Healthy *versus* inflamed lung environments differentially affect mesenchymal stromal cells

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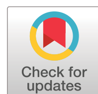

Shareable abstract (@ERSpublications)
MSCs exposed to a healthy lung environment induce an inflammatory response with increased gene/protein expression associated with self- *versus* non-self-recognition. These changes were absent or opposite in MSCs exposed to an inflamed ARDS environment. https://bit.ly/3eombO8

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Abstract

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

Aim The aim of this study was to comparatively assess how the inflammatory environment present in ARDS lungs *versus* the lung environment present in healthy volunteers alters MSC behaviour.

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 human leukocyte antigen expression, and activation of coagulation and complement pathways.

Results Pro-inflammatory, pro-coagulant and major histocompatibility complex (self-recognition) related gene expression was markedly upregulated in hMSCs exposed *ex vivo* to BALF obtained from healthy volunteers. 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 *versus* inflamed lung environments, and strongly suggest that the inflamed environment in ARDS induces hMSC responses that are potentially beneficial for cell survival and actions. This further highlights the need to understand how different disease environments affect hMSC functions.

Introduction

Mesenchymal stromal cells (MSCs) are being increasingly investigated as a cell-based therapy to suppress excessive inflammation in 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 a significant impact on MSC functions, including the profile of secreted mediators and downstream effects on macrophage functions, often enhancing anti-inflammatory actions [8–12]. For example, BALF from cystic fibrosis patients with 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 have deleterious effects on MSCs, with implications for potential therapeutic use. Moreover, contrary to previously held beliefs, systemically administered allogeneic MSCs rapidly undergo clearance and/or inactivation [14–17]. 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 controls (HCs) for comparison. Unexpectedly, hMSCs exposed to HC BALF developed an inflammatory response as well as increased gene and protein expression associated with self- *versus* non-self-recognition, notably increased class II human leukocyte antigen (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-*versus* 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 on 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, and Blood Institute (NHLBI) ARDS Network (ARDSNet) (ClinicalTrials.gov NCT0011216) [21]. ARDS BALF samples were obtained by mini-BAL from a phase 2 National Institutes of Health (NIH) trial conducted by the ARDSNet (Prevention and Treatment of Acute Lung Injury (PETAL) trial) [21]. A standard 40 mL mini-BAL was performed using sterile saline in intubated ARDS patients. BALF samples were subsequently centrifuged and stored at −80°C. The healthy volunteers underwent standard fibre-optic bronchoscopy of the right middle lobe at Dartmouth under appropriate institutional review board protocols, using 20 cm^3 of sterile saline. Samples were comparably centrifuged and supernatants stored at −70°C. These samples were more recent, having been obtained between January and July 2018. Healthy volunteers were excluded if they had any history of cardiopulmonary disease, if they smoked or vaped regularly or if they were taking any immunomodulatory medication.

Ex vivo *exposure of hMSCs*

hMSCs were obtained from the NHLBI's Production Assistance for Cellular Therapies (PACT) programme and routinely cultured. The hMSCs utilised 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 utilised in the recent Stem Cells for ARDS Treatment (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 h. Serum-free media only or with 20% PBS was added to control and unstimulated hMSCs, respectively. After 24 h 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 an Agilent fragment analyser instrument and quantified on a Qubit fluorometer (Thermo Fisher Scientific). 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], normalised in edgeR based on library size to create counts per million (CPM) for each gene and differential gene expression assessed. Pathway analysis genes that differed significantly from unstimulated control samples were identified using Ingenuity Pathway Analysis (www.ingenuity.com).

Statistical analyses

A 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 $\leqslant 0.05$ were considered significant, except in the case of RNA-sequencing data analysed in edgeR, where a multiple hypothesis corrected false discovery rate (FDR) <0.05 was 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 supplementary material.

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 using a Human Magnetic Luminex Assay kit (R&D Systems). The BALF samples used for each assay in the overall study are depicted in supplementary table S1. Although there were variations between the different clinical isolates, the levels of total protein, double-stranded DNA (dsDNA) and a range of inflammatory mediators were significantly elevated in ARDS compared to HC BALF samples (supplementary table S2). There were no significant differences between ARDS and HC BALF in the levels of anti-inflammatory and T-helper 2 (Th2) mediators such as interleukin (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–c) and by lactate dehydrogenase (LDH) release (figure 1d). To further determine toxicity, mitochondrial respiration in hMSCs exposed to different BALFs was assessed. Neither HC nor ARDS BALF significantly altered hMSC basal respiration rate, maximal respiration rate, spare respiratory capacity or other mitochondrial functions compared to PBS-exposed hMSCs (figure 1e–g, supplementary figure S1). 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 1g). 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

In comparison to that of PBS-exposed hMSCs, hMSC-CM exposed to HC or ARDS BALF samples had increased levels 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) (figure 2a–c, table 1). Moreover, significantly increased levels of CD44 (p=0.0041 and p=0.0342, respectively) and surfactant protein D (p=0.0162 and p=0.0055, respectively) were comparably observed in hMSCs exposed to both ARDS and HC BALF compared to PBS-exposed hMSCs (figure 2d, e, table 1). hMSCs exposed to ARDS but not HC BALF samples induced significantly higher levels of hepatocyte growth factor (p=0.0180) and, in particular, matrix metalloproteinase-3 (MMP3) (p=0.0041) compared to controls (figure 2f, g). In contrast, hMSCs exposed to HC but not ARDS BALF induced significantly higher chemokine (C-C motif) ligand 2 (CCL2) levels (p=0.0208) compared to controls (figure 2h). These data suggest that hMSCs can acquire both pro- and anti-inflammatory phenotypes in response to specific mediators present or absent in the BALF.

BALF IL-1β predicts hMSC cytokine secretion

We next determined whether specific BALF cytokines correlated (Spearman correlations) with hMSC inflammatory mediator production. Notably, IL-1β in both ARDS and 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 cell surface death receptor (FAS) (p=0.0427) and IL-8 (p=0.0346) (figure 3a, supplementary table S3, supplementary figure S2). As shown in figure 3b, expression of IL-1β was not correlated with other cytokines measured in BALF

