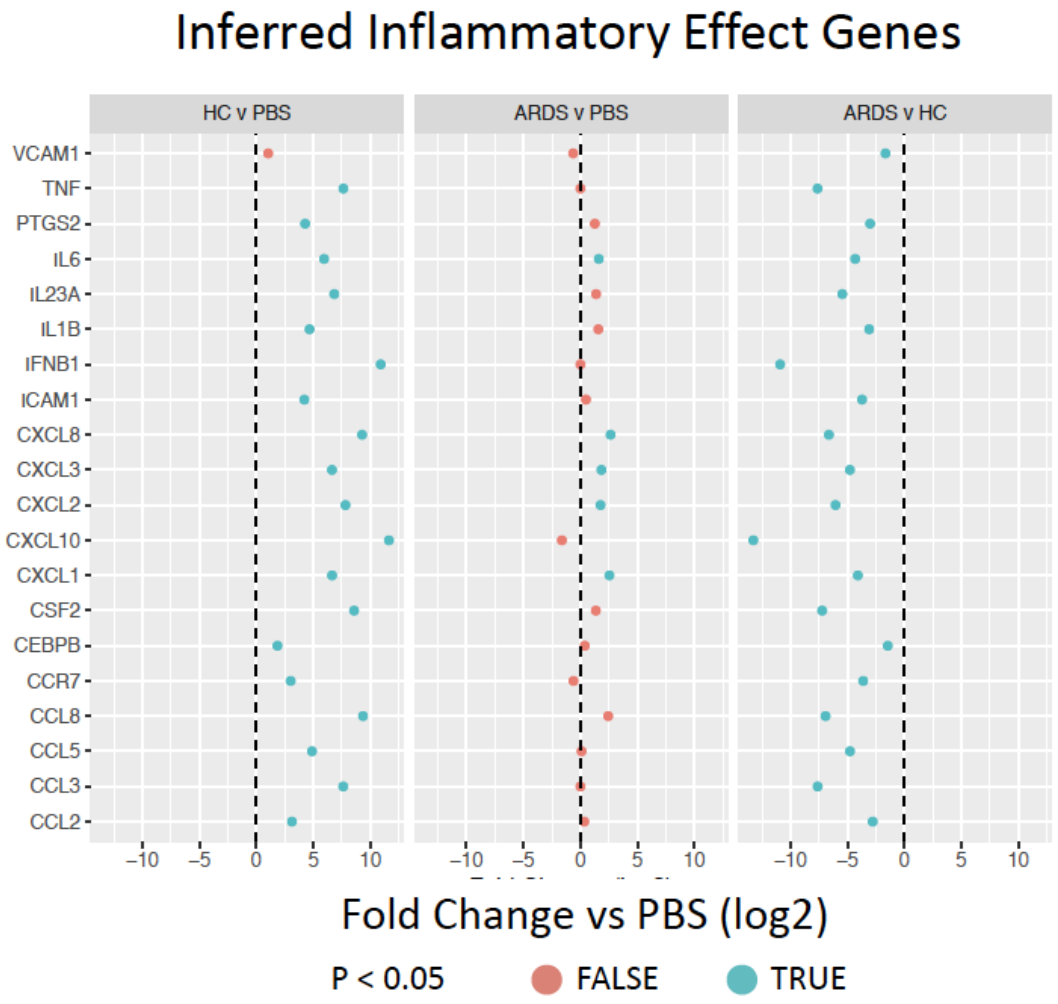

A
BAL Rank
GM-CSF
CCL3
CCL4
Osteopontin
FAS Ligand
IL-4
IL-36
TNF-α
IL-12 p70
IL-13
Leptin
G-CSF
IL-2
MMP-3
HGF
FAS
IL-6
CCL-2
CXCL8/IL-8
ADAMTS13
IL-18
CD44
MIF
IL-1β
IL-10
SP-D
HC
ARDS
B
HC vs PBS
ARDS vs PBS
ARDS vs HC
P-value (-log 10)
240
1062
267
323
1100
283
Fold change (log 2)

