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### Authors

Enes, Sara Rolandsson  
Hampton, Thomas H  
Barua, Jayita  
[et al.](#)

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## Healthy versus Inflamed Lung Environments Differentially Effect MSCs

Sara Rolandsson Enes<sup>1,2</sup>, Thomas H. Hampton<sup>3</sup>, Jayita Barua<sup>1</sup>, David H. McKenna<sup>4</sup>, Claudia C. dos Santos<sup>5</sup>, Eyal Amiel<sup>6</sup>, Alix Ashare<sup>3,7</sup>, Kathleen D. Liu<sup>8</sup>, Anna D. Krasnodembskaya<sup>9</sup>, Karen English<sup>10</sup>, Bruce A. Stanton<sup>3</sup>, Patricia R. M. Rocco<sup>11,12</sup>, Michael A. Matthay<sup>8</sup>, Daniel J. Weiss<sup>1,\*</sup>

<sup>1</sup>Department of Medicine, Larner College of Medicine, University of Vermont, Burlington, VT, USA

<sup>2</sup>Department of Experimental Medical Science, Lung Biology Unit, Lund University, Lund, Sweden

<sup>3</sup>Department of Microbiology and Immunology, Geisel School of Medicine at Dartmouth, Hanover, NH, USA

<sup>4</sup>Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, USA

<sup>5</sup>Interdepartmental Division of Critical Care, Department of Medicine and the Keenan Center for Biomedical Research, St. Michael's Hospital, University of Toronto, Toronto, Canada

<sup>6</sup>Department of Biomedical and Health Sciences, College of Nursing and Health Sciences, University of Vermont, Burlington, VT, USA

<sup>7</sup>Section of Pulmonary and Critical Care Medicine, Dartmouth-Hitchcock Medical Center, Lebanon, NH, USA

<sup>8</sup>Departments of Medicine and Anesthesiology and the Cardiovascular Research Institute, University of California San Francisco

<sup>9</sup>Wellcome-Wolfson Institute for Experimental Medicine, School of Medicine, Dentistry, and Biomedical Sciences, Queens University, Belfast, UK

<sup>10</sup>Cellular Immunology Laboratory, Biology Department, Kathleen Lonsdale Institute for Human Health Research, Maynooth University, Maynooth, Co. Kildare, Ireland

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\*To whom correspondence may be addressed: Daniel J. Weiss, MD, PhD, Department of Medicine, University of Vermont, Burlington, VT USA, Phone: +1 802-847-1158, [daniel.weiss@med.uvm.edu](mailto:daniel.weiss@med.uvm.edu).

Author's contributions to the study:

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. All authors read and approved the final version of the manuscript.

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<sup>11</sup>Laboratory of Pulmonary Investigation, Carlos Chagas Filho Institute of Biophysics, Federal University of Rio de Janeiro

<sup>12</sup>National Institute of Science and Technology for Regenerative Medicine, Rio de Janeiro, Brazil

## 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 volunteers 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 volunteers. 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 expression was markedly up-regulated in hMSCs exposed *ex vivo* to BALF obtained from healthy volunteers. 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 beneficial for cell survival and actions. This further highlights the need to understand how different disease environments affect hMSC functions.

## Keywords

Cell Therapy; Inflammation; Acute Respiratory Distress Syndrome; Mesenchymal Stromal Cells; Bronchoalveolar Lavage Fluid

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## 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, 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]. *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 clearance and/or inactivation [14–17]. 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 [18–20].

Thus, to investigate the effects of the ARDS inflammatory lung environment on MSC 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 (HC) for comparison. Unexpectedly, hMSCs exposed to HC 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, changes in gene and protein expression associated with self- vs non-self recognition 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.

## METHODS

### BALF samples

BALF samples from ARDS patients without sepsis were collected prospectively as part of an unrelated clinical investigation conducted by the National Heart Lung Blood Institute (NHLBI) ARDSNET ([ClinicalTrials.gov NCT0011216](https://clinicaltrials.gov/ct2/show/study/NCT0011216)) [21]. ARDS BALF samples were obtained by mini-BAL from a phase 2 NIH trial conducted by the ARDSNET (PETAL) (ref 21, published in 2008). The technique utilized was a standard 40 ml mini-BAL utilizing sterile saline in intubated ARDS patients. BALF samples were subsequently centrifuged and stored at  $-80^{\circ}\text{C}$ . The healthy volunteers underwent standard fiberoptic bronchoscopy of the RML at Dartmouth under appropriate institutional IRB protocols. Twenty cc sterile saline was utilized and samples were comparably centrifuged and supernatants stored at  $-70^{\circ}\text{C}$ . These samples are more recent, being obtained between January and July 2018, and stored at  $-70^{\circ}\text{C}$ . Healthy volunteers were excluded if they had any history of cardiopulmonary

disease, if they smoked or vaped regularly, or were taking any immunomodulatory medications.

### Ex vivo exposure of hMSCs

Human MSCs (hMSCs) were obtained from the NHLBI's PACT program and routinely cultured. The hMSCs utilized were obtained from a single volunteer (a second donor was also used in the complement and flow cytometry experiments) and were the same as those utilized in the recent START trial [3, 4]. Cells at passage 3–5 were used. hMSCs were exposed to individual ARDS or HC BALF samples diluted into serum free media (20% BALF as delineated in prior studies [11, 13]) for 24 hours. Serum free media only or with 20% PBS was added to control and unstimulated hMSCs, respectively. After 24 hours incubation, cells and conditioned media (CM) was collected.

### RNA Sequencing Analysis

Total RNA was extracted using standard Trizol extraction followed by a cleaning step using RNeasy spin columns (Qiagen). RNA was quality assessed on a Fragment Analyzer instrument (Agilent) and quantified on a qubit fluorometer. RNA sequencing analyses were performed on RNA extracted from hMSCs exposed to PBS (n=4), ARDS BALF (n=5), and HC samples (n=5) and were aligned to human genes using salmon [22]. Transcript level information from salmon was imported into R using tximport [23], 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](http://www.ingenuity.com)).

### Statistical Analyses

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  $\leq 0.05$  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. Additional detailed descriptions of these and other methods used in this study are provided in the online supplement.

## 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 utilizing a human magnetic Luminex Assay kit (R&D Systems). The BALF samples utilized for each assay in the overall study are depicted in Supplementary 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 (Supplementary 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.

### **BALF from both ARDS and HC 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* 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 (Figure 1B). To further determine toxicity, mitochondrial respiration in hMSCs exposed to different BALFs was assessed. Neither HC or ARDS BALF significantly altered hMSC basal respiration rate, maximal respiration rate, spare respiratory capacity, or other mitochondrial functions compared to PBS-exposed hMSCs (Figure 1C–E, Supplementary Figure 1). 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) ( $p=0.030$  and  $p=0.034$ , respectively, Figure 1E). However, this reduction was also observed in PBS-exposed hMSCs compared to control hMSCs ( $p=0.059$ ).