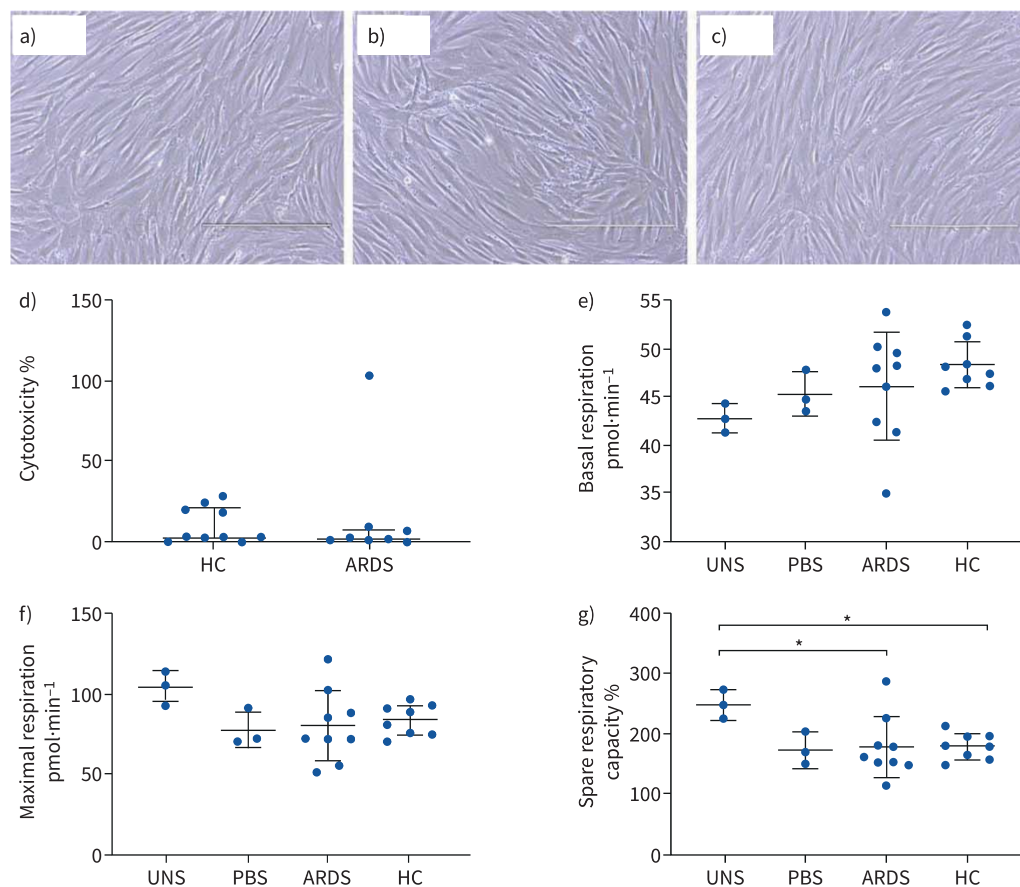

FIGURE 1 Exposure to bronchoalveolar lavage fluid (BALF) from acute respiratory distress syndrome (ARDS) patients and healthy controls (HCs) are non-toxic to human mesenchymal stromal cells (hMSCs). a–c) Representative phase contrast photomicrographs (×10) of PBS-exposed (20%) control hMSCs (a) and hMSCs exposed for 24 h to 20% BALF samples obtained from HCs (b) and ARDS patients (c). Scale bar: 400 µm. Photomicrographs have been brightness/contrast adjusted. d) Cytotoxicity was evaluated in conditioned medium using a standard lactate dehydrogenase assay following 24 h exposure (ARDS: n=8; HC: n=10). Data are presented as median (interquartile range) of % cytotoxicity. e–g) Impact of ARDS (n=9) and HC (n=8) BALF samples on hMSC mitochondrial function. Basal respiration (e), maximal respiration (f) and spare respiration capacity (g) were measured in pre-exposed hMSCs (24 h) using an XF-96e Extracellular Flux Analyser and compared to PBS-exposed (n=3) and unstimulated (serum-free media only, n=3) hMSCs. Data are presented as mean±SD, and statistical analysis was performed by Shapiro–Wilk test, followed by a one-way ANOVA with Dunnett's *post hoc* test. UNS: unstimulated (serum-free media only). *: $p \leqslant 0.05$.

samples. However, there was frequent correlation between the expression of different cytokines detected in CM (figure 3c). None of the cytokines measured in CM was co-expressed with FAS (figure 3c). These data suggest that the presence of higher concentrations of IL-1β in BALF may be used to predict the 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 analysed by RNA sequencing and compared to PBS-exposed hMSCs. A heat map demonstrates the cytokine profiles of the individual BALF samples utilised for hMSC exposures prior to RNA-sequencing analyses (figure 4a). These data demonstrate that both the HC and ARDS BALF samples utilised were representative of the full set of BALF samples analysed (supplementary table S2). 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 control PBS exposure (figure 4b). Notably, many of the genes that had increased expression with HC BALF exposure had