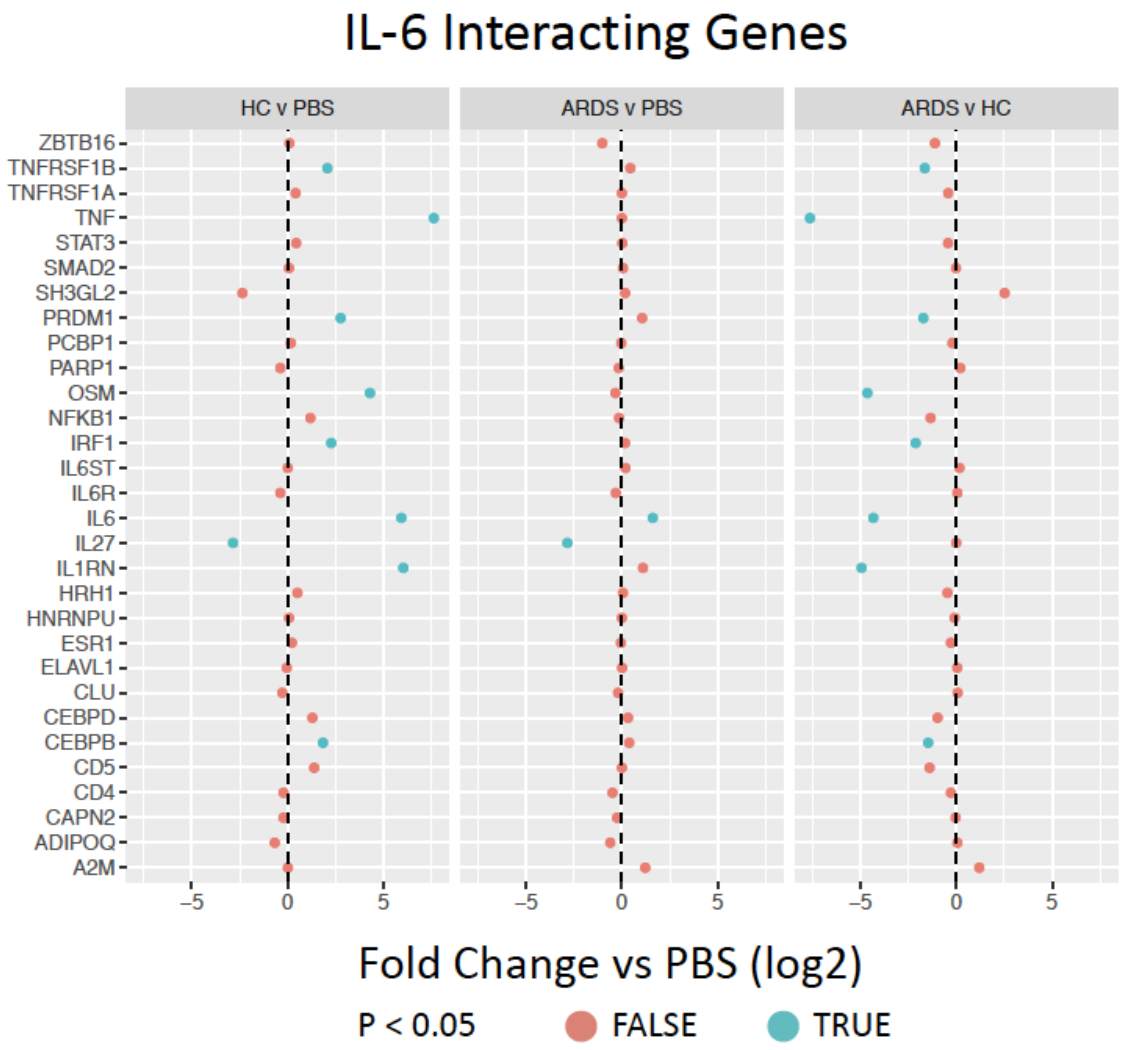
Figure 4. Healthy control-exposed hMSCs demonstrate increased gene expression compared to ARDS- and PBS-exposed hMSCs. Heat map clustering of ranked cytokines measured in ARDS (n=5) and healthy control (n=5) BALF samples utilized in the RNA sequencing analysis. Red color indicates lower and yellow color higher concentrations of cytokines (**A**). Volcano plots of the total number of genes identified by RNA sequencing of ARDS BALF (n=5), healthy control BALF (n=5), or PBS-exposed hMSCs (n=4). Red color indicates decreased and yellow color increased gene expression. Y-axis is -log10 p-value and X-axis is log2 fold change. Numbers indicate the numbers of up- or downregulated genes (**B**). Abbreviations: HC, healthy control; ARDS, acute respiratory distress syndrome; PBS, phosphate-buffered saline.

Figure 5.

