Mesenchymal Stem Cells Reconditioned in Their Own Serum Exhibit Augmented Therapeutic Properties in the Setting of Acute Respiratory Distress Syndrome

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Abstract

Mesenchymal stem cells (MSCs) are a promising form of therapy for acute respiratory distress syndrome (ARDS). The objective of this study was twofold: (a) to characterize cytokine expression in serum from ARDS subjects receiving MSCs and (b) to determine MSC function following "preconditioning" with ARDS serum. In phase I, serum from three cohorts of animals (uninjured [no ARDS, $n = 4$], injured untreated [$n = 5$], and injured treated with approximately 6 million per kilogram MSCs [$n = 7$]) was analyzed for expression of inflammatory mediators. In phase II, the functional properties of bone marrow porcine MSCs were assessed following "preconditioning" with serum from the three cohorts. In phase III, the findings from the previous phases were validated using human bone marrow MSCs (hBM-MSCs) and lipopolysaccharide (LPS). Serum from injured treated animals had significantly lower levels of interferon-γ and significantly higher levels of interleukin (IL)-1 receptor antagonist (IL-1RA) and IL-6. Similarly, upon exposure to the injured treated serum ex vivo, the MSCs secreted higher levels of IL-1RA and IL-10, dampened the secretion of proinflammatory cytokines, exhibited upregulation of toll-like receptor 4 (TLR-4) and vascular endothelial growth factor (VEGF) genes, and triggered a strong immunomodulatory response via prostaglandin E2 (PGE$_2$). hBM-MSCs demonstrated a similar augmented therapeutic function following reconditioning in a LPS milieu. Administration of MSCs modulated the inflammatory milieu following ARDS. Exposure to ARDS serum ex vivo paralleled the trends seen in vivo, which appear to be mediated, in part, through TLR-4 and VEGF and PGE$_2$. Reconditioning MSCs in their own serum potentiates their immunotherapeutic function, a technique that can be used in clinical applications. Stem Cells Translational Medicine 2019;8:1092–1106

Significance Statement

Treatment with bone marrow mesenchymal stem cells (MSCs) mitigates the inflammatory milieu following acute respiratory distress syndrome (ARDS) because of the smoke inhalation and large surface area burns. Preconditioning MSCs with serum from subjects with ARDS negatively impacts their functional properties. Interestingly, MSCs preconditioned with serum that was previously exposed to MSCs potentiate their regenerative function. Therefore, this "pre-exposure" technique can be used to develop MSCs with augmented function for clinical applications.

Introduction

Acute respiratory distress syndrome (ARDS) is a rapidly progressive disease characterized by diffuse inflammation and increased vascular permeability of the lung parenchyma, leading to impaired alveolar gas exchange [1]. ARDS mortality has been estimated to be in the range of 35% to 46% [2]. Although health care advancements have improved patient outcomes, no pharmacological treatment has shown therapeutic effectiveness to date.

As one of the defining features of ARDS is an imbalance between proinflammatory and anti-inflammatory mediators [3], cytokine regulation has been targeted as a potential therapeutic approach [4, 5]. In the setting of ARDS, mesenchymal stem cells (MSCs) have been shown to treat impaired alveolar fluid clearance, decrease the lung permeability, combat infection, and

regulate inflammation [6]. Previous studies show that MSCs secrete a myriad of soluble paracrine factors, including the key anti-inflammatory cytokines interleukin (IL)-1 receptor antagonist (IL-1RA) and IL-10. However, because of the large heterogeneity in the field of MSCs [7, 8], these therapeutic effects are not always demonstrated in preclinical models or clinical trials [9].

One approach that has been proposed to enhance their immunomodulatory capacity is to activate or "precondition" the MSCs prior to administration. One form of preconditioning involves MSC exposure to serum obtained from subjects with ARDS, which has been shown to significantly mitigate ARDS in a small animal model [10]. Recently, we have shown that the ARDS milieu impairs the functional characteristics of endogenous bone-marrow MSCs [11]; however, preconditioning the MSCs with an augmented ARDS milieu ex vivo may offer downstream benefits in vivo. The primary purpose of this study was twofold: (a) to characterize cytokine expression in relation to disease progression using serum obtained from swine with ARDS and (b) to evaluate MSC function following exposure to ARDS serum and "precondition" them for administration to a hostile ARDS microenvironment. To accomplish this, MSCs were delivered to swine with ARDS (phase I). Next, serum from these animals was used to precondition MSCs, and their therapeutic efficacy was evaluated ex vivo (phase II); finally, the therapeutic mechanism of action (MOA) was verified using human MSCs in vitro (phase III; Supporting Information Figure S1).

MATERIALS AND METHODS

Research was conducted in compliance with the Animal Welfare Act, the implementing Animal Welfare Regulations, and the principles of the Guide for the Care and Use of Laboratory Animals, National Research Council. The facility's Institutional Animal Care and Use Committee approved all research conducted in this study. The facility where this research was conducted is fully accredited by AAALAC International.

Porcine Model of ARDS

Sixteen female nonpregnant, cross-bred Yorkshire pigs (3–6 months, 42.5 $\pm$ 1.67 kg; Midwest Research Swine, Gibbon, MN) were housed for at least 1 week to allow for acclimation and testing for any preexisting disease. Animals were fed standard chow and were fasted for 12 to 18 hours with access to water ad libitum. The animals were divided into three cohorts: "uninjured" (no ARDS, $n = 4$), "injured untreated" ($n = 5$), and "injured treated" with allogeneic MSCs ($n = 7$). ARDS was induced via (wood bark) smoke inhalation and large surface area burns, as previously described [12, 13].

Administration of MSCs

From the stem cell library developed at the U.S. Army Institute of Surgical Research Multi-Organ Support Technology Task Area, allogeneic MSCs were obtained from the bone marrow of donor swine. The allogeneic MSCs were characterized along different passages for their multilineage differentiation capacity as well as surface marker expression of CD29, CD90, and CD105 and the absence of CD45 using flow cytometry, as previously described [11, 14]. The allogeneic passage 3 MSCs were harvested fresh using 0.25% trypsin-EDTA (Thermo Fisher Scientific, Waltham, MA) from standard cell culture flasks, resuspended in 60 ml of

PlasmaLyte, and administered intravenously at an approximate rate of 2 ml/minute. The MSCs were administered via the pulmonary artery (through a Swan-Ganz catheter) at an average dose of 6.1 ($\pm$1.85 SEM) million cells per kilogram at three time points: postinjury (PI; within 30 minutes after injury to accommodate transfer to the animal intensive care unit), 24 hours PI, and 48 hours PI.