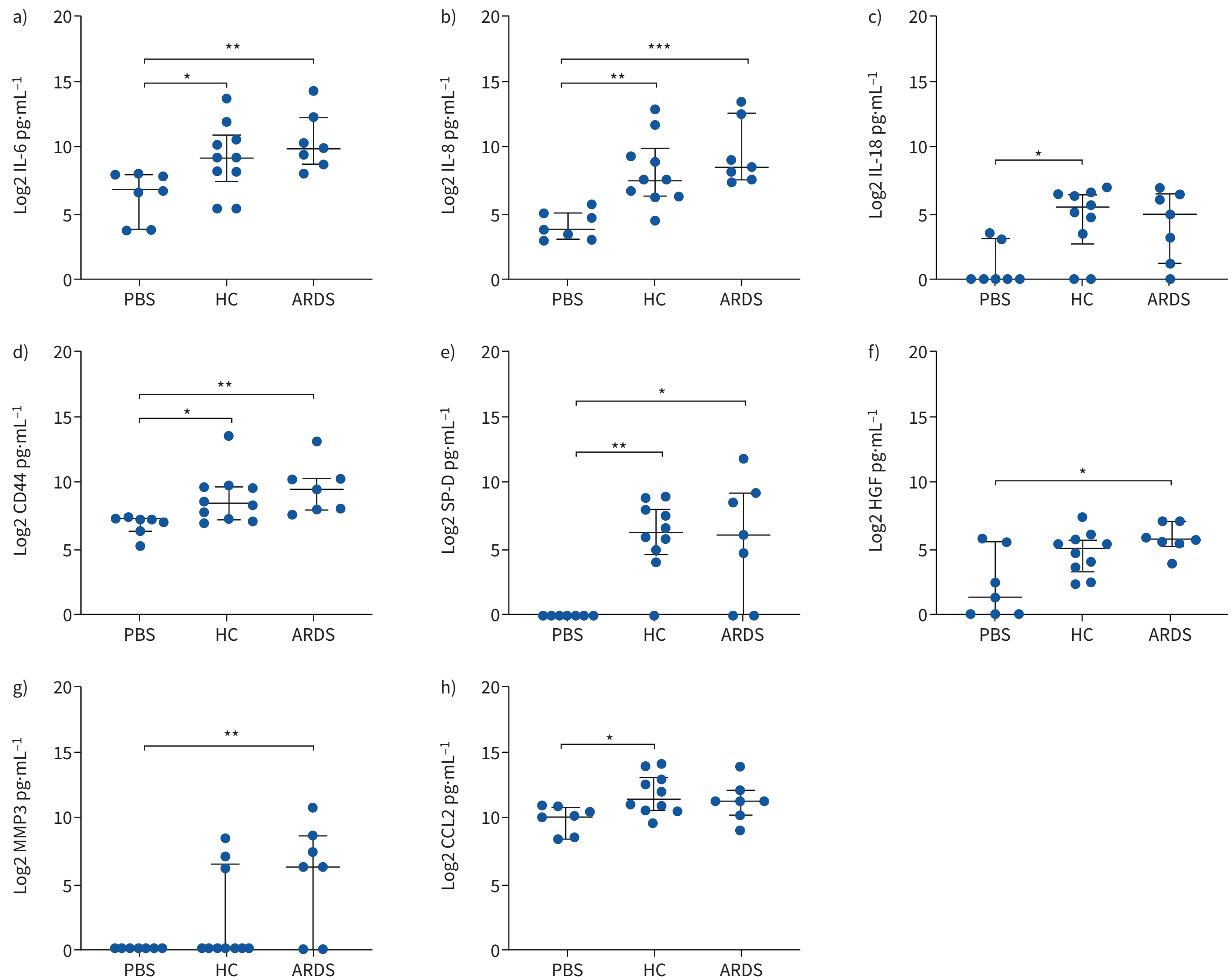

FIGURE 2 Acute respiratory distress syndrome (ARDS) bronchoalveolar lavage fluid (BALF) and healthy control (HC) BALF exposure activates human mesenchymal stromal cells (hMSCs) to release a spectrum of mostly pro- but some anti-inflammatory mediators. To assess if the secretome profiles of hMSCs exposed to ARDS BALF (n=7) samples differed from those of 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 a) interleukin (IL)-6, b) IL-8, c) IL-18, d) CD44 molecule/hyaluronate receptor (CD44), e) surfactant protein D (SP-D), f) hepatocyte growth factor (HGF), g) matrix metalloproteinase-3 (MMP3) and h) chemokine (C-C motif) ligand 2/monocyte chemoattractant protein 1 (CCL2). Data are presented as median (interquartile range) of log2 normalised 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. *: $p \leqslant 0.05$; **: $p \leqslant 0.01$.

decreased expression with ARDS BALF exposure (figure 4b). Interestingly, the severe acute respiratory syndrome coronavirus 2 binding and entry receptors angiotensin converting enzyme 2 (ACE2) and transmembrane serine protease 2 (TMPRSS2) were expressed in the hMSCs, although only at minimal levels and with no differences observed between the BALF exposure groups (supplementary table S4).

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 compared to PBS-exposed hMSCs, including tumour necrosis factor (*TNF*), intercellular adhesion molecule 1 (*ICAM1*), chemokine (C-X-C motif) ligand 10 (*CXCL10*), *CCL2*, *CCL8* and interferon-β1 (*IFN-B1*) (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 increased expression of cytokines known

TABLE 1 Cytokines detected in CM from hMSCs exposed to ARDS and HC BALF samples

Cytokine	Cytokines in CM pg·mL^{-1}			Kruskal–Wallis with Dunn's compared to UNS	
	UNS Mean±SD	HC Mean±SD	ARDS Mean±SD	HC p-value	ARDS 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	20 575.8±30710.7	14 619.7±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 CM from hMSCs exposed to ARDS BALF samples (n=7), HC BALF samples (n=10) and PBS (n=7) were analysed using a 27-plex Luminex assay with plates. Data are presented as mean±SD of extrapolated values. Values out of range below were set to 1.0. CM: conditioned media; hMSCs: human mesenchymal stromal cells; ARDS: acute respiratory distress syndrome; HC: healthy controls; BALF: bronchoalveolar lavage fluid; UNS: unstimulated; NA: not available. [#]: IFN-γ was measured on a separate ELISA on CM from hMSCs exposed to ARDS BALF (n=3), HC BALF (n=5) or PBS (n=4). *: $p \leqslant 0.05$; **: $p \leqslant 0.01$; ***: $p \leqslant 0.001$.

to be involved in neutrophil trafficking, including *CXCL1*, *CXCL2* (macrophage inflammatory protein 2α), *CXCL3*, *CXCL8*/*IL8* and *IL6* (figure 5a). However, these genes were expressed at levels lower than those observed following exposure to HC BALF.

Further evaluations were undertaken to assess the effect of BALF exposure on hMSC expression of other inter-related genes associated with prominent inflammatory markers and other mediators that had increased gene (figure 3b, f) and protein expression (figure 2a, supplementary figure S3) after BALF exposure, specifically IL-6, IL-8 and FAS. HC BALF exposure significantly induced a range of IL-6 interacting genes whereas ARDS BALF exposure resulted in similar expression to that observed in PBS-exposed hMSCs, with only a significant increase in IL-6 gene expression itself. Both HC and ARDS BALF exposure had an inhibitory effect on secretion of the anti-inflammatory cytokine IL-27 compared to PBS exposure (figure 5b). Similarly, a marked induction of a wide range of FAS interacting 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, for which hMSC gene but not protein expression was increased after BALF exposure. There was increased expression of a range of IL-8 interacting genes after 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.