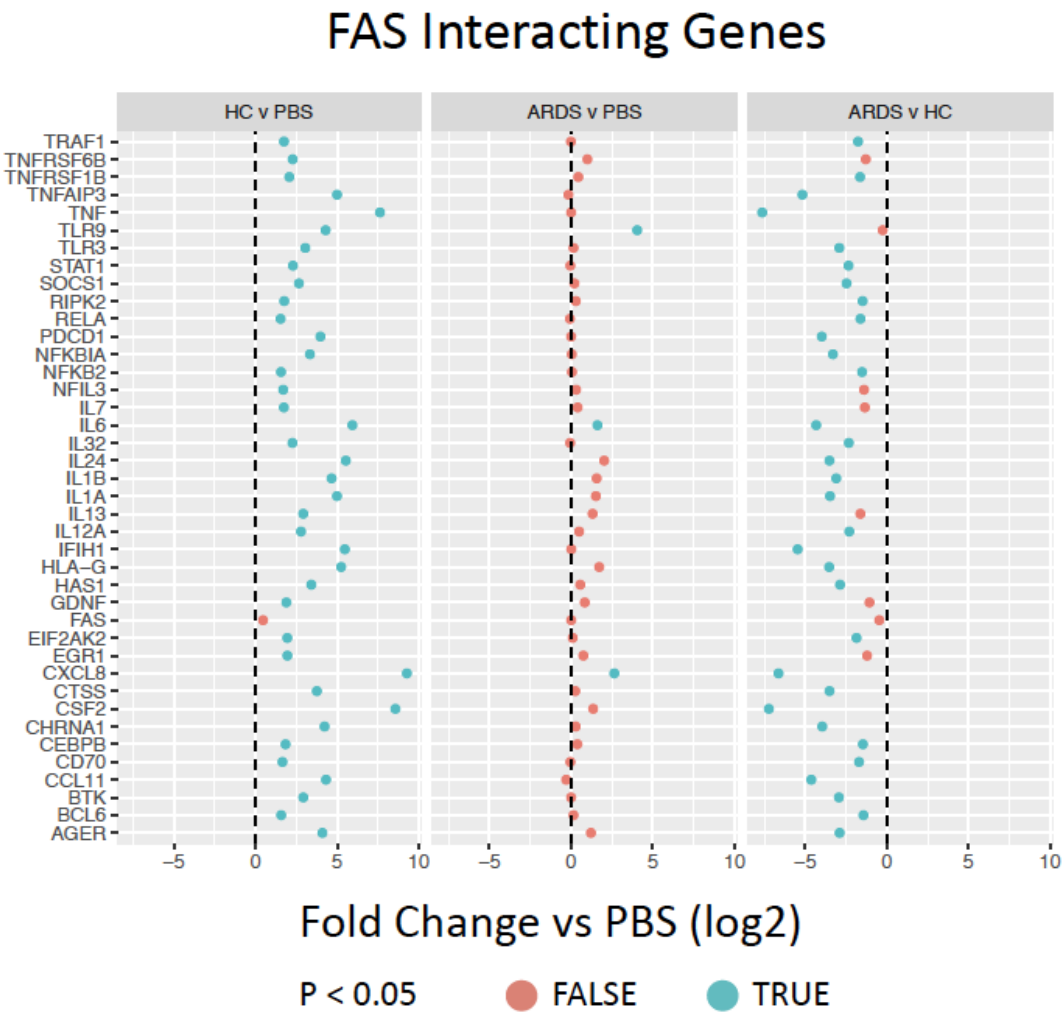
A


Inferred Inflammatory Effect Genes
HC v PBS
ARDS v PBS
ARDS v HC
VCAM1
TNF
PTGS2
IL6
IL23A
IL1B
IFNB1
ICAM1
CXCL8
CXCL3
CXCL2
CXCL10
CXCL1
CSF2
CEBPB
CCR7
CCL8
CCL5
CCL3
CCL2
−10
−5
0
5
10
Fold Change vs PBS (log2)
P < 0.05
FALSE
TRUE

B


IL-6 Interacting Genes
HC v PBS
ARDS v PBS
ARDS v HC
ZBTB16
TNFRSF1B
TNFRSF1A
TNF
STAT3
SMAD2
SH3GL2
PRDM1
PCBP1
PARP1
OSM
NFKB1
IRF1
IL6ST
IL6R
IL6
IL27
IL1RN
HRH1
HNRNPU
ESR1
ELAVL1
CLU
CEBPD
CEBPB
CD5
CD4
CAPN2
ADIPOQ
A2M
−5
0
5
Fold Change vs PBS (log2)
P < 0.05
FALSE
TRUE

C


FAS Interacting Genes
HC v PBS
ARDS v PBS
ARDS v HC
TRAF1
TNFRSF6B
TNFRSF1B
TNFAIP3
TNF
TLR9
TLR3
STAT1
SOCS1
RIPK2
RELA
PDCD1
NFKBIA
NFKB2
NFIL3
IL7
IL6
IL32
IL24
IL1B
IL1A
IL13
IL12A
IFIH1
HLA−G
HAS1
GDNF
FAS
EIF2AK2
EGR1
CXCL8
CTSS
CSF2
CHRNA1
CEBPB
CD70
CCL11
BTK
BCL6
AGER
−5
0
5
10
Fold Change vs PBS (log2)
P < 0.05
FALSE
TRUE

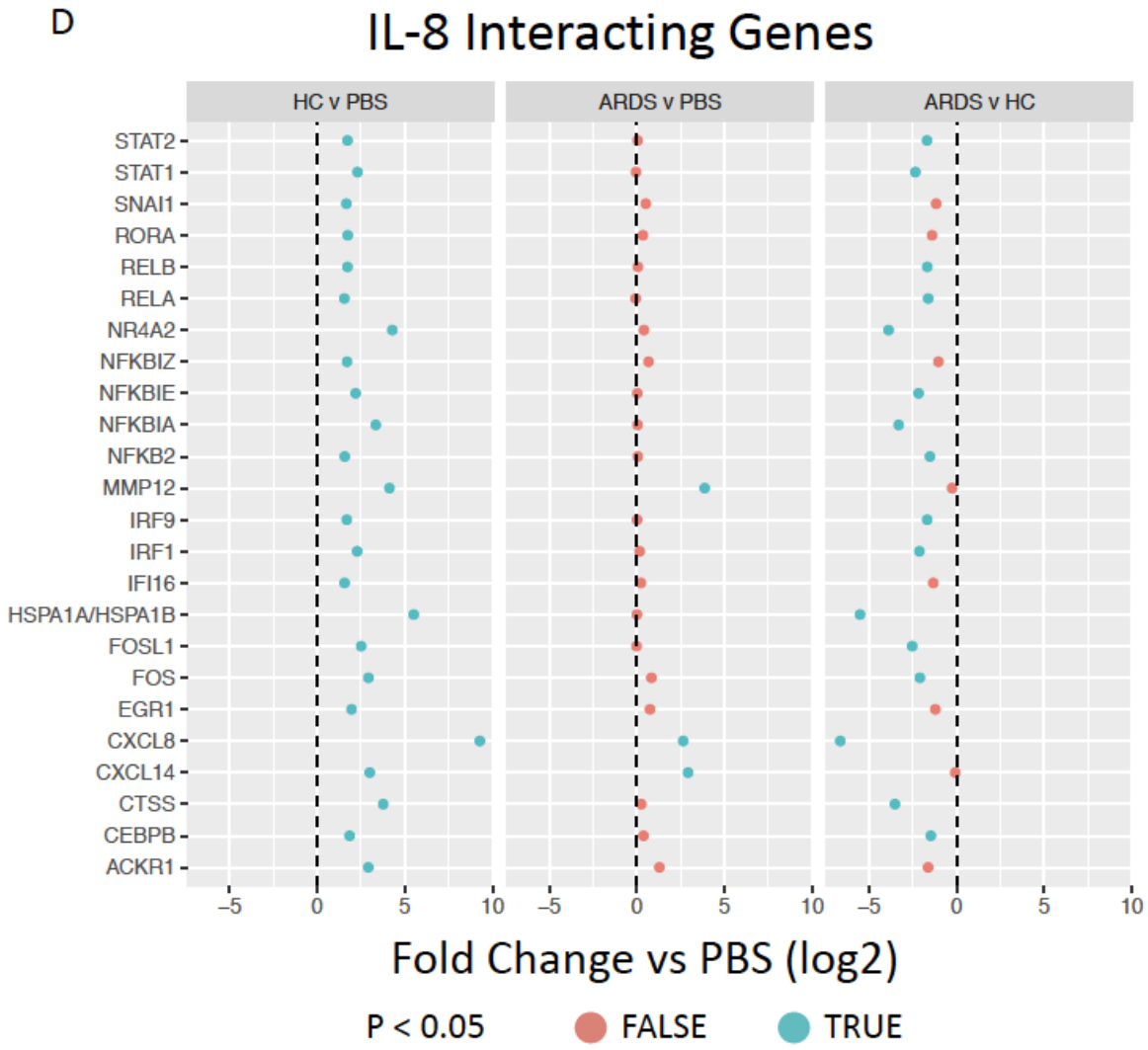
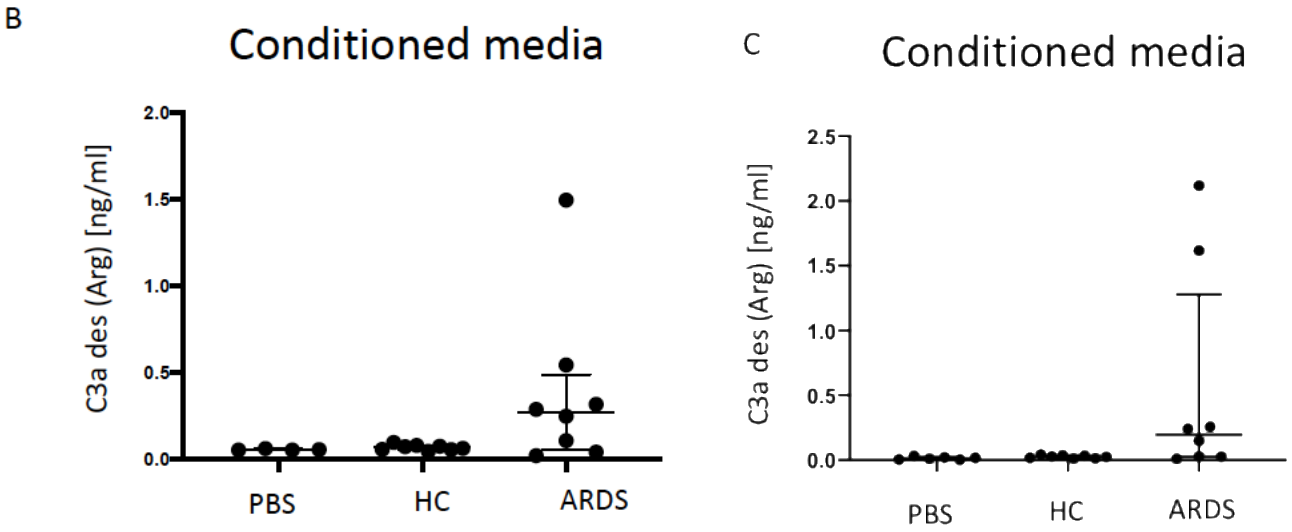