### **ARDS and HC BALF activate hMSCs to release a spectrum of 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-CM following exposure to either HC or ARDS BALF samples compared to PBS-exposed hMSCs (Figure 2A–C, Table 1). 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 hMSCs (Figure 2D–E, Table 1). 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). 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-1 $\beta$ predicts hMSC cytokine secretion**

We next determined whether specific BALF cytokines correlated (Spearman correlations) with hMSC inflammatory mediator production. Notably, IL-1 $\beta$  in both ARDS or HC BALF samples was predictive of the presence of several inflammatory and apoptosis-inducing mediators in hMSC-CM 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, Supplementary Table 3, Supplementary Figure 2). As shown in Figure 3B, expression of IL-1 $\beta$  was not correlated with other cytokines measured in BALF samples. However, expression of cytokines detected in CM were frequently correlated with each other (Figure 3C). Interestingly, none of the cytokines measured in CM were co-expressed with FAS (Figure 3C). These data suggest that the presence of higher concentrations of IL-1 $\beta$  in BALF may be used to predict presence of pro-inflammatory mediators.

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

To further probe BALF exposure effects on hMSC functions, 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 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 (Supplementary Table 2). Despite high levels of inflammatory mediators in ARDS BALF samples, RNA sequencing 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). 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 4).

## Exposure to HC but not ARDS BALF increases pro-inflammatory cytokine genes

hMSCs exposed to HC BALF samples demonstrated an overall increased expression of genes involved in multiple immune-regulatory pathways as compared to PBS-exposed hMSCs, including TNF, ICAM-1, CXCL10, CCL2, CCL8, and IFN- $\beta$ 1 (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-2 $\alpha$ ), 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, 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 CM 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, 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, hMSC HLA-ABC and DR protein expression was evaluated by flow cytometry following 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 ARDS BALF behaved quite differently from the same hMSCs exposed to HC BALF. Not only was the pro-inflammatory gene response observed with HC BALF exposure blunted with ARDS BALF exposure but also the increased expression of genes and proteins associated with self- vs non-self recognition following HC BALF exposure. 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 HC BALF, 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 [19, 20]. 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 [24]. 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 [25]. Paradoxically, some of the ARDS but not HC BALF samples stimulated detectable levels of complement (C3a) in hMSC-CM. At present, the significance of these findings remains unknown.

Human MSCs generally express low levels of HLA class I molecules and no constitutive expression of Class II molecules [18, 26, 27], attributes that have long been thought to minimize recognition of systemically administered allogeneic hMSCs by host immune surveillance mechanisms [28]. However, HLA-ABC and HLA-DR expression can be induced by exposure to several factors, including IFN- $\gamma$ , which contributes to increased

immune recognition and clearance of hMSCs [29, 30]. Thus, the observed results demonstrating increased 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 [24, 31, 32]. However, although IFN- $\gamma$  was detected at low levels in both groups there was no significant difference. As such, even though exposure to HC BALF samples triggers increased Class II HLA expression, this does not appear to be correlated with BALF IFN- $\gamma$ . One possible explanation is that low doses of IFN- $\gamma$  have been demonstrated to have less effects on MSC immunosuppressive potency compared to higher doses [33]. An increased expression of the IFN- $\gamma$ -dependent HLA class II transactivator C2Ta was observed, however the significance of this finding remains unknown [34].

Notably, BALF IL-1 $\beta$  was significantly predictive of hMSC production of a range of important pro-inflammatory mediators. This suggests that IL-1 $\beta$ , commonly elevated in ARDS lungs, can drive hMSC behaviors and that blocking IL-1 $\beta$  may potentially alter hMSC actions. However, the overall picture is complex, as demonstrated in Figure 3 and Supplemental Figure 2. This suggests that healthy lung environments, are capable of provoking inflammatory behaviors in allogeneic bone marrow-derived hMSCs.

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. 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-derived MSC isolates obtained from different HC. 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 fully demonstrate uniformity in either bronchoscopy procedures utilized or in any potential sample dilutions. Further, the BALF samples utilized were obtained using different isolation protocols, were stored differently both at the originating institutions  $-80^{\circ}\text{C}$  (ARDS) and  $-70^{\circ}\text{C}$  (HC) and also in our laboratory at  $-20^{\circ}\text{C}$  (ARDS) and  $-70^{\circ}\text{C}$  (HC) respectively. As such, although we feel these difference unlikely to account for the current observations, further prospective studies will attempt to better control for these variable. 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 [21]. Similarly, limited clinical information is available for the healthy volunteers. 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 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 (15). 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.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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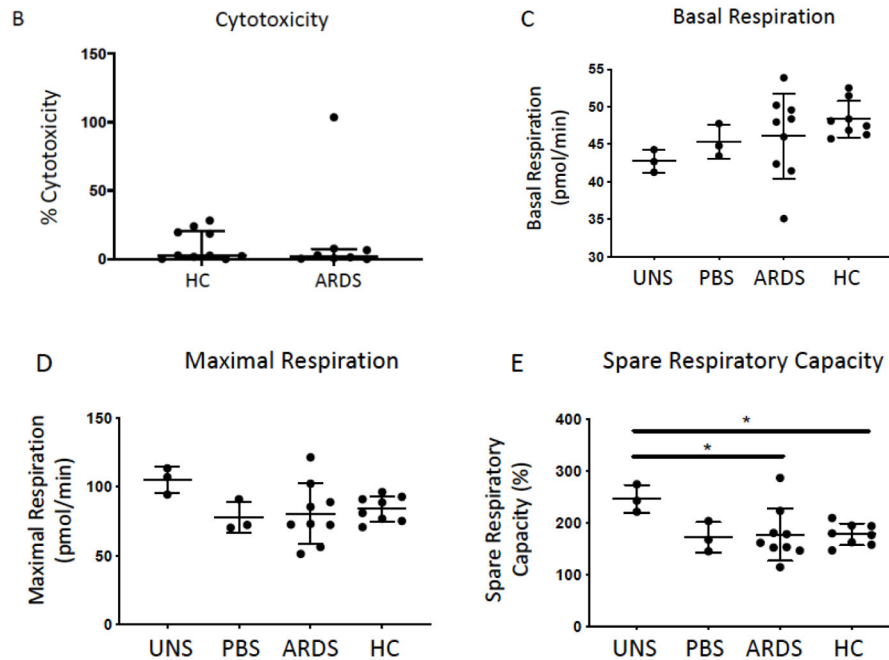
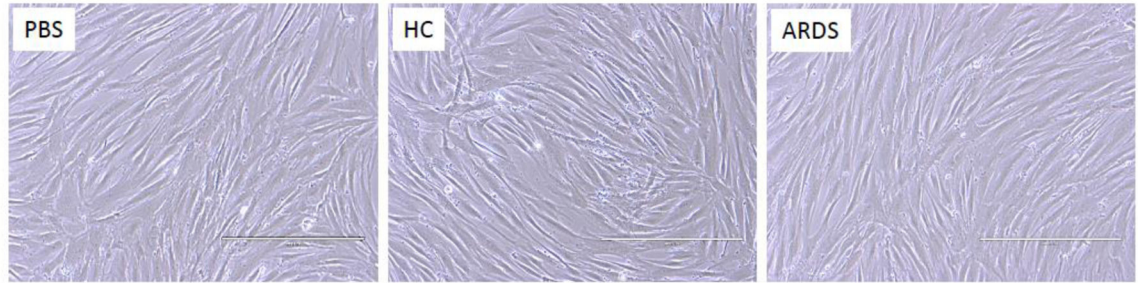
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**Take home message:**