Evaluation of Inflammatory Mediators: Phase I

Whole blood was collected in serum collection tubes from all animals at five time points: preinjury baseline (BL), PI, 24 hours, 48 hours, and before euthanasia at 72 hours. After 1-hour incubation time, samples were centrifuged at 3000g and the serum transferred to a 1.5 ml centrifuge tube for storage at -80°C until batch analysis.

For cytokine measurements, the serum samples were slowly thawed on ice and subsequently analyzed on a BioPlex 200 system (Bio-Rad, Hercules, CA; http://www.bio-rad.com). The porcine cytokine-chemokine 13-plex Milliplex kit (Millipore, Billerica, MA) was used to evaluate Granulocyte-macrophage colony stimulating factor (GM-CSF), interferon-γ (IFN-γ), IL-1α, IL-1β, IL-1RA, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18, and tumor necrosis factor-α (TNF-α). Serum samples ($n = 80$, five time points per animal, 16 animals) were processed following the manufacturer's instructions. Data were analyzed using Bio-Rad BioPlex Manager software, version 6.0, and expressed in ng/ml. Subsequently, data were normalized to total protein and expressed as relative quotients (RQs).

Preconditioning of MSCs: Phase II

Phase I serum was pooled for each time point within each cohort to yield 15 samples ($n = 15$, three groups $\times$ five time points per group). Passage 2 porcine bone marrow MSCs were thawed and resuspended in complete culture medium (CCM) comprised of minimal essential medium-alpha formulation supplemented with 15% heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA), 1% L-glutamine, and 1% antibiotic/antimycotic. Unless noted, all reagents were purchased from Thermo Fisher Scientific. Cells were incubated at 37°C for a few hours to allow for initial attachment. Following attachment, the CCM was replaced with media supplemented with either 5% serum or 10% FBS as control. The cells were then incubated for 48 hours, after which preconditioned MSCs and their conditioned media (CM) were evaluated. The 48-hour time point was selected as it has been shown to be the optimal window for preconditioning MSCs [15].

Evaluation of Preconditioned MSCs

Viability and Cell Counts
The preconditioned MSCs were enzymatically detached from the plates using 0.25% trypsin-EDTA. Triplicates were pooled ($n = 16$), and cell yields/viabilities were recorded using a hemocytometer and trypan blue exclusion test. All time points were run in triplicates to examine differences between cohorts.

Clonogenicity, Metabolism, and Proliferation
Clonal capacity was measured via a colony-forming unit fibroblast (CFU-F) assay, as previously described [11, 16]. As BL levels varied, time points were normalized to BL values and reported as RQ.

Preconditioned MSCs were evaluated for their metabolic activity using the Vybrant assay (Thermo Fisher Scientific), according to the manufacturer's instructions and as previously described
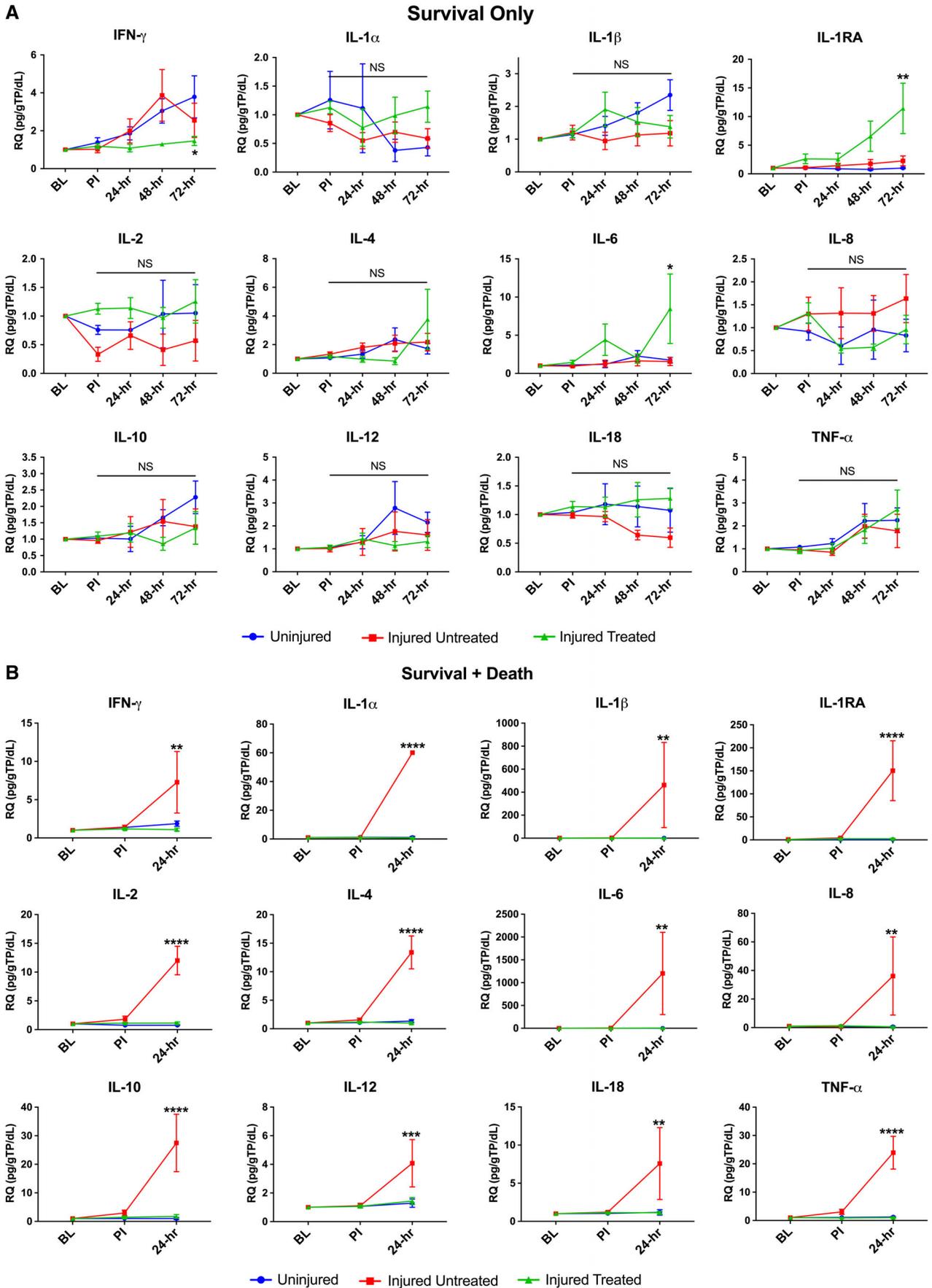
Figure 1. *(Legend appears on next page.)*

[11, 15]. To perform this, MSCs were seeded at 1000 cells per centimeter square in triplicates and evaluated along three different time points: day 3, day 7, and day 10. Time points were normalized to BL values and reported as RQ.