Figure 5. Exposure to healthy control but not ARDS BALF increases pro-inflammatory cytokine gene and interacting gene expression. Inferred inflammatory genes resulting from Ingenuity software data analysis including (**A**), IL-6 (**B**), CXCL8/IL-8 (**C**), and FAS (**D**) interacting genes expressed by ARDS-exposed (left, n=5) and healthy control BALF (right, n=5) samples were compared to PBS-exposed hMSCs (n=4). Red color indicates no significant different ($p>0.05$) and blue color indicates significantly difference ($p<0.05$). Data are presented as mean of log2 fold change.

Figure 6.


A
Complement Genes
HC v PBS
ARDS v PBS
ARDS v HC
SERPING1
MCP
MASP2
MASP1
ITGB2
ITGAX
ITGAM
IF
HF1
DF
DAF
CR2
CR1
CD59
C9
C7
C5AR
C5
C4a
C3b
C3AR
C2a
C1s
C1r
C1QBP
BFb
−3
0
3
6
Fold Change vs PBS (log2)
P < 0.05
FALSE
TRUE


B
Conditioned media
C3a des (Arg) [ng/ml]
2.0
1.5
1.0
0.5
0.0
PBS
HC
ARDS
C
Conditioned media
C3a des (Arg) [ng/ml]
2.5
2.0
1.5
1.0
0.5
0.0
PBS
HC
ARDS