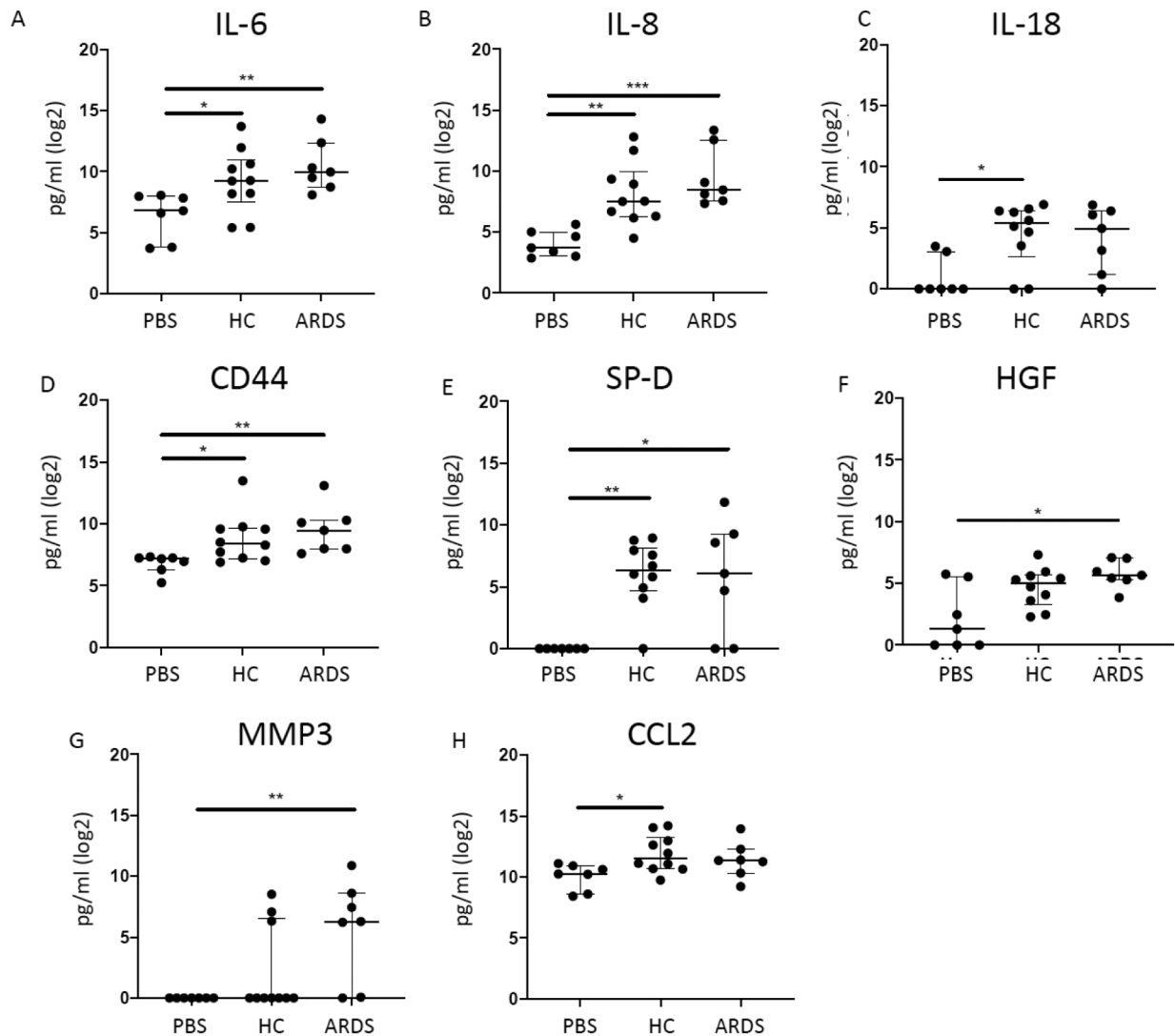
MSCs exposed to a healthy lung environment induces an inflammatory response with increased gene/protein expression associated with self vs non-self-recognition. These changes were either absent or opposite in MSCs exposed to an inflamed ARDS environment.

A



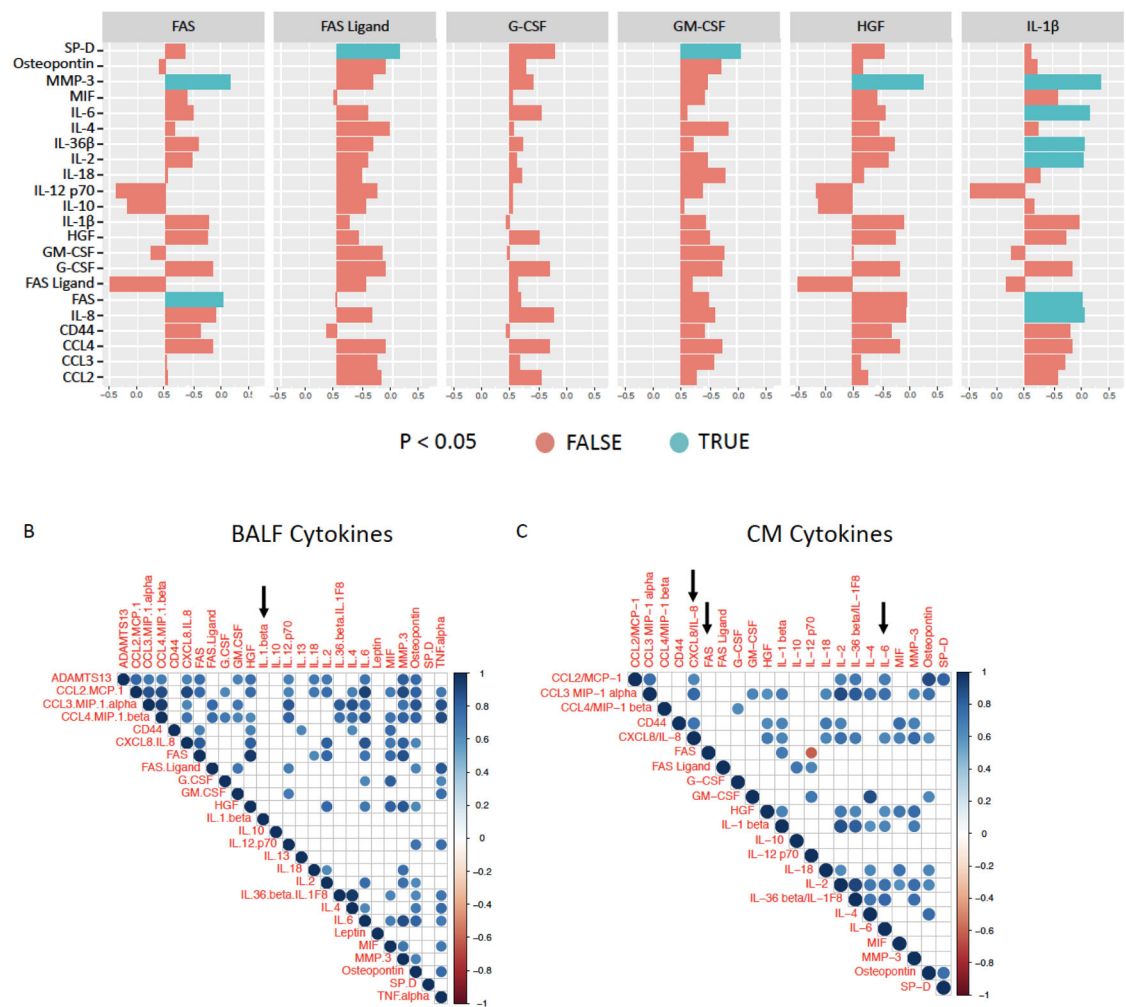
**Figure 1. Exposure to BALF from ARDS patients and healthy controls are non-toxic to hMSCs.** Representative phase contrast photomicrographs (10X) of hMSCs exposed for 24 hours to 20% BALF samples obtained from ARDS patients or from healthy control subjects (HC). PBS-exposed (20%) hMSCs were used as 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 0.05.