Following the metabolic assay, the MSCs from the different groups were placed in a cell lysis buffer (Cell Signaling Technology, Danvers, MA). Following lysis, DNA concentration was measured using the Quant-iT PicoGreen assay (Invitrogen) to evaluate cell proliferation on days 3, 7, and 10, as previously described [11, 16]. Time points were normalized to BL values and reported as RQ.

Cytokine Measurement
After the 48-hour incubation period, the preconditioned media were collected and cell debris removed before subsequent concentration using Amicon Ultra-2 Centrifugal Filters with a 3K molecular weight cut-off (Millipore, Burlington, MA). Cytokine levels were then determined via the multiplex assay and standardized to total protein. Time points were normalized to BL values and reported as RQ.

Quantitative Real-Time Polymerase Chain Reaction
Gene expression was evaluated via quantitative real-time polymerase chain reaction (qRT-PCR), as previously described [11, 15, 16]. Amplification and detection were carried out with a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA) for the following genes: angiopoietin 1 (ANG-1), B cell leukemia/lymphoma 2 (BCL2)-associated X (BAX), BCL-2, caspase 3 (CASP3), cytochrome c (CYCS), high-mobility group box protein 1 (HMGB1), NANOG, catalase (CAT), heme oxygenase-1 (HMOX1), porcine beta-defensin 2, prostaglandin-endoperoxide synthase 2, sex-determining region Y-box 2 (SOX-2), toll-like receptor 4 (TLR-4), tissue factor (TF), and vascular endothelial growth factor (VEGF). For porcine, gene expression was normalized to BL levels, and housekeeping gene was β-actin. For human, gene expression was normalized to the uninjured group ("−lipopolysaccharide" [LPS]), and housekeeping gene was 18 seconds. Levels are expressed as a RQ.

Coculture with Mononuclear Cells
Preconditioned MSCs were plated in triplicates at a density of 50,000 cells per well and incubated in CCM. The media were then replaced with RPMI1640 supplemented with 10% heat inactivated FBS. Phytohaemagglutinin (PHA)-activated mononuclear cells (MNCs) were added to each well at a seeding density of 250,000 cells per well. This coculture system was incubated at 37°C for 3 days. At day 3, the media were replaced with media containing either 5% pooled serum or 10% FBS as control. The cells were incubated for additional 24 hours, and the media were collected and analyzed with the Multiplex assay. Time points were normalized to BL values and reported as RQ.

To further elucidate MSC's paracrine activity and its therapeutic function, CM from the injured treated group was used in a coculture with MNCs, as described above. For this purpose, the CM was added at a dilution of 1:20 (v/v) to MNCs. After 96 hours, the CM was collected and analyzed with the Milliplex assay, as described above.

Evaluation of prostaglandin E2
To inform on MSC's MOA, prostaglandin E2 (PGE_2) levels were measured in the CM of the injured treated group using an enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN). Samples were analyzed according to the manufacturer's instructions. Measurements were normalized to total protein and reported as pg/ml.

Total Protein
All multiplex data were standardized to the total amount of protein in each sample to account for differences in BL cytokine levels. We used the Pierce protein assay (Thermo Fisher Scientific) according to the manufacturer's instructions. Protein concentrations were estimated by referencing samples' absorbance at 660 nm to values obtained for a series of standard solutions.

Flow Cytometry
Human and porcine MSCs were incubated with phosphate-buffered saline containing 1% bovine serum albumin or porcine serum, respectively. Additionally, Fc blocker (BioLegend, San Diego, CA) was added to cells for 10 minutes at a concentration of 1×10^6 cell per milliliter in order to reduce nonspecific binding. Anti-human CD3 APC (BD, Biosciences, San Jose, CA) and anti-porcine CD3e AF647 (BD, Biosciences) were used for assessing pan T cells. Cells were stained with preconjugated antibodies according to manufacturer's instruction. Following antibody binding, 7AAD viability staining solution (eBioscience, Grand Island, NY) was added for an additional 5 minutes to exclude dead cells. Acquisition was carried out on a BD FACSCelesta using the BDFACS Diva software (BD, Biosciences). Analysis was completed using FlowJo analysis software.

Verification of MOA Using Human MSCs: Phase III
Step 1: To validate findings from the above in vivo methods, we repeated the experimental design in vitro. For this purpose, human MNCs were isolated from peripheral blood of consenting donors using Ficoll-Paque in accordance with an institutional review board-approved protocol. The human MNCs were cultured at 0.5 million per milliliter in RPMI under standard conditions and exposed to 100 ng/ml LPS for 72 hours to form the following six groups: (a) not exposed to LPS, designated as the control uninjured group and labeled as "MNCs −LPS"; (b) exposed to LPS, designated as the injured untreated group and labeled as "MNCs +LPS"; (c) exposed to LPS and treated with MSCs for 6 hours, designated as the "injured treated PI" group and labeled as "PI"; (d) exposed to LPS and treated with MSCs for 24 hours, designated as the "injured treated 24 hours" group