D

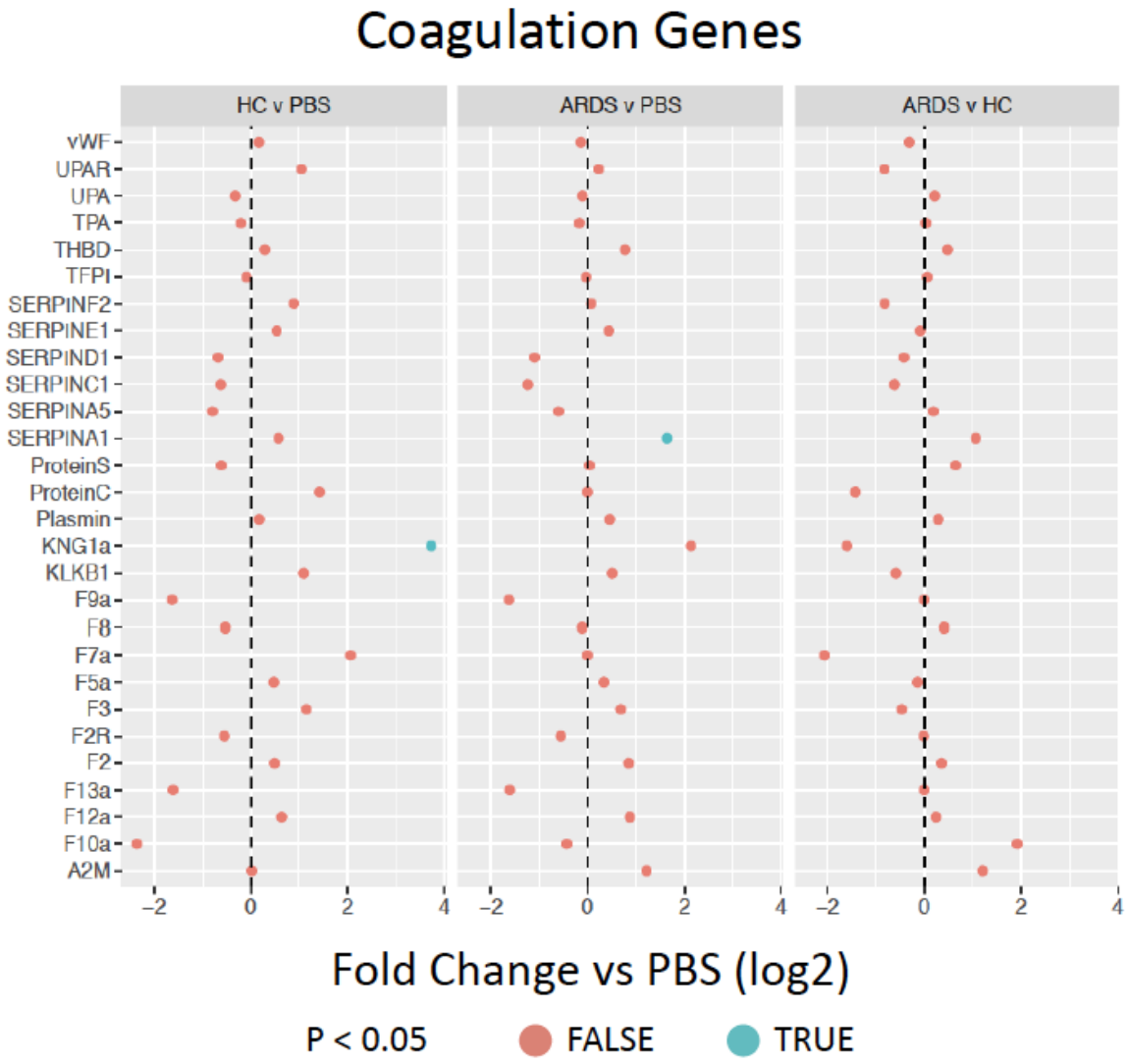


Figure 6. Exposure to healthy control but not ARDS BALF increases complement gene and protein but not coagulation cascade gene expression. Complement genes (**A**) expressed by ARDS-exposed (n=5) and healthy control BALF (n=5) samples were compared to PBS-exposed hMSCs (n=4). Levels of complement secreted into the conditioned media by HC or ARDS BALF compared to PBS-exposed hMSCs are depicted in **B-C**. Two different hMSC donors were used. Abbreviations: HC, healthy control; ARDS, acute respiratory distress syndrome; PBS, phosphate-buffered saline; TFP1, tissue factor. Coagulation cascade genes (**D**) expressed by ARDS-exposed (n=5) and healthy control BALF (n=5) samples were compared to PBS-exposed hMSCs (n=4). Gene expression data are presented as mean of log2 fold change. Complement (C3a) levels are depicted media with interquartile range.

Figure 7.

A


HLA Genes
HC v PBS
ARDS v PBS
ARDS v HC
HLA–G
HLA–DRB1
HLA–DRA
HLA–C
HLA–B
HLA–A
−5.0
−2.5
0.0
2.5
5.0
Fold Change vs PBS (log2)
P < 0.05
FALSE
TRUE