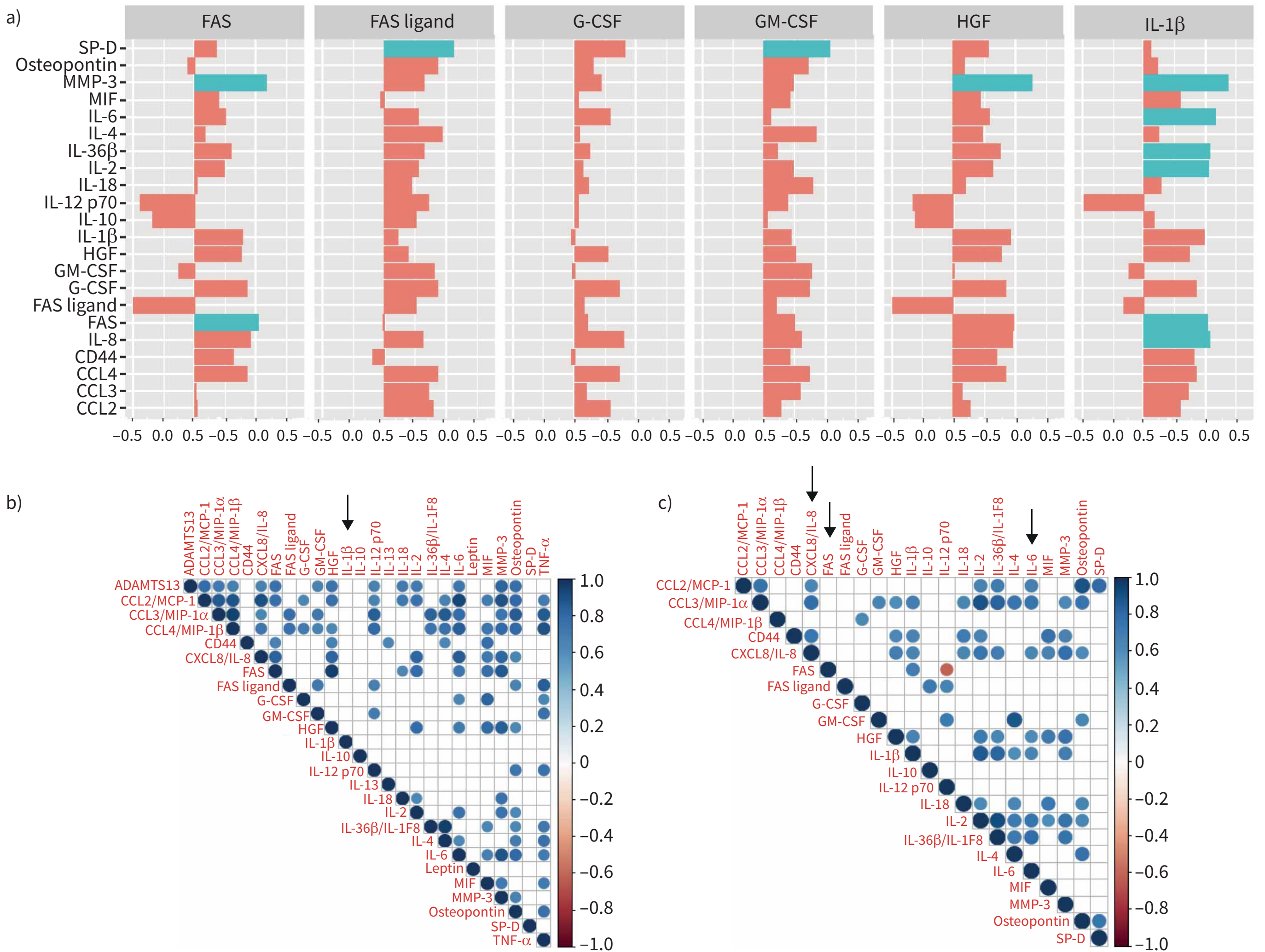

FIGURE 3 Bronchoalveolar lavage fluid (BALF) interleukin (IL)-1β predicts human mesenchymal stromal cell (hMSC) pro-inflammatory cytokine secretion. a) Cytokines measured in BALF samples (top) were correlated with cytokines detected in conditioned media (left) from acute respiratory distress syndrome (ARDS) (n=6) and healthy control (HC) (n=10) BALF-exposed hMSC cultures. Red indicates no significant difference ($p<0.05$) and blue indicates significant difference ($p<0.05$). b) Cytokines measured in BALF samples were correlated with each other and c) cytokines measured in conditioned media from BALF-exposed hMSC cultures were correlated with each other. Red indicates significantly decreased expression ($p<0.01$) and blue 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.

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 an increase in C3b expression only, with other complement cascade genes expressed at levels similar to those observed in PBS-exposed hMSCs (figure 6a). Direct comparison of ARDS *versus* HC BALF-exposed hMSCs demonstrated a respective decrease in C4a and C3AR as well as C2a and BFb gene expression. Assessment of the CM from BALF- and PBS-exposed 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 CM from hMSCs exposed to ARDS BALF (figure 6b, c).

Parallel assessment of coagulation-associated gene expression demonstrated no changes in tissue factor pathway inhibitor (*TFP1*) expression in hMSCs exposed to either HC or ARDS BALF as compared to PBS-exposed cells (figure 6d). An isolated increase in gene expression of kininogen 1a, part of the