Figure 1. Systemic inflammatory mediators in serum from three groups of swine: "uninjured" (*n* = 4), "injured untreated" (*n* = 5), and "injured treated" (*n* = 7). Injured treated swine received an intravascular infusion of approximately 6 million cells per kilogram immediately after injury and 24 hours and 48 hours PI. **(A):** Animals that survived, termed "survival only," exhibited significantly higher levels of IL-1RA (*p* < .01) and IL-6 (*p* < .05) and inhibition of IFN-γ at 72 hours. **(B):** Animals that succumbed to acute respiratory distress syndrome and did not survive demonstrated significant increases in all tested cytokines. *, *p* < .05; **, *p* < .01; ***, *p* < .001; ****, *p* < .0001. Abbreviations: BL, baseline; IFN, interferon-γ; IL, interleukin; IL-1RA, interleukin-1 receptor antagonist; PI, postinjury; RQ, relative quotient; TNF-α, tumor necrosis factor-α.
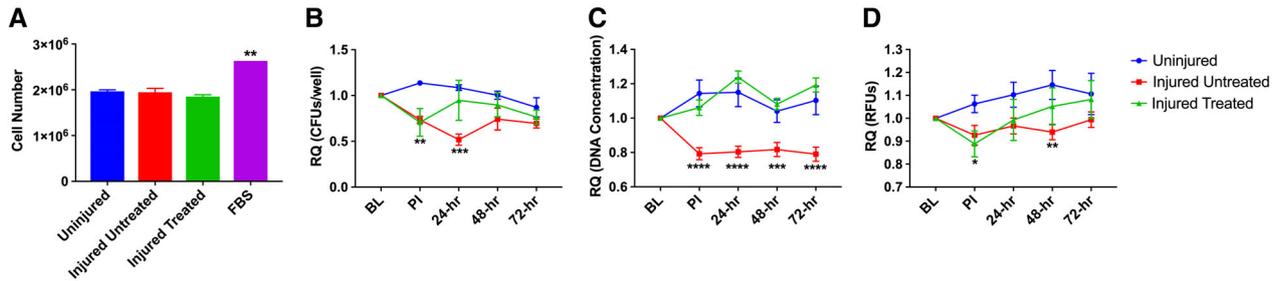
Figure 2. Characteristics of preconditioned mesenchymal stem cells (MSCs) following exposure to acute respiratory distress syndrome serum. **(A):** Cell numbers showing significantly higher counts ($p < .01$) for MSCs cultured in FBS compared with MSCs cultured in 5% pooled serum. **(B):** CFU-fibroblast assay showed diminished clonogenic capacity in "injured" MSCs. **(C):** Reduced proliferation in MSCs from the "injured untreated" group on day 10. **(D):** Reduction in metabolic activity for "injured" MSCs on day 10. *, $p < .05$; **, $p < .01$; ***, $p < .001$; ****, $p < .0001$. Abbreviations: BL, baseline; CFU, colony-forming unit; FBS, fetal bovine serum; PI, postinjury; RQ, relative quotient; RFUs, relative fluorescent units.
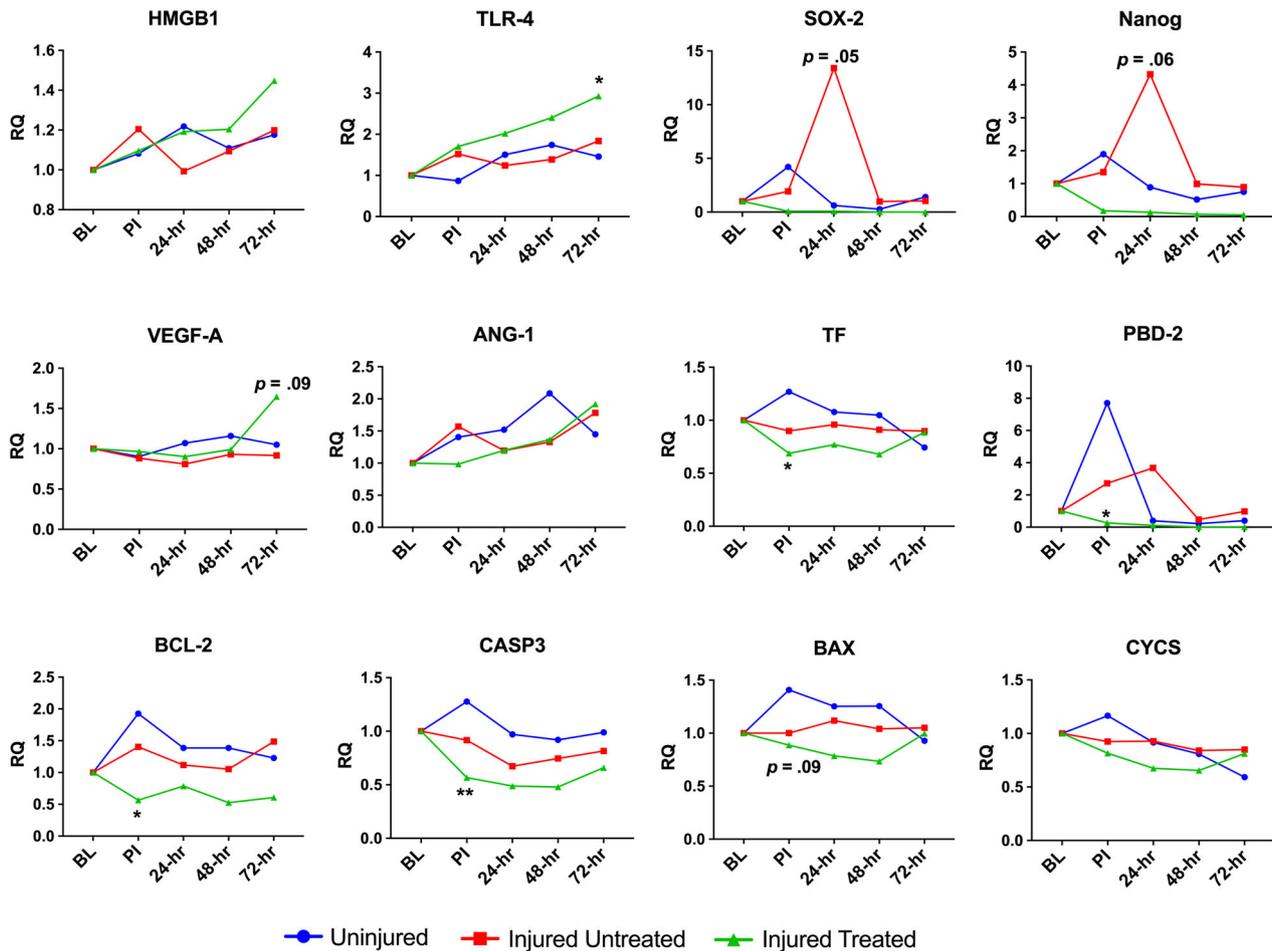