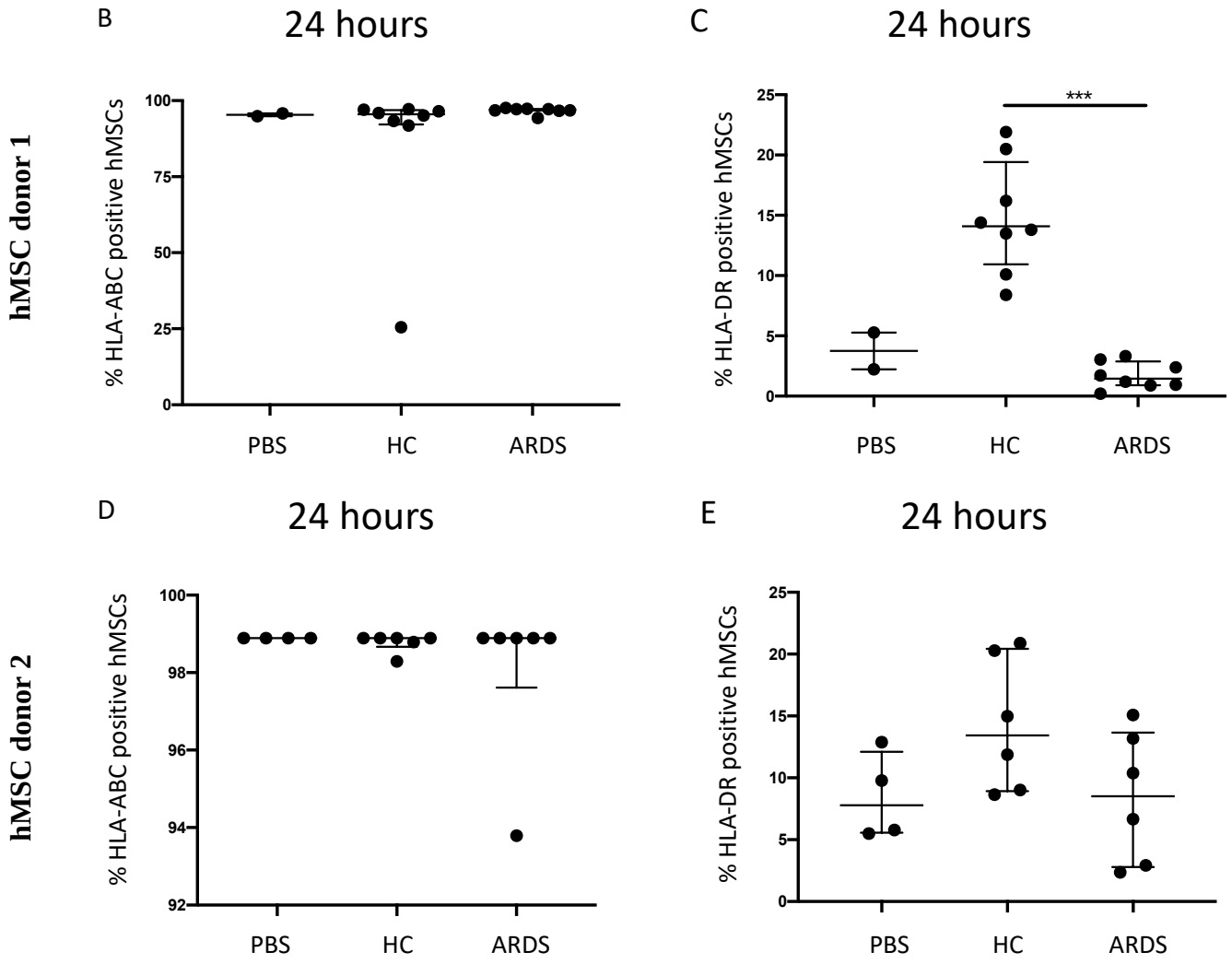


Figure 7. Exposure to healthy control but not ARDS BALF increases Class II hMSC HLA gene and protein expression. HLA genes expressed by ARDS-exposed (n=5) and healthy control BALF (n=5) samples were compared to PBS-exposed hMSCs (n=4) (**A**). Data are presented as mean of log2 fold change. HLA-ABC (**B, D**) and HLA-DR (**C**, **E**) surface marker expression was measured with flow cytometry on hMSCs exposed to PBS, HC BALF samples, and ARDS BALF samples for 24 hours. Two different hMSC donors were used. Data are presented as median with interquartile range of % positive cells – % positive cells on DAPI only stainings. Statistical analysis was performed by Kruskal-Wallis followed by Dunn's post hoc test. Abbreviations: HC, healthy control; ARDS, acute respiratory distress syndrome; PBS, phosphate-buffered saline; HLA, human leukocyte antigen.

Table 1. Delineation of each sample and how it was utilized in this study

Sample #	Status	Location	Study
1	HC	Dartmouth	RNA seq, CM luminex, BALF luminex, Seahorse, LDH, stimulation exp., dsDNA, osmolality, total protein, IFNγ BALF and CM, Flow cytometry, and C3a ELISA
2	HC	Dartmouth	RNA seq, CM luminex, BALF luminex, Seahorse, LDH, stimulation exp., dsDNA, osmolality, total protein, IFNγ BALF and CM, Flow cytometry, and C3a ELISA
3	HC	Dartmouth	RNA seq, CM luminex, BALF luminex, LDH, stimulation exp., dsDNA, osmolality, total protein, IFNγ BALF and CM, Flow cytometry, and C3a ELISA
4	HC	Dartmouth	RNA seq, CM luminex, BALF luminex, Seahorse, LDH, stimulation exp., dsDNA, osmolality, total protein, IFNγ BALF and CM, Flow cytometry, and C3a ELISA
5	HC	Dartmouth	RNA seq, CM luminex, BALF luminex, Seahorse, LDH, stimulation exp., dsDNA, osmolality, total protein, IFNγ BALF and CM
6	HC	Dartmouth	CM luminex, BALF luminex, Seahorse, LDH, stimulation exp., dsDNA, osmolality, total protein, Flow cytometry, and C3a ELISA
7	HC	Dartmouth	CM luminex, BALF luminex, Seahorse, LDH, stimulation exp., dsDNA, osmolality, total protein, IFNγ BALF, Flow cytometry, and C3a ELISA
8	HC	Dartmouth	CM luminex, BALF luminex, LDH, stimulation exp., dsDNA, osmolality, total protein, IFNγ BALF, Flow cytometry, and C3a ELISA, Flow cytometry, and C3a ELISA
9	HC	Dartmouth	CM luminex, BALF luminex, Seahorse, LDH, stimulation exp., dsDNA, osmolality, total protein, IFNγ BALF, Flow cytometry, and C3a ELISA, Flow cytometry, and C3a ELISA
10	HC	Dartmouth	CM luminex, BALF luminex, Seahorse, LDH, stimulation exp., dsDNA, osmolality, total protein, IFNγ BALF, Flow cytometry, and C3a ELISA, Flow cytometry, and C3a ELISA
11	HC	Dartmouth	BALF luminex, dsDNA, osmolality, total protein, IFNγ BALF
12	HC	Dartmouth	BALF luminex, dsDNA, osmolality, total protein, IFNγ BALF, Flow cytometry, and C3a ELISA, Flow cytometry, and C3a ELISA
13	HC	Dartmouth	BALF luminex, dsDNA, osmolality, total protein, IFNγ BALF, Flow cytometry, and C3a ELISA
14	HC	Dartmouth	BALF luminex, dsDNA, osmolality, total protein, IFNγ BALF
15	HC	Dartmouth	BALF luminex, dsDNA, osmolality, total protein, IFNγ BALF, Flow cytometry, and C3a ELISA
16	ARDS	UCSF	BALF luminex, LDH, stimulation exp., dsDNA, osmolality, IFNγ BALF, Flow cytometry (day3), and C3a ELISA (day3)
17	ARDS	UCSF	BALF luminex, dsDNA, osmolality, IFNγ BALF
18	ARDS	Fresno	RNA seq, CM luminex, BALF luminex, LDH, stimulation exp., dsDNA, osmolality, total protein, IFNγ BALF and CM, Flow cytometry (day3), and C3a ELISA (day3), Flow cytometry, and C3a ELISA
19	ARDS	Fresno	RNA seq, CM luminex, BALF luminex, Seahorse, LDH, stimulation exp., dsDNA, osmolality, total protein, IFNγ BALF and CM, Flow cytometry (day3), and C3a ELISA (day3)
20	ARDS	Fresno	BALF luminex, Seahorse, IFNγ BALF, Flow cytometry (day3), and C3a ELISA (day3)
21	ARDS	Fresno	CM luminex, BALF luminex, Seahorse, LDH, stimulation exp., dsDNA, osmolality, IFNγ BALF
22	ARDS	Fresno	BALF luminex, IFNγ BALF, Flow cytometry, and C3a ELISA
23	ARDS	Fresno	BALF luminex, Seahorse, IFNγ BALF
24	ARDS	OHSU	RNA seq, CM luminex, BALF luminex, Seahorse, LDH, stimulation exp., dsDNA, osmolality, total protein, IFNγ BALF, Flow cytometry (day3), and C3a ELISA (day3)
25	ARDS	Oregon	RNA seq, CM luminex, BALF luminex, Seahorse, LDH, stimulation exp., dsDNA, osmolality, total protein, IFNγ BALF
26	ARDS	Stanford	BALF luminex, IFNγ BALF
27	ARDS	USC	BALF luminex, IFNγ BALF
28	ARDS	USC	CM luminex, BALF luminex, Seahorse, LDH, stimulation exp., dsDNA, osmolality, total protein, IFNγ BALF, Flow cytometry (day3), and C3a ELISA (day3)
29	ARDS	USC	RNA seq, CM luminex, BALF luminex, Seahorse, LDH, stimulation exp., dsDNA, osmolality, total protein, IFNγ BALF and CM, Flow cytometry (day3), and C3a ELISA (day3)
30	ARDS	Bstate	BALF luminex, Seahorse, IFNγ BALF
31	ARDS	Oregon	Flow cytometry (day3), and C3a ELISA (day3)
32	ARDS	Oregon	Flow cytometry, and C3a ELISA
33	ARDS	Yale	Flow cytometry, and C3a ELISA
34	ARDS	Stanford	Flow cytometry, and C3a ELISA
35	ARDS	Stanford	Flow cytometry, and C3a ELISA
36	ARDS	USC	Flow cytometry, and C3a ELISA
37	ARDS	Bstate	Flow cytometry, and C3a ELISA