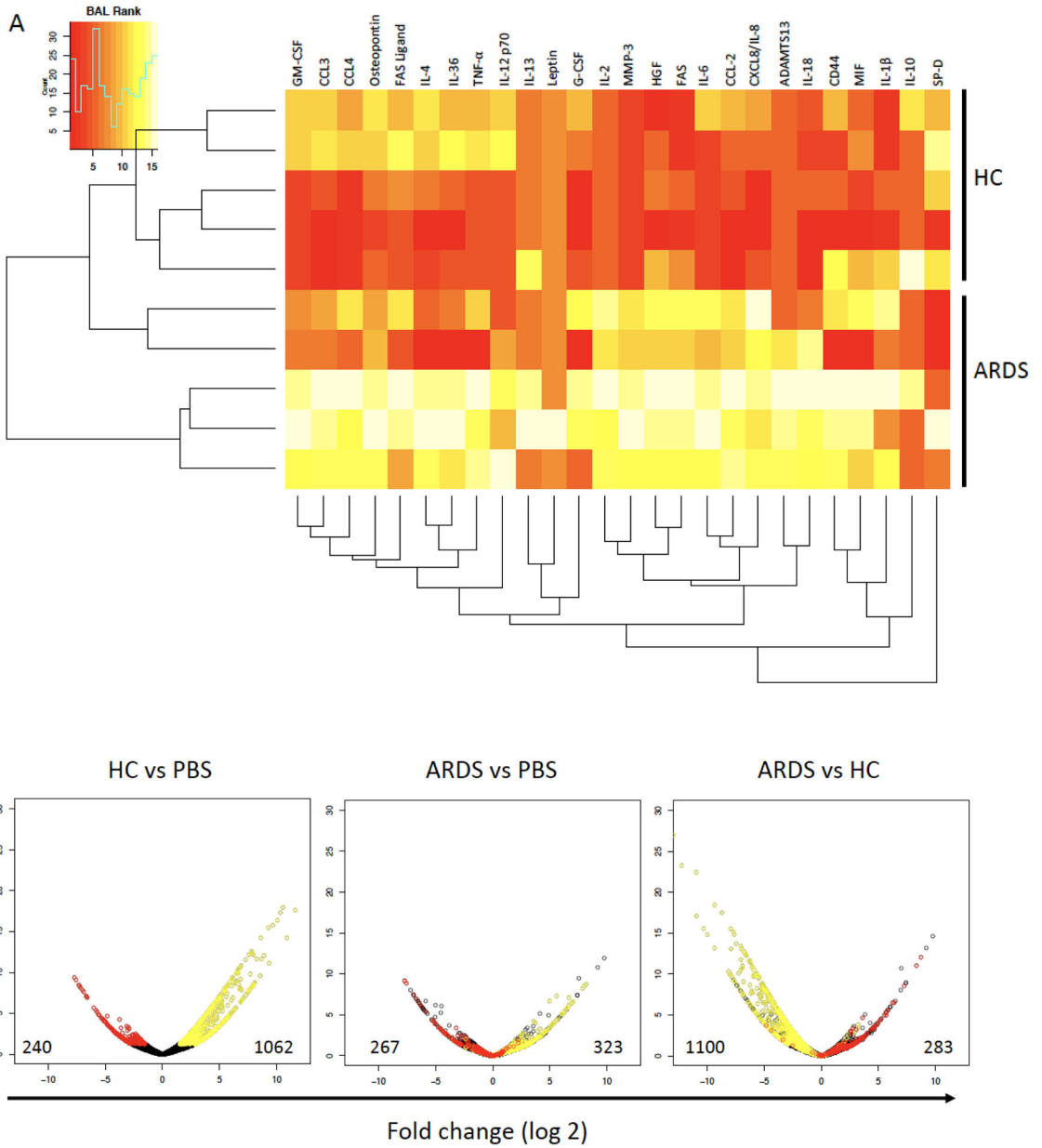
**Figure 2. ARDS BALF as well as healthy control BALF exposure activates hMSCs to release a spectrum of mostly pro- but some anti-inflammatory mediators.**

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 log<sub>2</sub> 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 0.05; \*\*, p 0.01. 0.01.



**Figure 3. BALF IL-1 $\beta$  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-1 $\beta$ , 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. 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 genes identified by RNA sequencing of ARDS BALF (n=5), healthy control BALF (n=5), or PBS-exposed hMSCs (n=4). Each volcano plot shows a different comparison. Y-axis shows  $-\log_{10}$  p-value and X-axis is  $\log_2$  fold change for each gene. Genes highlighted in red were significantly repressed in the comparison between HC BALF and PBS or ARDS vs PBS. Genes highlighted in yellow were significantly induced in the comparison between HC BALF and PBS or ARDS vs PBS. The number of genes that were significantly repressed (left) or induced (right) in each of the three comparisons is also shown. Abbreviations:

HC, healthy control; ARDS, acute respiratory distress syndrome; PBS, phosphate-buffered saline.