Figure 3. Gene expression of mesenchymal stem cells (MSCs) following 48 hours of preconditioning with 5% serum from the three groups of swine. Upregulation of TLR-4 ($p < .05$) and VEGF genes ($p = .089$) in MSCs conditioned with the "injured treated" serum with concomitant downregulation of the proapoptotic genes BCL-2 ($p < .05$), CASP3 ($p < .01$), and BAX ($p = .09$). Moreover, upregulation of the stem cell genes SOX-2 ($p = .052$) and Nanog ($p = .06$) for MSCs conditioned with the "injured untreated" serum. *, $p < .05$; **, $p < .01$. Abbreviations: ANG-1, angiopoietin 1; BAX, BCL2-associated X; BCL-2, B cell leukemia/lymphoma 2; BL, baseline; CASP3, caspase 3; CYCS, cytochrome c; HMGB1, high-mobility group box protein 1; PBD-2, porcine beta-defensin 2; PI, postinjury; RQ, relative quotient; SOX-2, sex-determining region Y-box 2; TF, tissue factor; TLR-4, toll-like receptor 4; VEGF-A, vascular endothelial growth factor A.

and labeled as "24 hours"; (e) exposed to LPS and treated with MSCs for 48 hours, designated as the "injured treated 48 hours" group and labeled as "48 hours"; (f) exposed to LPS and treated with MSCs for 72 hours, designated as the "injured treated 72 hours" group and labeled as "72 hours."

Human bone marrow-MSCs were isolated and characterized according to the International Society of Cellular Therapy, as previously described [15, 16]. In the MSC-containing groups, passage 2 MSCs were cultured at a 1:10 ratio to MNCs (1×10^5 MSCs to 1×10^6 MNCs). At each endpoint (PI, 24 hours,
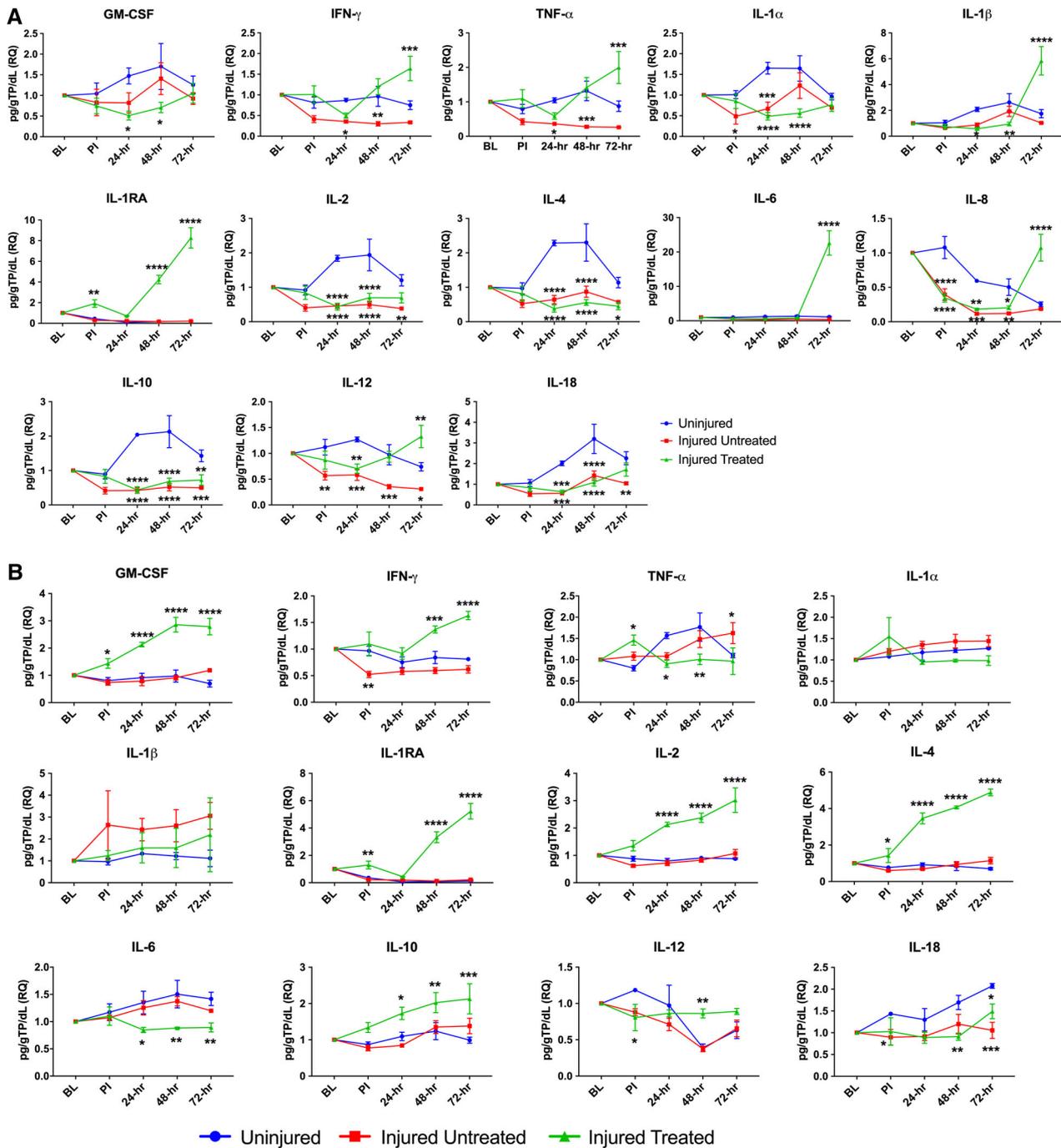
Figure 4. (*continued on next page*)

48 hours, and 72 hours), CM was collected and analyzed with the Milliplex and total protein assays. Additionally, PGE_2 levels were measured in the CM via ELISA. This first step created the injurious environment using LPS (similar to the smoke inhalation and burn environment in vivo) and the serum-like CM, as in the first two phases of the study.

Step 2: In the second verification step, MSCs were preconditioned in the serum-like CM. For this purpose, MSCs were plated at a density of 0.75×10^6 cells per well and cultured using the six groups of serum-like CM (diluted 1:10). As an additional control group, the MSCs were cultured in CCM

containing the standard 10% FBS. After 48 hours, the CM was analyzed again using the Milliplex assay, total protein, and levels of PGE_2. In addition, the preconditioned MSCs from the different groups were lifted for both qRT-PCR and coculture with MNCs.