HC, healthy control subjects; ARDS, acute respiratory distress syndrome; BALF, bronchoalveolar lavage fluid; RNA Seq, RNA sequencing; CM, conditioned media; LDH, lactate dehydrogenase; dsDNA, double stranded DNA; IFNγ, Interferon gamma; Dartmouth, Dartmouth Hitchcock Medical Centre; UCSF, University of California, San Francisco; Fresno, University of California Fresno; USC, Univ. of Southern California; OHSU, Oregon Health & Science University; Stanford, Stanford University; Bstate, Bay State Medical Center, Day3, samples collected on study day 3.

Table 2. Mediators detected in BALF from ARDS patients and healthy controls

Cytokine	Cytokines in BALF (pg/ml)		Mann-Whitney
	HC BALF	ARDS BALF	
	Mean (SD)	*Mean (SD)*	*P-value*
ADAMTS13	606 (2342)	40454 (49330)	<0.0001 (****)
CXCL8/IL-8	85 (69)	3540 (2487)	<0.0001 (****)
Fas Ligand	3.1 (2.3)	20 (30)	0.0143 (*)
GM-CSF	4.4 (3.5)	55 (157)	0.0100 (*)
IL-10	3.4 (5.2)	2.0 (2.3)	0.5545
IL-13	25 (93)	843 (2140)	0.2241
IL-2	1.0 (0.0)	34 (34)	0.0002 (***)
IL-4	34 (15)	50 (40)	0.3295
Leptin	1.0 (0.0)	736 (1908)	0.2241
MIF	20415 (11140)	100999 (150610)	0.0408 (*)
CCL4	210 (167)	50575 (192241)	0.0006 (***)
Osteopontin	5926 (860)	47322 (129511)	<0.0001 (****)
TNF-α	2.0 (1.3)	42 (125)	0.0017 (**)
CD44	356 (215)	1099 (1557)	0.0408 (*)
Fas	99 (111)	2509 (3626)	<0.0001 (****)
G-CSF	50 (48)	505 (569)	0.0497 (*)
HGF	18 (9.9)	1081 (1605)	<0.0001 (****)
IL-1β	7.7 (8.6)	151 (377)	0.0068 (**)
IL-12 p70	43 (42)	86 (70)	0.0825
IL-18	34 (31)	67 (77)	0.2854
IL-36β	7.5 (3.9)	8.1 (5.7)	0.9589
IL-6	3.6 (5.8)	1268 (2599)	<0.0001 (****)
CCL2	68 (41)	4667 (5018)	<0.0001 (****)
CCL3	171 (98)	617 (981)	0.0168 (*)
MMP-3	16 (22)	988 (798)	<0.0001 (****)
SP-D	89435 (36372)	38750 (50805)	0.0017 (**)
IFN-γ #	4.6 (7.7)	25 (40)	0.1751

Other mediators	HC BALF	ARDS BALF	Unpaired t-test
	Mean (SD)	*Mean (SD)*	*P-value*
dsDNA (ng/ul)	0.3 (0.1)	10.2 (9.9)	0.0007 (***)
Total Protein (ug/ml)	110 (73.6)	1894 (2007)	0.0020 (**)

Cytokines detected in clinical ARDS BALF samples (n=15) and healthy control subjects (n=15) using 27-plex Luminex assay. Samples were analyzed on two luminex plates. Mean with SD of extrapolated values are presented and statistical analysis was performed by Mann-Whitney test. Values out of range below were set to 1.0. IFN-γ was measured on a separate ELISA on BALF samples from ARDS patients (n=15) and healthy control subjects (n=14) and presented as mean with SD and statistical analysis was performed by Mann-Whitney test. To further assess what inflammatory mediators might be present in ARDS BALF samples, dsDNA content (HC: n=15 and ARDS: n=9) and total protein (HC: n=15 and ARDS: n=6), were assessed and compared to those found in BALF obtained from normal healthy controls. Data are presented as mean with SD and statistical analysis was performed by unpaired t-test. BALF: bronchoalveolar lavage fluid; ARDS: acute respiratory distress syndrome; HC: healthy control subjects; # IFN-γ was measured on a separate ELISA; SD: standard deviation; * $p < 0.05$; **; $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Table 3. Cytokines detected in CM from hMSCs exposed to ARDS and healthy controls BALF samples