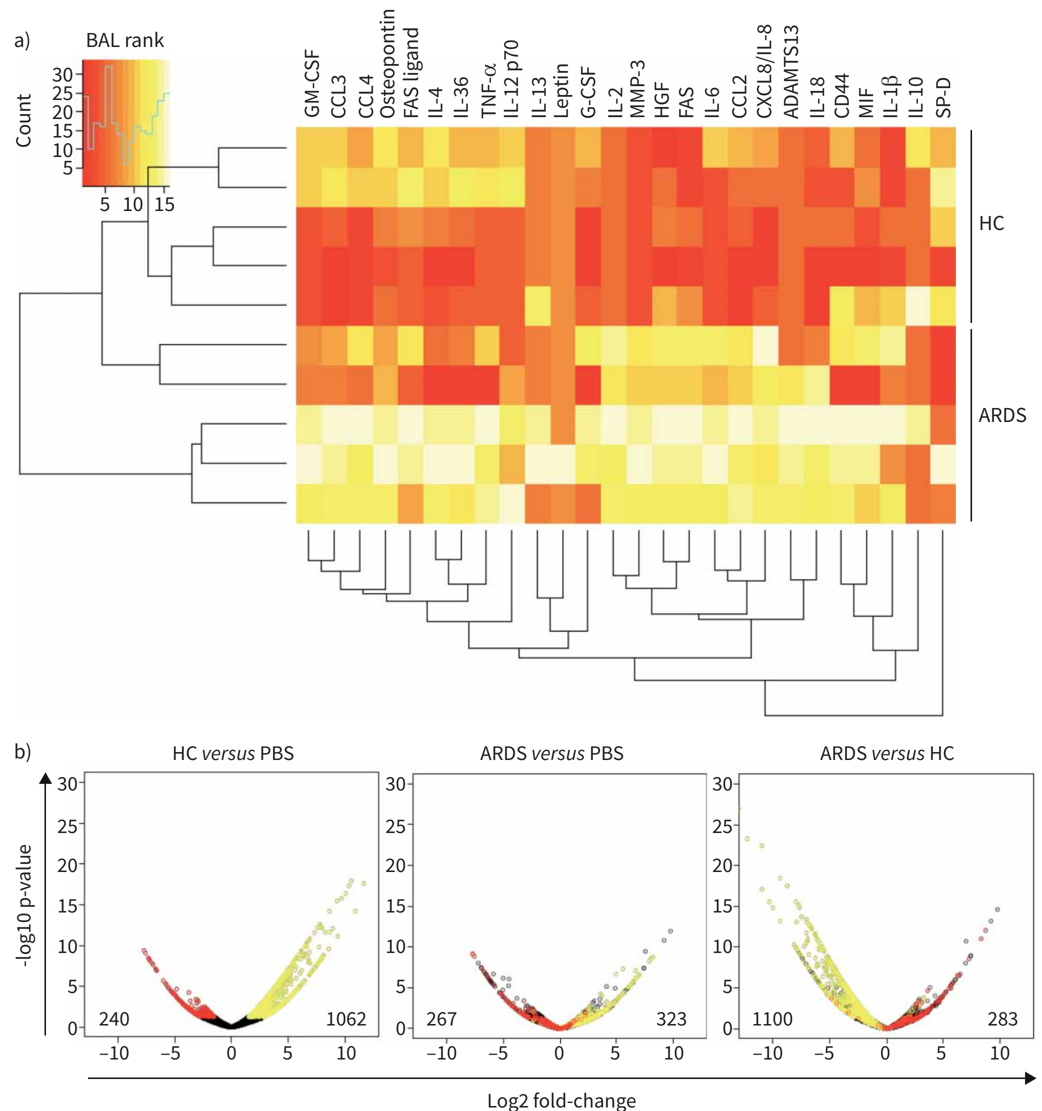

FIGURE 4 Human mesenchymal stromal sells (hMSCs) exposed to healthy control (HC) bronchoalveolar lavage fluid (BALF) demonstrate increased gene expression compared to those exposed to acute respiratory distress syndrome (ARDS) BALF and PBS. a) Heat map clustering of ranked cytokines measured in ARDS (n=5) and HC (n=5) BALF samples utilised in the RNA-sequencing analysis. Red indicates lower and yellow higher concentrations of cytokines. b) Volcano plots of genes identified by RNA sequencing of ARDS BALF (n=5), HC BALF (n=5) or PBS-exposed hMSCs (n=4). Each volcano plot shows a different comparison. y-axis shows −log10 p-value and x-axis is log2 fold-change for each gene. Genes highlighted in red were significantly repressed in the comparison between HC BALF *versus* PBS and ARDS BALF *versus* PBS. Genes highlighted in yellow were significantly induced in the comparison between HC BALF *versus* PBS and ARDS BALF *versus* PBS. The number of genes that were significantly repressed (left) or induced (right) in each of the three comparisons is also shown.

intrinsic coagulation cascade, was observed in HC-exposed hMSCs whereas an 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 in 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 the expression of genes and proteins that might result in recognition of the MSCs by the host immune system, notably HLA expression. HC BALF exposure

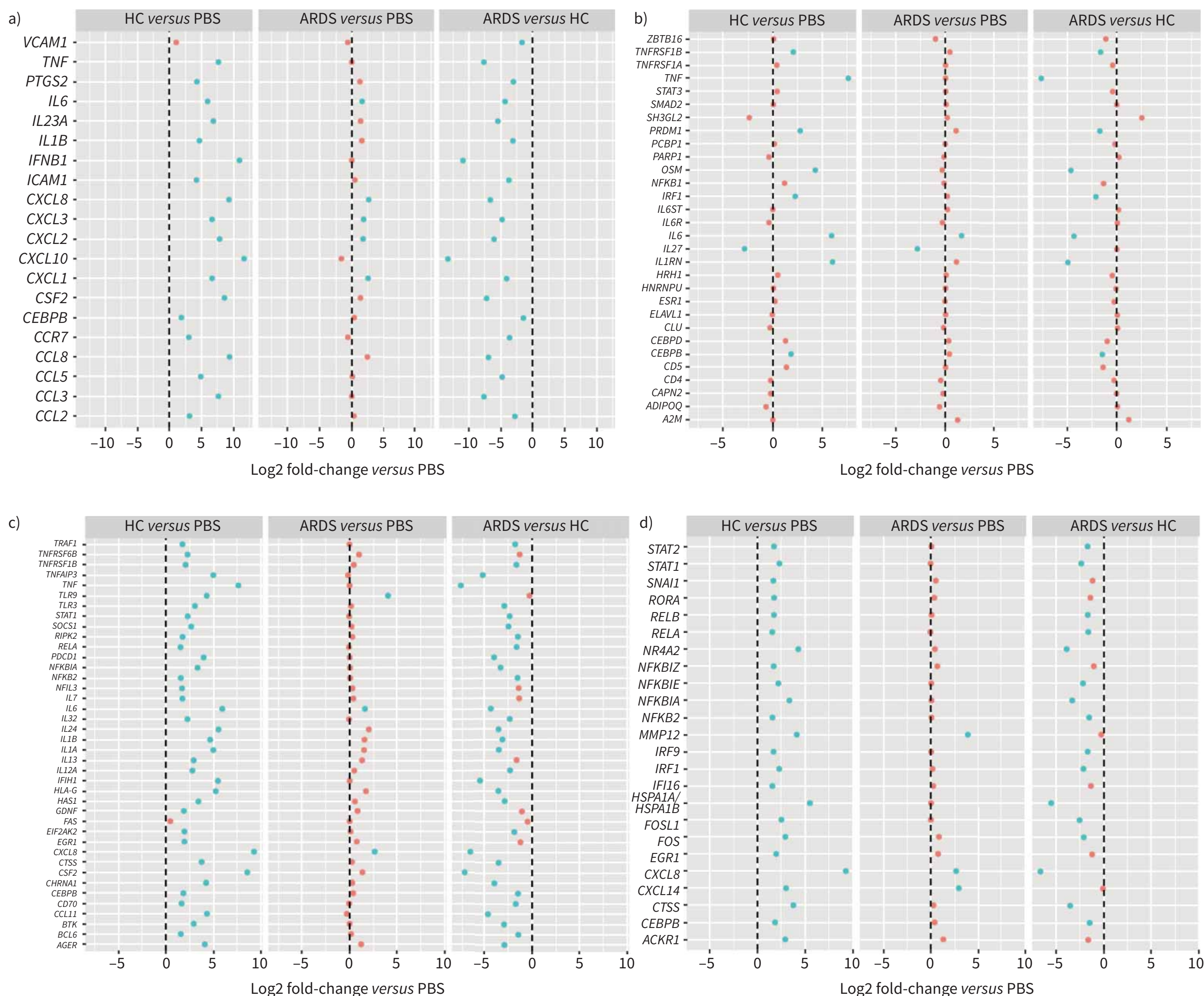

FIGURE 5 Exposure to healthy control (HC) but not acute respiratory distress syndrome (ARDS) bronchoalveolar lavage fluid (BALF) increases pro-inflammatory cytokine gene and interacting gene expression. a) Inferred inflammatory genes resulting from Ingenuity software data analysis, including genes interacting with b) interleukin (IL)-6, c) FAS and d) chemokine (C-X-C motif) binding ligand 8 (CXCL8)/IL-8, expressed by ARDS BALF-exposed (n=5) and HC BALF-exposed (n=5) human mesenchymal stromal cells (hMSCs) were compared to PBS-exposed hMSCs (n=4). Red indicates no significant difference ($p>0.05$) and blue indicates a significant difference ($p<0.05$). Data are presented as mean of log2 fold-change.