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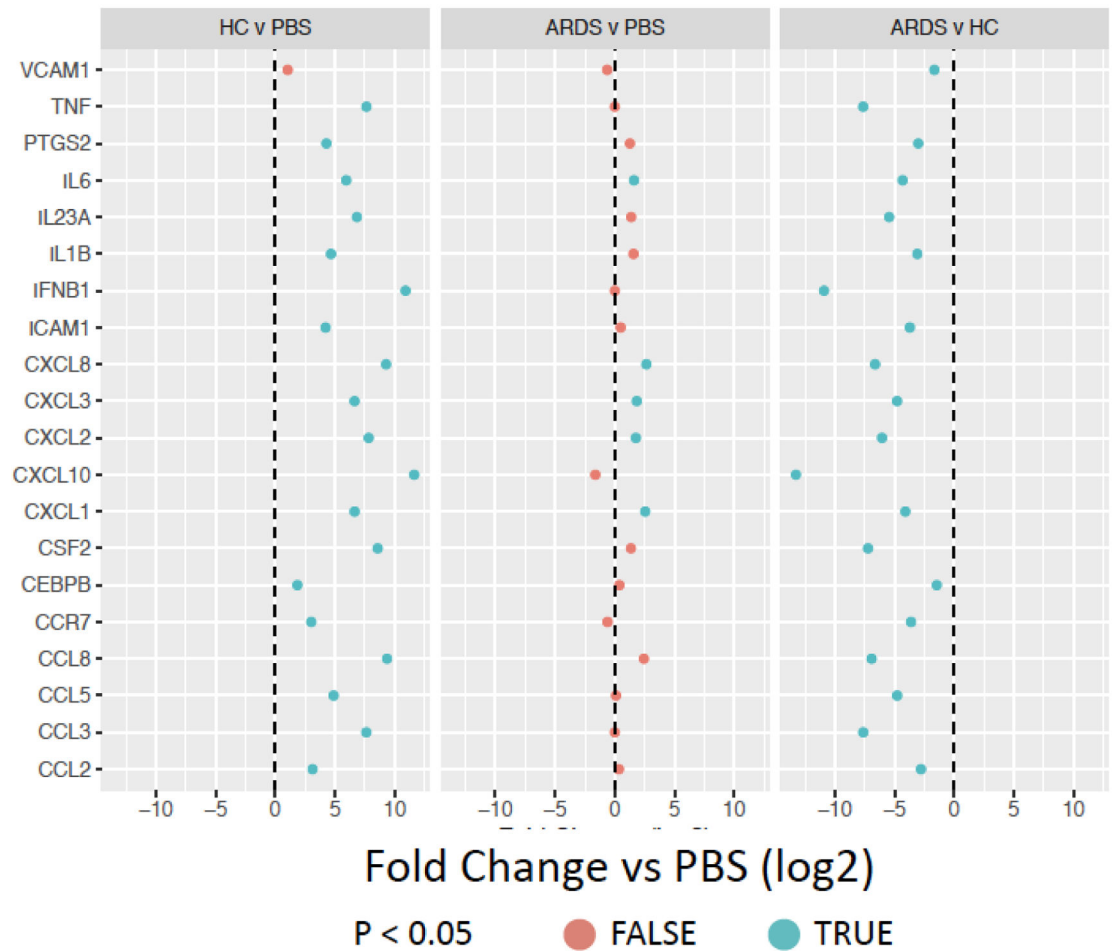
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A

# Inferred Inflammatory Effect Genes



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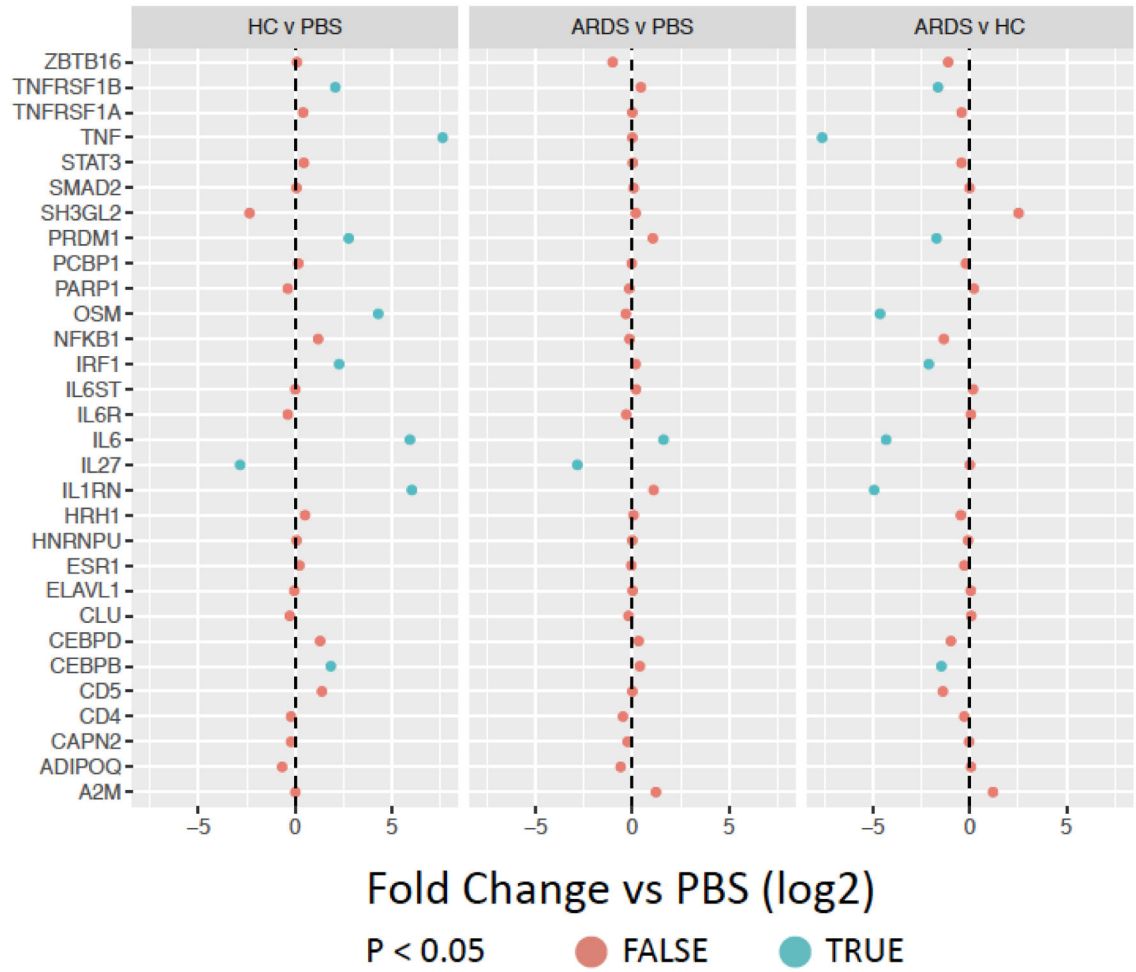
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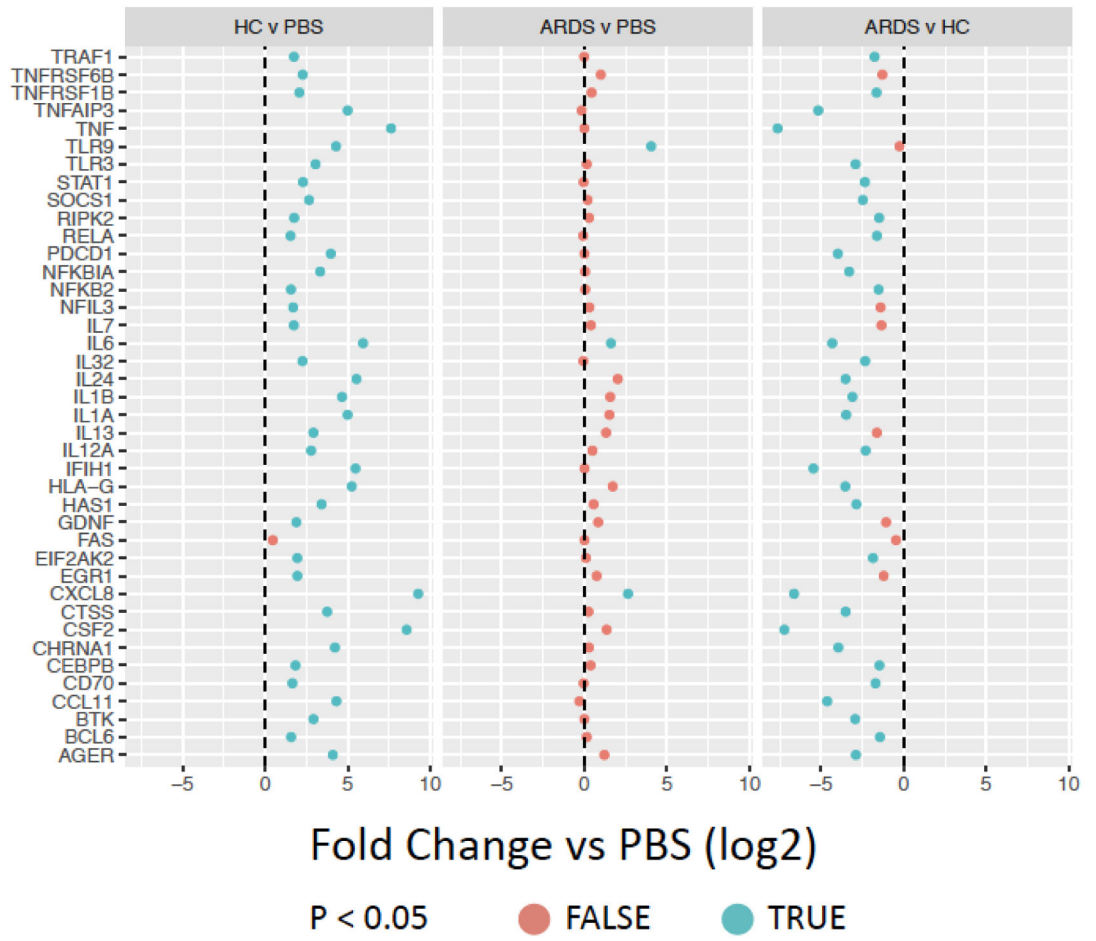
**B**