Step 3: To evaluate the "reconditioned" CM and MSCs via MNC coculture, the reconditioned MSCs were seeded at a density of 0.5×10^5 cells per well in one plate, whereas the reconditioned CM was diluted (1:10 V/V) and added to another plate. Human MNCs were then added at a density of 0.5×10^6 cells per well to each well. As with the porcine coculture assay, the MNCs were
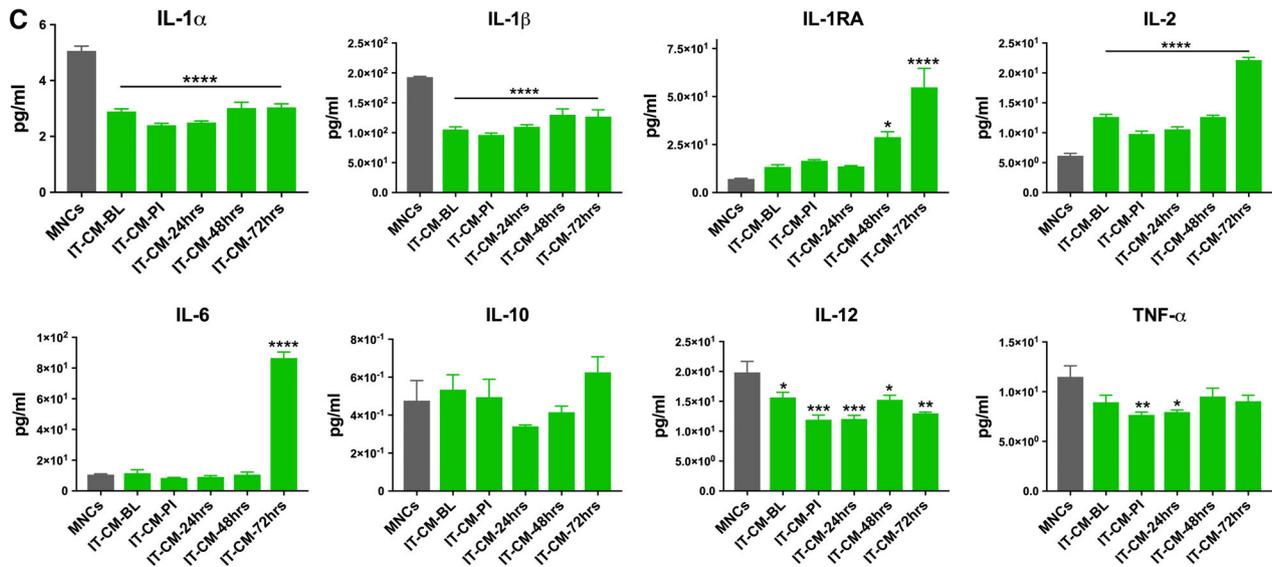
Figure 4. *(continued)* Levels of inflammatory mediators from preconditioned mesenchymal stem cells (MSCs) and following cocultures of the preconditioned MSCs or their CM with MNCs. **(A):** Overall suppression of various inflammatory mediators from MSCs treated with "injured" serum along the different time points with significant spikes in secretion of MSCs exposed to injured treated serum. Note the similar expression profiles of IL-1RA and IL-6, as seen in vivo. **(B):** Various cytokines show similar profiles as in single cultures, whereas other cytokines, such as GM-CSF, TNF-α, IL-2, IL-4, IL-6, and IL-10, demonstrate inverse relationship following a coculture with MNCs. **(C):** Secretion levels of the injured treated CM compared with control (+/+ MNCs) show significant suppression of inflammatory mediators and upregulation of IL-1RA and IL-6 as seen in vivo. *, $p < .05$; **, $p < .01$; ***, $p < .001$; ****, $p < .0001$. Abbreviations: BL, baseline; CM, conditioned media; GM-CSF, granulocyte-stimulating factor; IFN-γ, interferon-γ; IL, interleukin; IL-1RA, interleukin-1 receptor antagonist; MNCs, mononuclear cells; PI, postinjury; RQ, relative quotient; TNF-α, tumor necrosis factor-α.

stained with Carboxyfluorescein succinimidyl ester and stimulated with PHA. These stained and stimulated MNCs were designated as controls and labeled as "+/+." After 96 hours, floating cells were collected for flow cytometry analysis of T-cell proliferation, and the media were collected for measurement of PGE_2 levels and Milliplex analysis of inflammatory mediators (identical to the porcine panel) as well as the secretion of epidermal growth factor (EGF), fibroblast growth factor-2 (FGF-2), platelet-derived growth factor-(PDGF-AB/BB), and VEGF-A.

Statistical Analysis

Results are presented as means ± SEM. All statistical tests were performed with the aid of GraphPad Prism (San Diego, CA) version 7.01. In phases I and II, a one- or two-way analysis of variance (ANOVA) with multiple comparisons to the uninjured group and Bonferroni correction was used to examine differences between the three cohorts. In phase III, a one-way ANOVA with a Dunnett multiple comparison post hoc test was used to compare differences between the experimental groups to the control groups ("−LPS" in steps 1 and 2 and "+/+" [stained and stimulated] in step 3). A $p < .05$ was considered statistically significant. Cytokine data were standardized to total protein and all assays normalized to BL values and reported as RQ to focus on the effect of ARDS on MSCs and exclude the inherent differences between animals.

RESULTS

Phase I: In Vivo

ARDS Inflammatory Milieu

To elucidate the effects of ARDS due to smoke inhalation and burn on the systemic inflammatory milieu in vivo, we examined the

serum at five time points from the three cohorts of swine. First, animals that succumbed to ARDS and died were removed from analysis to compare cytokine expression from those who survived. At the 72-hour time point, the serum from the injured treated animals exhibited decreased levels of the proinflammatory cytokine IFN-γ ($p < .05$) and higher levels of the anti-inflammatories IL-1RA ($p < .01$) and IL-6 ($p < .05$). For all cohorts, levels of TNF-α increased with ARDS progression but did not show significance. There were no significant deviations from BL for IL-1α, IL-1β, IL-2, IL-4, IL-8, IL-10, IL-12, and IL-18 (Fig. 1A).

Animals that succumbed to ARDS and did not survive exhibited exuberant increases in cytokine levels prior to death, typical of the "cytokine storm" seen in ARDS. Dramatic differences can be seen between the injured untreated to the injured treated animals with significant differences in all tested cytokines at the 24-hour time point (Fig. 1B).