resulted in significantly increased expression of a number of HLA class I and II genes and pseudogenes when compared to PBS exposure. These included classical (*HLA-A*, *HLA-B*, *HLA-C*) and non-classical (*HLA-E*, *HLA-F*, *HLA-G*) HLA class I genes and HLA class II genes including *HLA-DRA*, *HLA-DRB1*, *HLA-DMA*, *HLA-DMB* and *HLA-DPA1* (major class I and II genes depicted in figure 7a with all HLA genes evaluated depicted in supplementary figure S4). In contrast, exposure to ARDS BALF resulted in a significant increase in only *HLA-C* and *HLA-F* and a significant decrease in *HLA-G*, with no changes in *HLA-A*, *HLA-B* or *HLA-E* compared to PBS-exposed hMSCs (figure 7a, supplementary figure S4). Comparably, exposure to ARDS BALF resulted in a significant increase in only *HLA-DMA* and a significant decrease in *HLA-DRA*, with no changes in any of the other class II genes. Strikingly, a significant decrease in the expression of genes encoding for HLA class I genes *HLA-A*, *HLA-B* and *HLA-G*, and HLA class II *HLA-DRB1* was observed when comparing ARDS BALF- to HC BALF-exposed hMSCs (figure 7a).

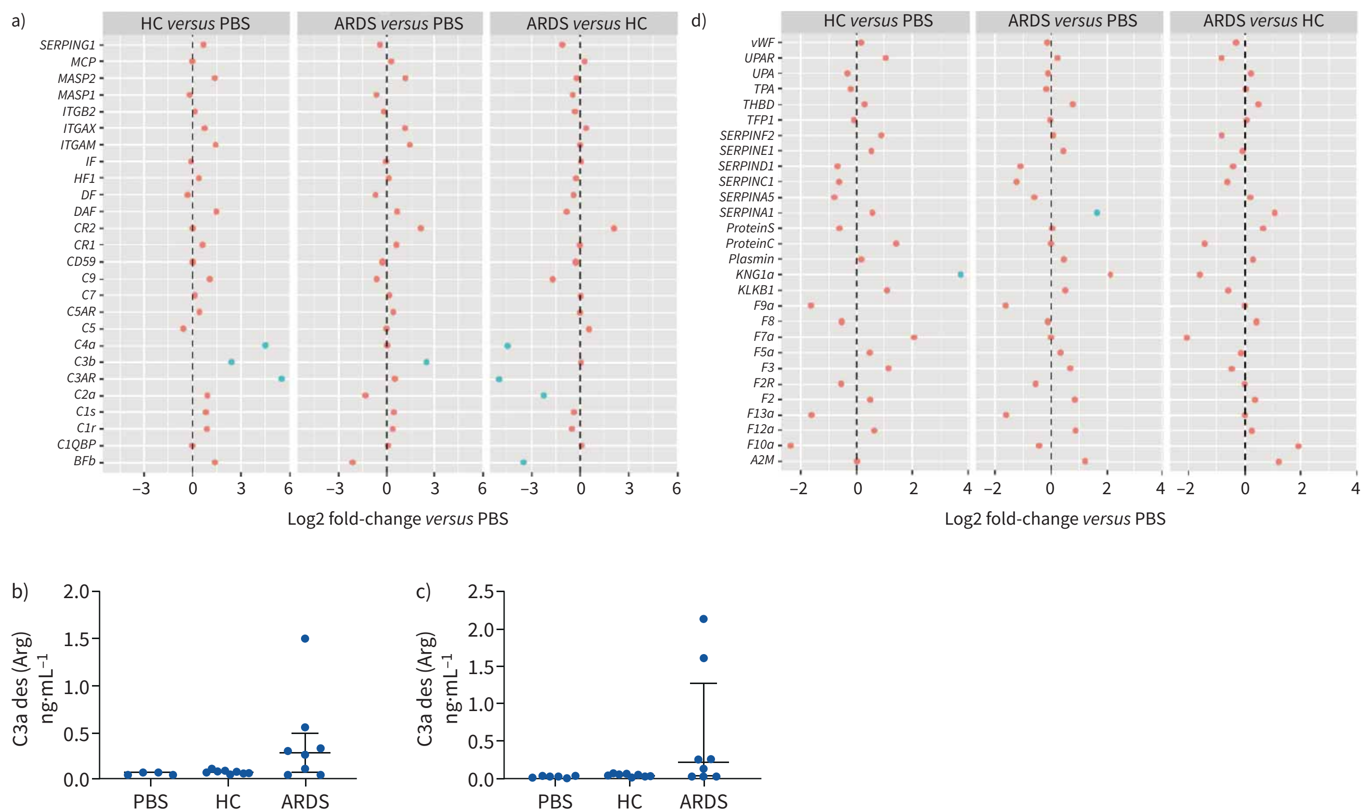

FIGURE 6 Exposure to healthy control (HC) but not acute respiratory distress syndrome (ARDS) bronchoalveolar lavage fluid (BALF) increases complement gene and protein but not coagulation cascade gene expression. a) Complement genes expressed by ARDS BALF-exposed (n=5) and HC BALF-exposed (n=5) human mesenchymal stromal cells (hMSCs) were compared to PBS-exposed hMSCs (n=4). b, c) Levels of complement secreted into the conditioned media by HC or ARDS BALF-exposed compared to PBS-exposed hMSCs from hMSC donor 1 (b) and 2 (c). d) Coagulation cascade genes expressed by ARDS BALF-exposed (n=5) and HC BALF-exposed (n=5) hMSCs were compared to PBS-exposed hMSCs (n=4). Gene expression data are presented as mean of log2 fold-change. Complement (C3a) levels are depicted as the medium (interquartile range).

To further validate these observations, hMSC HLA-ABC and HLA-DR protein expression was evaluated by flow cytometry following exposure to HC *versus* 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 S4. These results demonstrate that exposure of allogeneic hMSCs to a normal lung environment upregulates expression of a non-self antigen recognised 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. Exposure to ARDS BALF, compared with exposure to HC BALF, blunted not only the pro-inflammatory gene response but also the increased expression of genes and proteins associated with self- *versus* non-self-recognition. These are novel observations that provide insight into the potential mechanisms of action of MSC-based cell therapies in ARDS.