# IL-6 Interacting Genes



C

### FAS Interacting Genes

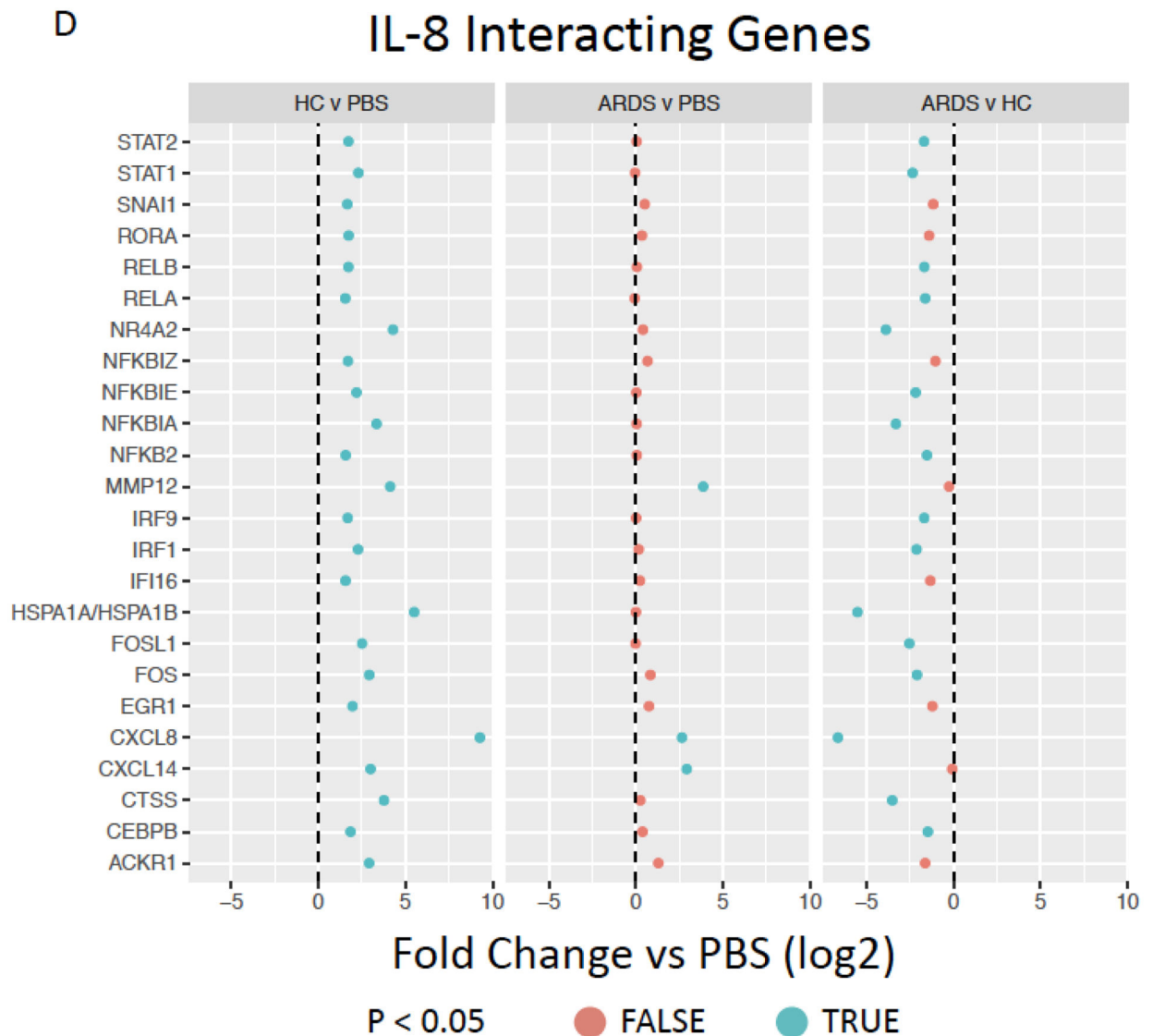


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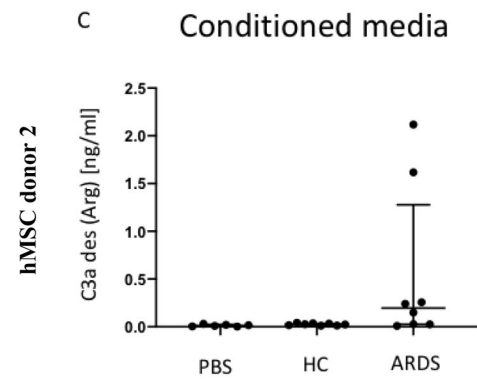
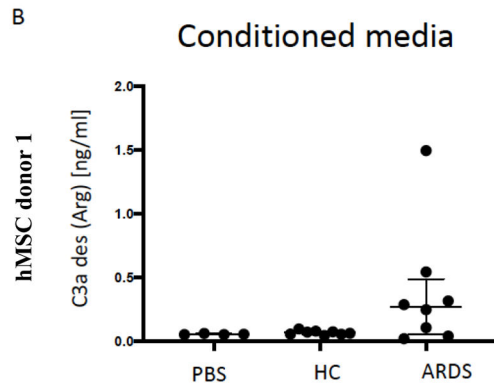
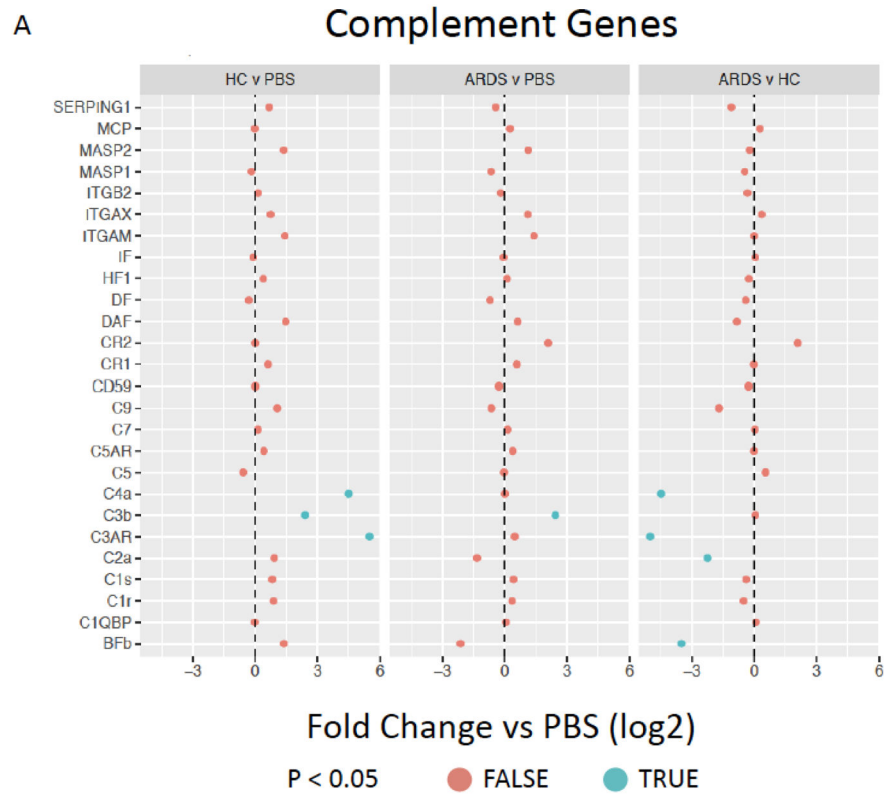
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**Figure 5. Exposure to healthy control but not ARDS BALF increases pro-inflammatory cytokine gene and interacting gene expression.**