Cytokine	Cytokines in conditioned media (pg/ml)			Kruskal-Wallis with Dunn's Compared to UNS	
	UNS	HC	ARDS	HC	ARDS
	Mean (SD)	*Mean (SD)*	*Mean (SD)*	*P-value*	*P-value*
ADAMTS13	1.0 (0.0)	1.0 (0.0)	1.0 (0.0)	NA	NA
CXCL8/IL-8	20.9 (15.9)	1227.8 (2307.8)	2583.7 (4101.4)	0.0084 (**)	0.0008 (***)
Fas Ligand	1.0 (0.0)	1.3 (1.0)	1.0 (0.0)	0.3019	>0.9999
GM-CSF	1.8 (1.0)	2.5 (2.4)	5.7 (9.3)	>0.9999	0.3280
IL-10	1.0 (0.0)	1.2 (0.6)	1.0 (0.0)	0.6403	>0.9999
IL-13	1.0 (0.0)	1.0 (0.0)	1.0 (0.0)	NA	NA
IL-2	1.0 (0.0)	10.7 (22.5)	23.7 (39.7)	0.6189	0.2913
IL-4	1.0 (0.0)	6.2 (7.3)	10.8 (10.6)	0.2390	0.0457 (*)
Leptin	1.0 (0.0)	1.0 (0.0)	1.0 (0.0)	NA	NA
MIF	2056.0 (2742.6)	20575.8 (30710.7)	14619.7 (34703.7)	0.2957	0.3239
CCL4	1.0 (0.0)	1.0 (0.0)	96.6 (166.5)	>0.9999	0.1171
Osteopontin	7638.4 (937.2)	9799.8 (3483.3)	9289.5 (3196.5)	0.4702	0.6699
TNF-α	1.0 (0.0)	1.0 (0.0)	1.0 (0.0)	NA	NA
CD44	121.7 (46.4)	1508.0 (3486.9)	1786.9 (3101.3)	0.0342 (*)	0.0041 (**)
Fas	1.0 (0.0)	47.3 (79.6)	260.2 (493.0)	0.1293	0.0477 (*)
G-CSF	1.0 (0.0)	1.0 (0.0)	10.1 (24.0)	>0.9999	0.3809
HGF	15.8 (23.4)	41.7 (45.7)	68.0 (47.0)	0.2356	0.0180 (*)
IL-1β	1.0 (0.0)	4.1 (9.8)	32.0 (74.2)	>0.9999	0.2291
IL-12 p70	11.9 (10.9)	16.8 (29.4)	8.7 (13.2)	>0.9999	>0.9999
IL-18	3.5 (4.4)	49.9 (42.0)	44.4 (45.5)	0.0157 (*)	0.0591
IL-36β	1.0 (0.0)	1.3 (0.7)	2.3 (2.3)	>0.9999	0.3667
IL-6	140.0 (108.4)	2202.3 (4080.5)	4179.4 (7296.9)	0.0257 (*)	0.0034 (**)
CCL2	1268.4 (716.0)	6290.1 (6588.2)	4377.0 (5283.3)	0.0208 (*)	0.0903
CCL3	1.0 (0.0)	38.8 (67.3)	47.6 (80.6)	0.3145	0.3667
MMP-3	1.0 (0.0)	58.8 (117.8)	372.4 (681.0)	0.4444	0.0041 (**)
SP-D	1.0 (0.0)	163.8 (176.5)	682.3 (13344.2)	0.0055 (**)	0.0162 (*)
IFN-γ #	0.07 (0.14)	1.5 (2.2)	0 (0)	0.9276	>0.9999

Cytokines detected in conditioned media from hMSCs exposed to ARDS BALF samples (n=7), healthy control BALF samples (n=10), or PBS-exposed (n=7) using 27-plex Luminex assay. Samples were analyzed on two luminex plates. Mean with SD of extrapolated values are presented. Values out of range below were set to 1.0. IFN-γ was measured on a separate ELISA on conditioned media from hMSCs exposed to ARDS BALF samples (n=3), healthy control BALF samples (n=5), or PBS-exposed (n=4). Data are presented as mean with SD. Kruskal-Wallis tests (Dunn's post hoc test) was used to assess differences between ARDS/HC exposed hMSCs and unstimulated hMSCs. CM, conditioned media; hMSCs, human mesenchymal stromal cells; BALF, bronchoalveolarlavage fluid; UNS, unstimulated; ARDS, acute respiratory distress syndrome; HC, healthy control subjects; #, IFN-γ was measured on a separate ELISA; SD, standard deviation; NA, not available; * ≤ 0.05; ** ≤ 0.01, *** ≤ 0.001.

Table 4. Correlations of IL-1β in BALF with cytokines detected in conditioned media

Cytokines in CM	Pearson-correlating ranks	Number	
	P-value	HC	ARDS
IL-6	0.0173 (*)	10	6
IL-36	0.0334 (*)	8	5
IL-2	0.0340 (*)	8	5
MMP-3	0.0034 (**)	8	5
FAS	0.0427 (*)	9	5
IL-8	0.0346 (*)	10	6

IL-1β levels (ranked) detected in BALF samples were Pearson correlated with IL-6, IL-36, IL-2, MMP-3, FAS, and CXCL8/IL-8 levels (ranked) detected in conditioned media from BALF-exposed hMSCs. CM: conditioned media, HC: healthy control, ARDS: acute respiratory distress syndrome.