Our data support the tantalising hypothesis that a mechanism similar to IBMIR may be responsible for the aggressive removal of hMSCs in healthy lungs. Similar to the IBMIR literature concerning blood exposure, stimulating hMSCs with HC BALF resulted in behaviours that provoke an acute innate immune response, including a 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

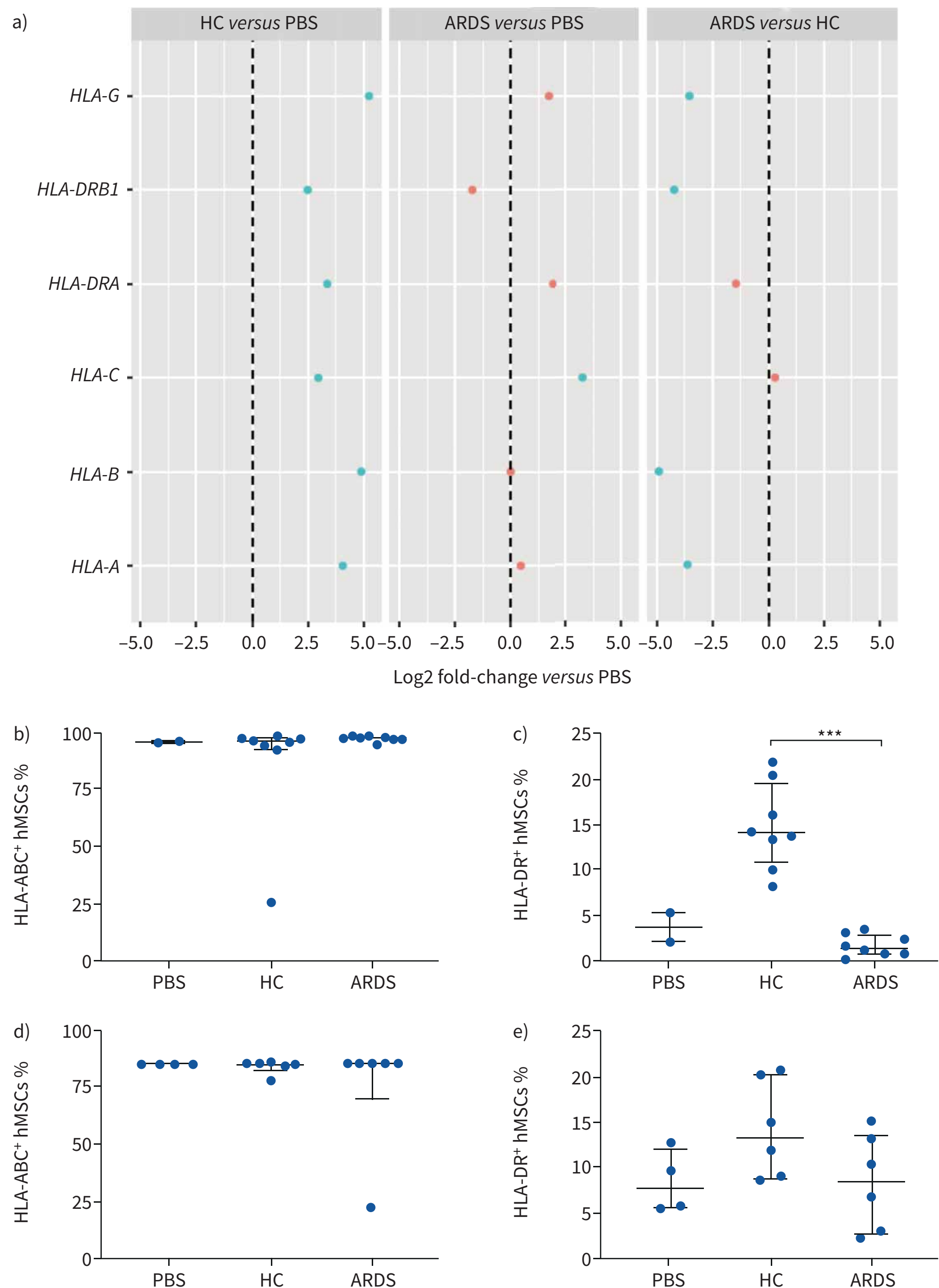

FIGURE 7 Exposure to healthy control (HC) but not acute respiratory disease syndrome (ARDS) bronchoalveolar lavage fluid (BALF) increases class II human mesenchymal stromal cell (hMSC) human leukocyte antigen (HLA) gene and protein expression. a) HLA genes expressed by ARDS BALF-exposed (n=5) and HC BALF-exposed (n=5) hMSCs were compared to PBS-exposed hMSCs (n=4). Data are presented as mean of log2 fold-change. b, d) HLA-ABC and c, e) HLA-DR surface marker expression was measured with flow cytometry on hMSCs exposed to PBS, HC BALF samples and ARDS BALF samples for 24 h. Two different hMSC donors were used. Data are presented as median (interquartile range) of % HLA-ABC$^+$ cells – % HLA-ABC$^+$ cells on DAPI-only stainings. Statistical analysis was performed by Kruskal–Wallis followed by Dunn's *post hoc* test. ***: $p \leqslant 0.001$.

to upregulate expression or resulted in comparatively decreased expression of these self-antigen-encoding genes and proteins. 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 are few other data in this regard. hMSCs are not well recognised as producing complement factors although they do produce antibacterial 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 have no constitutive expression of class II molecules [18, 26, 27], attributes that have long been thought to minimise 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-γ, 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 suggest 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 in inflamed *versus* non-inflamed lungs, presumably allowing for more opportunity to exert effects on inflammatory pathways [24, 31, 32]. However, although IFN-γ 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-γ. One possible explanation is that low doses of IFN-γ have less effect on MSC immunosuppressive potency compared to higher doses [33]. Increased expression of the IFN-γ-dependent HLA class II transactivator C2Ta was observed; however, the significance of this finding remains unknown [34].

Notably, BALF IL-1β was significantly predictive of hMSC production of a range of important pro-inflammatory mediators. This suggests that IL-1β, commonly elevated in ARDS lungs, can drive hMSC behaviour and that blocking IL-1β may potentially alter hMSC actions. However, the overall picture is complex, as demonstrated in figure 3 and supplementary figure S2. This suggests that healthy lung environments are capable of provoking inflammatory behaviours in allogeneic bone marrow-derived hMSCs.

This study has several strengths. First, the hMSCs utilised are clinically relevant, having also been utilised 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 HCs. However, one caveat is that the BALF samples utilised 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 utilised or in any potential sample dilutions. Further, the BALF samples utilised were obtained using different isolation protocols and stored differently both at the originating institutions (−80°C for ARDS and −70°C for HC) and also in our laboratory (−20°C for ARDS and −70°C for HC). As such, although we feel these differences are unlikely to account for the current observations, further prospective studies will attempt to better control for these variables. Further, given the US Health Insurance Portability and Accountability Act (HIPAA) limitations, no clinical data, including microbiological data, on the underlying aetiology of the ARDS patients are available except for the ARDSNet clinical study under which these samples were obtained, in which 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 utilised 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, *e.g.* with mixed lymphocyte studies utilising BALF-exposed hMSCs or otherwise adding immune effector cells to the BALF samples, will provide important information on the lung inflammatory environment effects on hMSC behaviours.