Phase II: Ex Vivo—Porcine MSCs

Characteristics of Preconditioned MSCs

As the hostile microenvironment of ARDS could be deleterious for MSCs, their characteristics were evaluated in vitro following a 48-hour preconditioning period. Regardless of the type of serum applied, cell viability was greater than 97% for all samples, whereas cell yield was significantly higher in the 10% FBS-supplemented media serum than those treated with 5% serum ($p < .01$), with comparable cell yields among the three cohorts (Fig. 2A).

Following preconditioning with the pooled serum, the MSCs were harvested and subjected to CFU-F, proliferation, and metabolic assays. For CFU-F, the MSCs treated with ARDS serum exhibited decreased clonogenic abilities at PI ($p < .01$) and 24-hour ($p < .001$) time points (Fig. 2B). Similarly, in MSCs
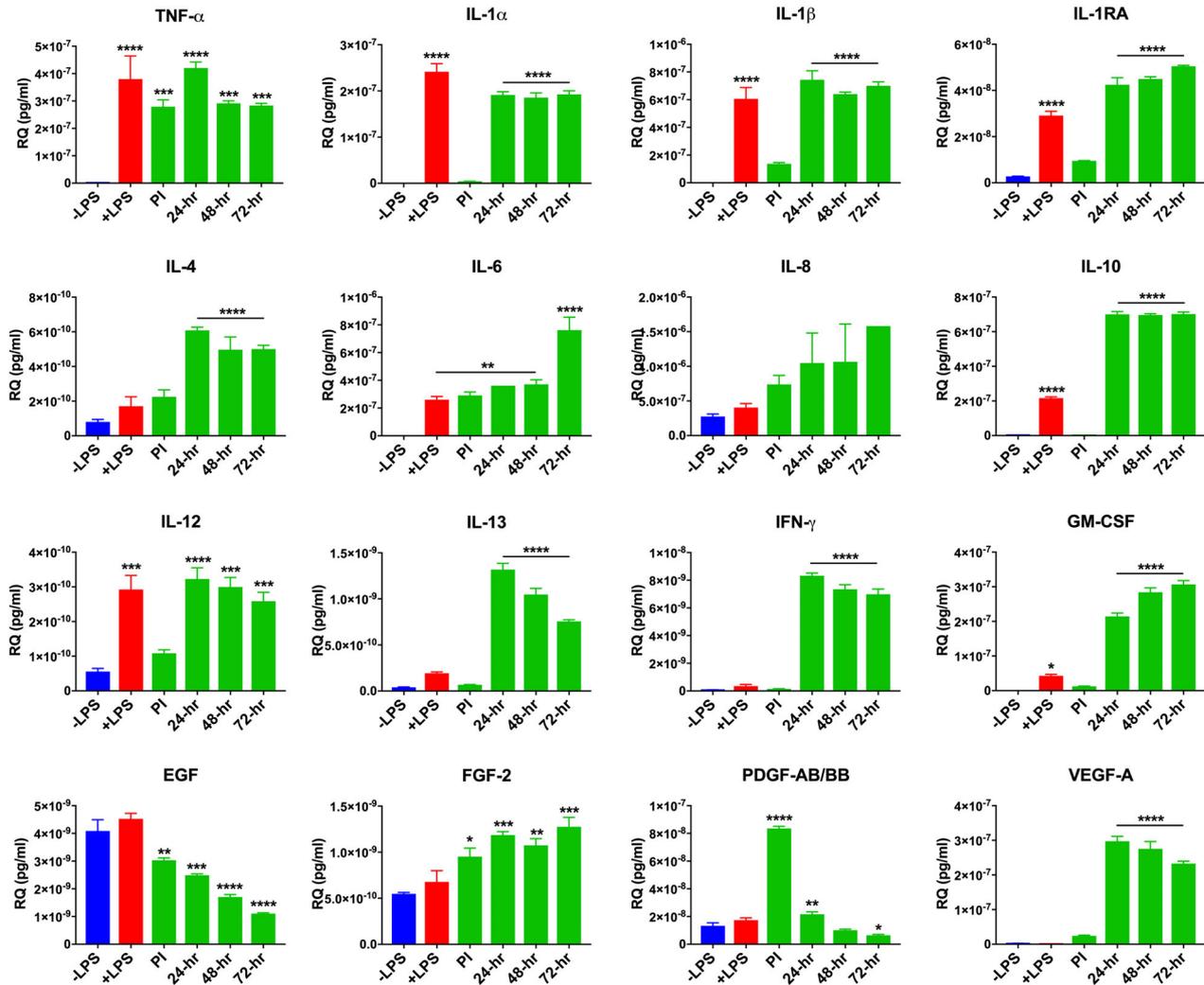
Figure 5. Secretion of inflammatory cytokines and bioactive factor from mesenchymal stem cells cocultured with mononuclear cells and challenged with LPS for 72 hours. At each time point, levels were measured in the conditioned media using the Milliplex assay. Note the significant increase in merely all of the inflammatory mediators as well as VEGF-A and FGF-2 beginning at the 24-hour time point. *, $p < .05$; **, $p < .01$; ***, $p < .001$; ****, $p < .0001$. Abbreviations: EGF, epidermal growth factor; FGF-2, fibroblast growth factor-2; GM-CSF, granulocyte-stimulating factor; IFN-γ, interferon-γ; IL, interleukin; IL-1RA, interleukin-1 receptor antagonist; LPS, lipopolysaccharide; PDGF-AB/BB, platelet-derived growth factor; PI, postinjury; RQ, relative quotient; TNF-α, tumor necrosis factor-α; VEGF-A, vascular endothelial growth factor.

preconditioned with the injured untreated serum, proliferation was significantly decreased at all time points ($p < .0001$). Their metabolic activity exhibited the same trend and was significantly lower at PI ($p < .05$) and 48-hour ($p < .01$) time points (Fig. 2C, 2D). Proliferation rates and metabolic capacities on days 3 and 7 revealed similar trends and are therefore not presented.

Gene Expression of Preconditioned MSCs

Following a 48-hour preconditioning period, gene expression levels of HMGB1, TLR-4, and VEGF showed overall upregulation in MSCs exposed to the injured treated serum, with significantly higher expression of TLR-4 at 72 hours ($p < .05$). In MSCs treated with the 24-hour injured untreated serum, the stem cell genes, SOX-2 and NANOG, were upregulated ($p = .052$ and $p = .06$, respectively). Additionally, significant downregulation were evident in the following genes: TF ($p < .05$), beta-defensin ($p < .05$), and the proapoptotic genes BCL-2 ($p < .05$), CASP3

($p < .01$), and BAX ($p = .09$) in the MSCs preconditioned with the injured treated (PI) serum (Fig. 3).