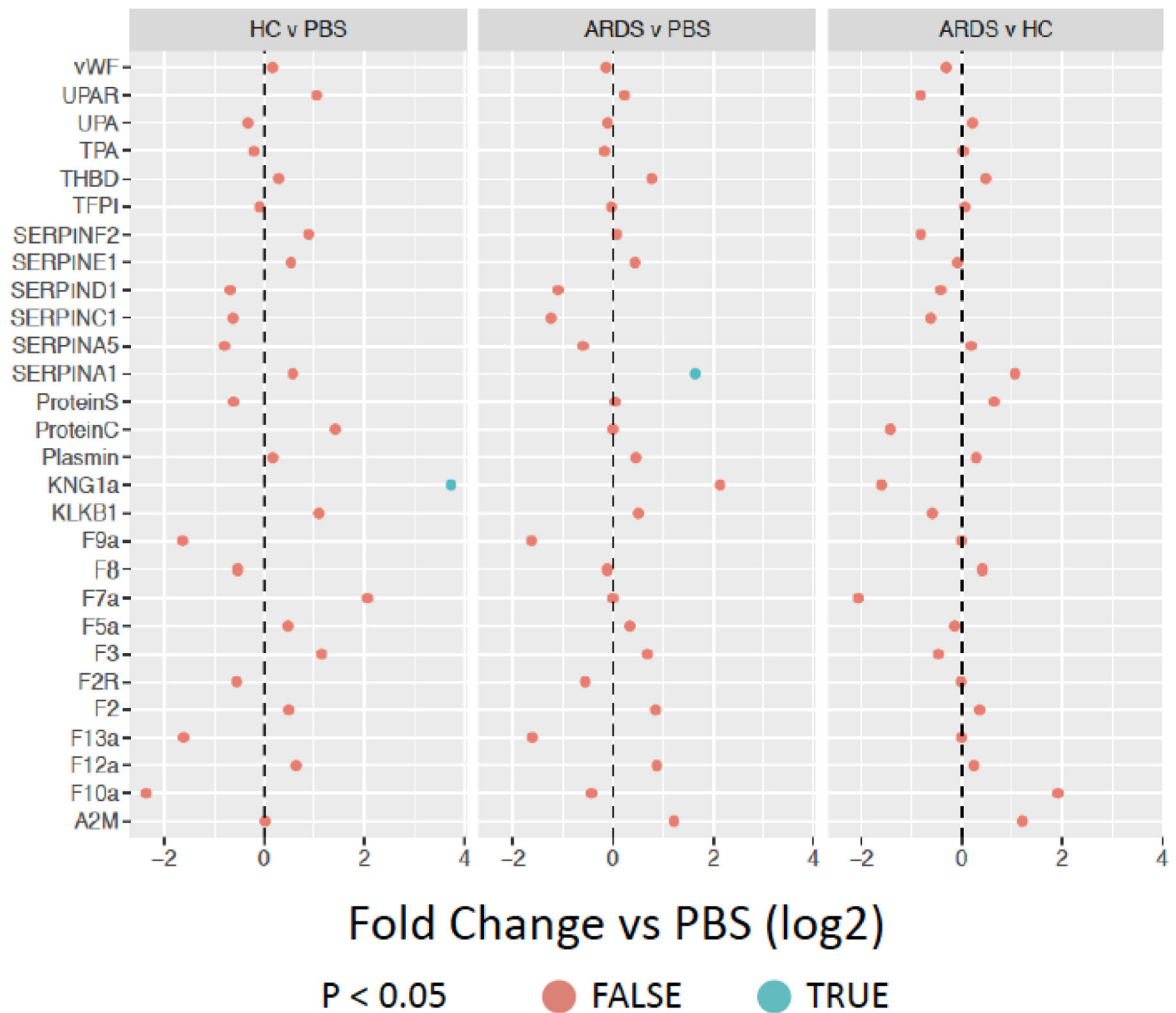
Inferred inflammatory genes resulting from Ingenuity software data analysis including (A), IL-6 (B), CXCL8/IL-8 (C), and FAS (D) interacting genes expressed by ARDS-exposed (left, n=5) and healthy control BALF (right, n=5) samples were compared to PBS-exposed hMSCs (n=4). Red color indicates no significant difference (p>0.05) and blue color indicates significant difference (p<0.05). Data are presented as mean of log<sub>2</sub> fold change.