In summary, hMSC exposure to healthy lung environments induces the expression of a range of genes encoding for inflammation and for recognition as foreign to the host immune system. These changes are not observed or, in some cases, are opposite after 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 for developing more effective MSC-based cell therapies for ARDS and other lung diseases.

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References

1 Matthay MA, Zemans RL, Zimmerman GA, *et al.* Acute respiratory distress syndrome. *Nat Rev Dis Primers* 2019; 5: 18.
2 Khoury M, Cuenca J, Cruz FF, *et al.* Current status of cell-based therapies for respiratory virus infections: applicability to COVID-19. *Eur Respir J* 2020; 55: 2000858.
3 Wilson JG, Liu KD, Zhuo H, *et al.* Mesenchymal stem (stromal) cells for treatment of ARDS: a phase 1 clinical trial. *Lancet Respir Med* 2015; 3: 24–32.
4 Matthay MA, Calfee CS, Zhuo H, *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: 154–162.
5 Simonson OE, Mougiakakos D, Heldring N, *et al. In vivo* effects of mesenchymal stromal cells in two patients with severe acute respiratory distress syndrome. *Stem Cells Transl Med* 2015; 4: 1199–1213.
6 Rolandsson Enes S, Westergren-Thorsson G. Comparison of the regenerative potential for lung tissue of mesenchymal stromal cells from different sources/locations within the body. In: Heijink IH, Burgess JK, eds. Stem Cell-Based Therapy for Lung Disease. New York, Springer, 2019.

7 Kusuma GD, Carthew J, Lim R, *et al.* Effect of the microenvironment on mesenchymal stem cell paracrine signaling: opportunities to engineer the therapeutic effect. *Stem Cells Dev* 2017; 26: 617–631.
8 Xu AL, Rodriguez LA, II, Walker KP, III, *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: 1092–1106.
9 Islam D, Huang Y, Fanelli V, *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: 1214–1224.
10 Bustos ML, Huleihel L, Meyer EM, *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: 884–895.
11 Abreu SC, Enes SR, Dearborn J, *et al.* Lung inflammatory environments differentially alter mesenchymal stromal cell behavior. *Am J Physiol Lung Cell Mol Physiol* 2019; 317: L823–L831.
12 Abreu SC, Xisto DG, de Oliveira TB, *et al.* Serum from asthmatic mice potentiates the therapeutic effects of mesenchymal stromal cells in experimental allergic asthma. *Stem Cells Transl Med* 2019; 8: 301–312.
13 Soraia THH, Abreu C, Hoffman E, *et al.* Differential effects of the cystic fibrosis lung inflammatory environment on mesenchymal stromal cells. *Am J Physiol Lung Cell Mol Physiol* 2020; 319: L908–L925.
14 Galleu A, Riffo-Vasquez Y, Trento C, *et al.* Apoptosis in mesenchymal stromal cells induces in vivo recipient-mediated immunomodulation. *Sci Transl Med* 2017; 9: eaam7828.
15 Weiss DJ, English K, Krasnodembskaya A, *et al.* The necrobiology of mesenchymal stromal cells affects therapeutic efficacy. *Front Immunol* 2019; 10: 1228.
16 Weiss ARR, Dahlke MH. Immunomodulation by mesenchymal stem cells (MSCs): mechanisms of action of living, apoptotic, and dead MSCs. *Front Immunol* 2019; 10: 1191.
17 Galipeau J, Sensebe L. Mesenchymal stromal cells: clinical challenges and therapeutic opportunities. *Cell Stem Cell* 2018; 22: 824–833.
18 Moll G, Hult A, von Bahr L, *et al.* Do ABO blood group antigens hamper the therapeutic efficacy of mesenchymal stromal cells? *PLoS One* 2014; 9: e85040.
19 Hoogduijn MJ, Roemeling-van Rhijn M, Engela AU, *et al.* Mesenchymal stem cells induce an inflammatory response after intravenous infusion. *Stem Cells Dev* 2013; 22: 2825–2835.
20 Moll G, Jitschin R, von Bahr L, *et al.* Mesenchymal stromal cells engage complement and complement receptor bearing innate effector cells to modulate immune responses. *PLoS One* 2011; 6: e21703.
21 Liu KD, Levitt J, Zhuo H, *et al.* Randomised clinical trial of activated protein C for the treatment of acute lung injury. *Am J Respir Crit Care Med* 2008; 178: 618–623.
22 Patro R, Duggal G, Love MI, *et al.* Salmon provides fast and bias-aware quantification of transcript expression. *Nat Methods* 2017; 14: 417–419.
23 Soneson C, Love MI, Robinson MD. Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences. *F1000Res* 2015; 4: 1521.
24 George MJ, Prabhakara K, Toledano-Furman NE, *et al.* Procoagulant *in vitro* effects of clinical cellular therapeutics in a severely injured trauma population. *Stem Cells Transl Med* 2020; 9: 491–498.
25 Krasnodembskaya A, Song Y, Fang X, *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: 2229–2238.
26 Wang Y, Huang J, Gong L, *et al.* The plasticity of mesenchymal stem cells in regulating surface HLA-I. *iScience* 2019; 15: 66–78.
27 Kot M, Baj-Krzyworzeka M, Szatanek R, *et al.* The importance of HLA assessment in “Off-the-Shelf” allogeneic mesenchymal stem cells based-therapies. *Int J Mol Sci* 2019; 20: 5680.
28 Ryan JM, Barry FP, Murphy JM, *et al.* Mesenchymal stem cells avoid allogeneic rejection. *J Inflamm (Lond)* 2005; 2: 8.
29 Chinnadurai R, Copland IB, Garcia MA, *et al.* Cryopreserved mesenchymal stromal cells are susceptible to T-cell mediated apoptosis which is partly rescued by IFNγ licensing. *Stem Cells* 2016; 34: 2429–2442.
30 Stagg J, Pommey S, Eliopoulos N, *et al.* Interferon-γ-stimulated marrow stromal cells: a new type of nonhematopoietic antigen-presenting cell. *Blood* 2006; 107: 2570–2577.
31 Moll G, Drzeniek N, Kamhieh-Milz J, *et al.* 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.
32 Reilly JP, Calfee CS, Christie JD. Acute respiratory distress syndrome phenotypes. *Semin Respir Crit Care Med* 2019; 40: 19–30.
33 Romieu-Mourez R, Francois M, Boivin MN, *et al.* Regulation of MHC class II expression and antigen processing in murine and human mesenchymal stromal cells by IFN-γ, TGF-β, and cell density. *J Immunol* 2007; 179: 1549–1558.
34 Tang KC, Trzaska KA, Smirnov SV, *et al.* Down-regulation of MHC II in mesenchymal stem cells at high IFN-γ can be partly explained by cytoplasmic retention of CIITA. *J Immunol* 2008; 180: 1826–1833.