D

## Coagulation Genes

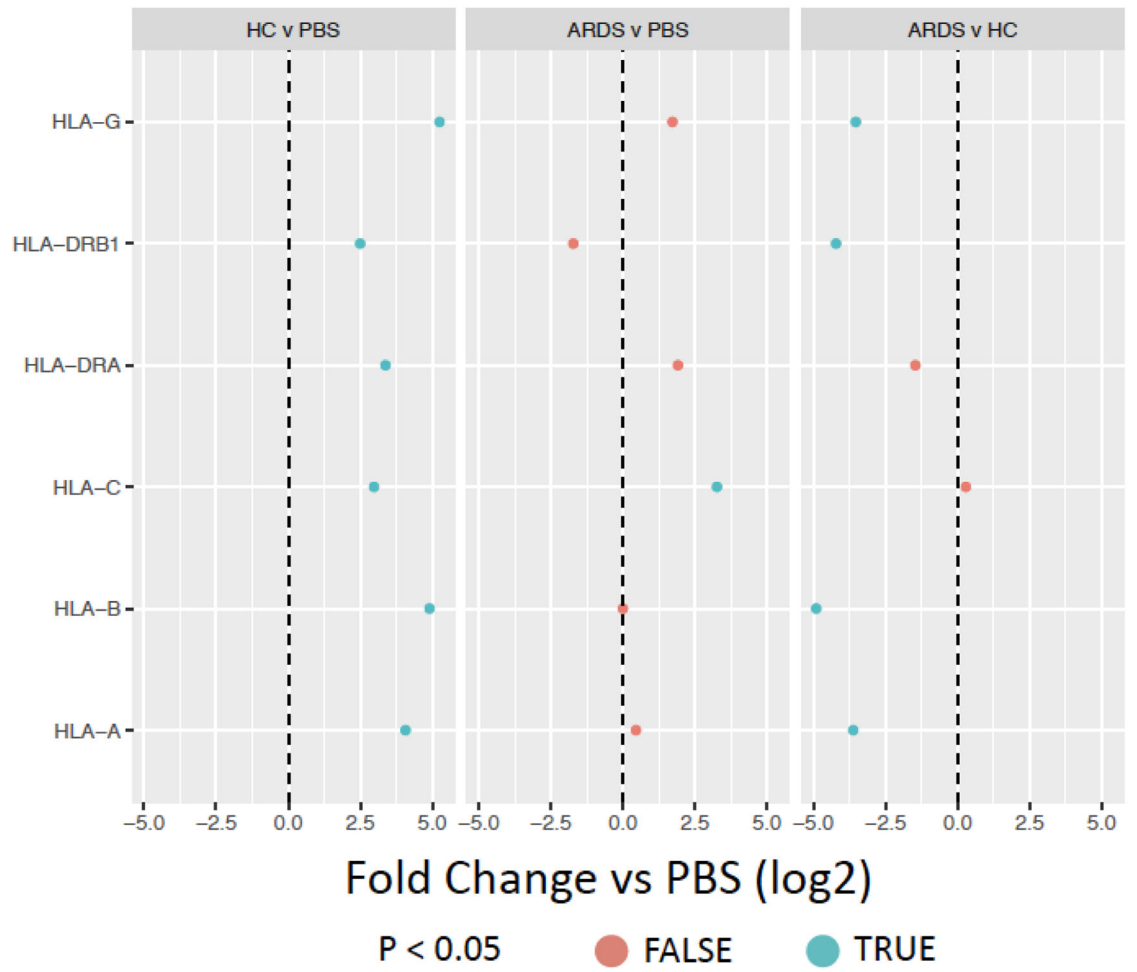


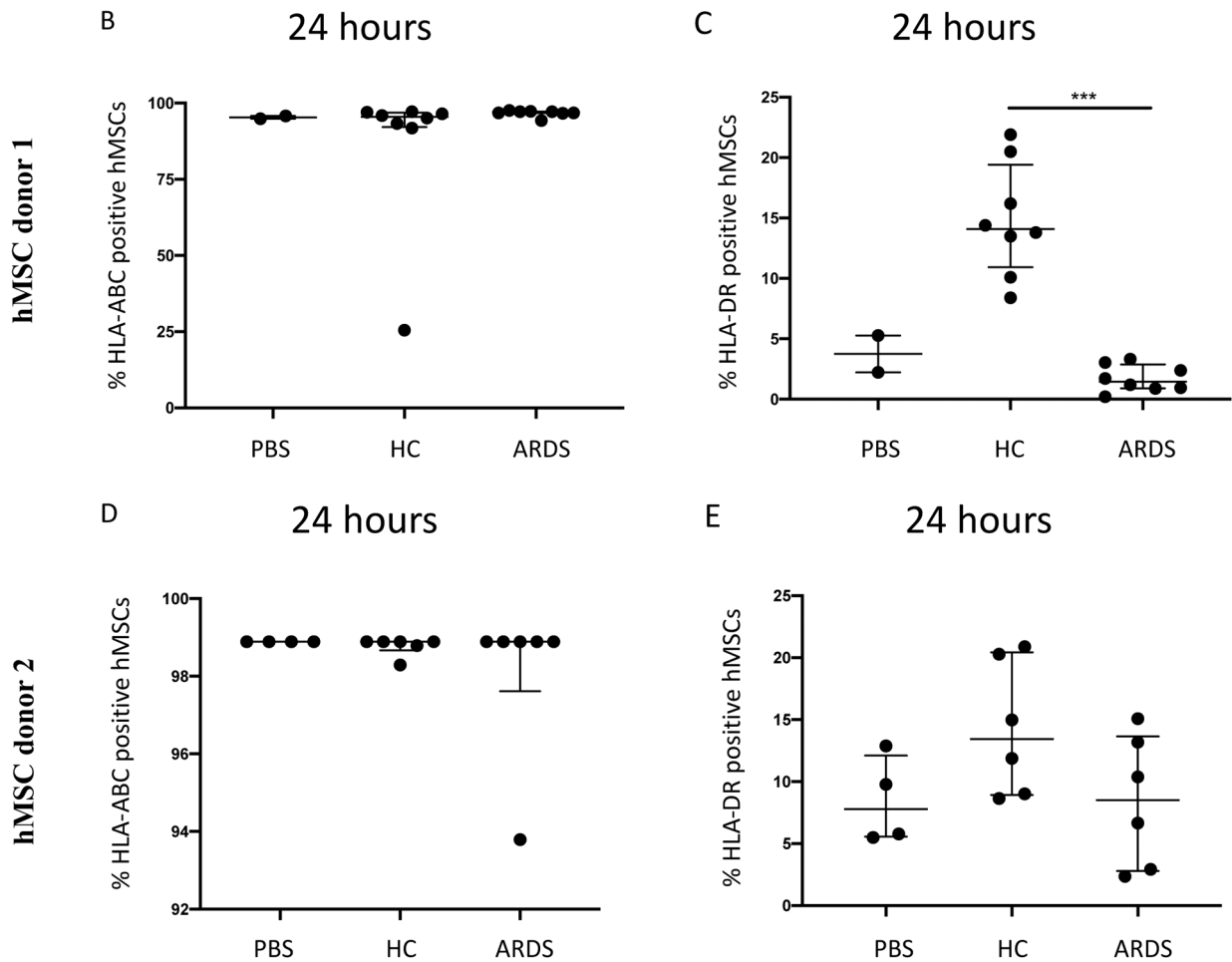
**Figure 6. Exposure to healthy control but not ARDS BALF increases complement gene and protein but not coagulation cascade gene expression.**

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 log<sub>2</sub> fold change. Complement (C3a) levels are depicted media with interquartile range.

A

# HLA Genes





**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 log<sub>2</sub> 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.**

Cytokines detected in CM from hMSCs exposed to ARDS and healthy volunteer 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- $\alpha$	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-1 $\beta$	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-36 $\beta$	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- $\gamma$ #	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- $\gamma$  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, bronchoalveolar lavage fluid; UNS, unstimulated; ARDS, acute respiratory distress syndrome; HC, healthy control subjects;

#. IFN- $\gamma$  was measured on a separate ELISA; SD, standard deviation; NA, not available;

\* 0.05;

\*\* 0.01,

\*\*\* 0.001.

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