MSC Secretion in an ARDS Microenvironment

To assess the paracrine function of MSCs, levels of mediators secreted by MSCs preconditioned with injured (treated and untreated) sera were compared with levels secreted by MSCs preconditioned with uninjured serum (Fig. 4A). Similar to the trends seen in vivo, after exposure to 5% serum ex vivo, the MSCs exposed to 72-hour injured treated serum secreted significantly higher levels of IL-6 ($p < .0001$) and IL-1RA ($p < .0001$). Unlike in vivo, higher levels at 72 hours were also seen for IFN-γ ($p < .001$), IL-1β ($p < .0001$), and IL-8 ($p < .0001$). Additionally, the MSCs preconditioned with injured treated serum exhibited suppressed secretion of the following cytokines: GM-CSF ($p < .05$), IL-1α ($p < .0001$), IL-1β ($p < .05$), IL-2 ($p < .0001$), IL-4 ($p < .05$), IL-8 ($p < .05$), IL-10 ($p < .01$), IL-12 ($p < .01$), and IL-18 ($p < .001$). In MSCs exposed to injured untreated serum, levels of
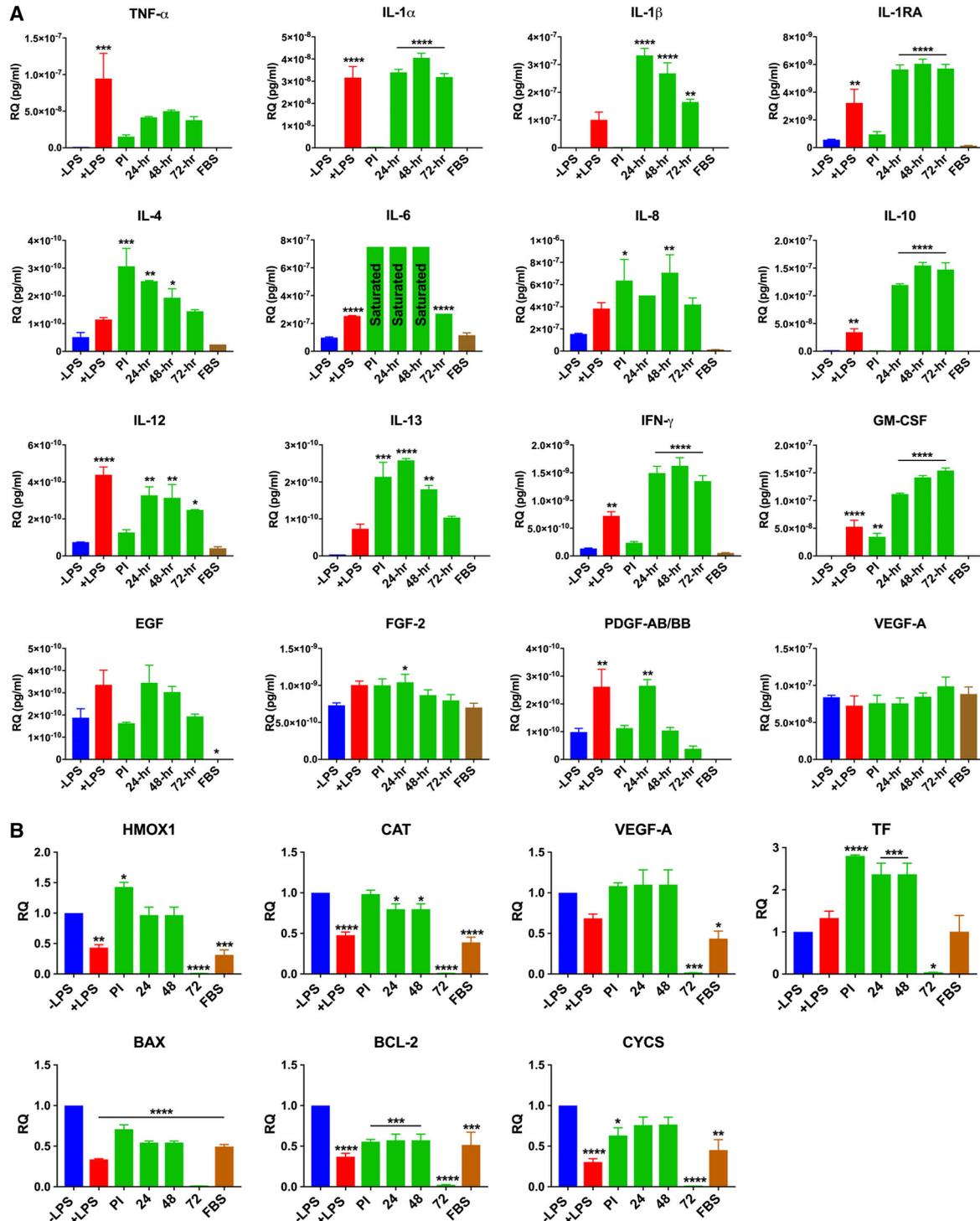
Figure 6. Secretion and gene expression profile of mesenchymal stem cells (MSCs) preconditioned in serum-like conditioned media. **(A):** Preconditioned MSCs showed increased secretion of the anti-inflammatory mediators IL-1RA, IL-4, IL-6, IL-10, and IL-13 as well as IL-1α, IL-1β, IL-12, IFN-γ, GM-CSF, FGF-2, and PDGF-AB/BB. **(B):** In treated MSCs, proapoptotic genes BAX, BCL2, and CYCS were significantly down-regulated, whereas HMOX, CAT, and TF were either upregulated or maintained levels similar to controls. *, $p < .05$; **, $p < .01$; ***, $p < .001$; ****, $p < .0001$. Abbreviations: BAX, BCL2-associated X; BCL-2, B cell leukemia/lymphoma 2; CAT, catalase; CYCS, cytochrome c; EGF, epidermal growth factor; FBS, fetal bovine serum; FGF-2, fibroblast growth factor-2; GM-CSF, granulocyte-stimulating factor; HMOX, heme oxygenase; IFN-γ, interferon-γ; IL, interleukin; IL-1RA, interleukin-1 receptor antagonist; LPS, lipopolysaccharide; PDGF-AB/BB, platelet-derived growth factor; PI, postinjury; RQ, relative quotient; TF, tissue factor; TNF-α, tumor necrosis factor-α; VEGF-A, vascular endothelial growth factor.
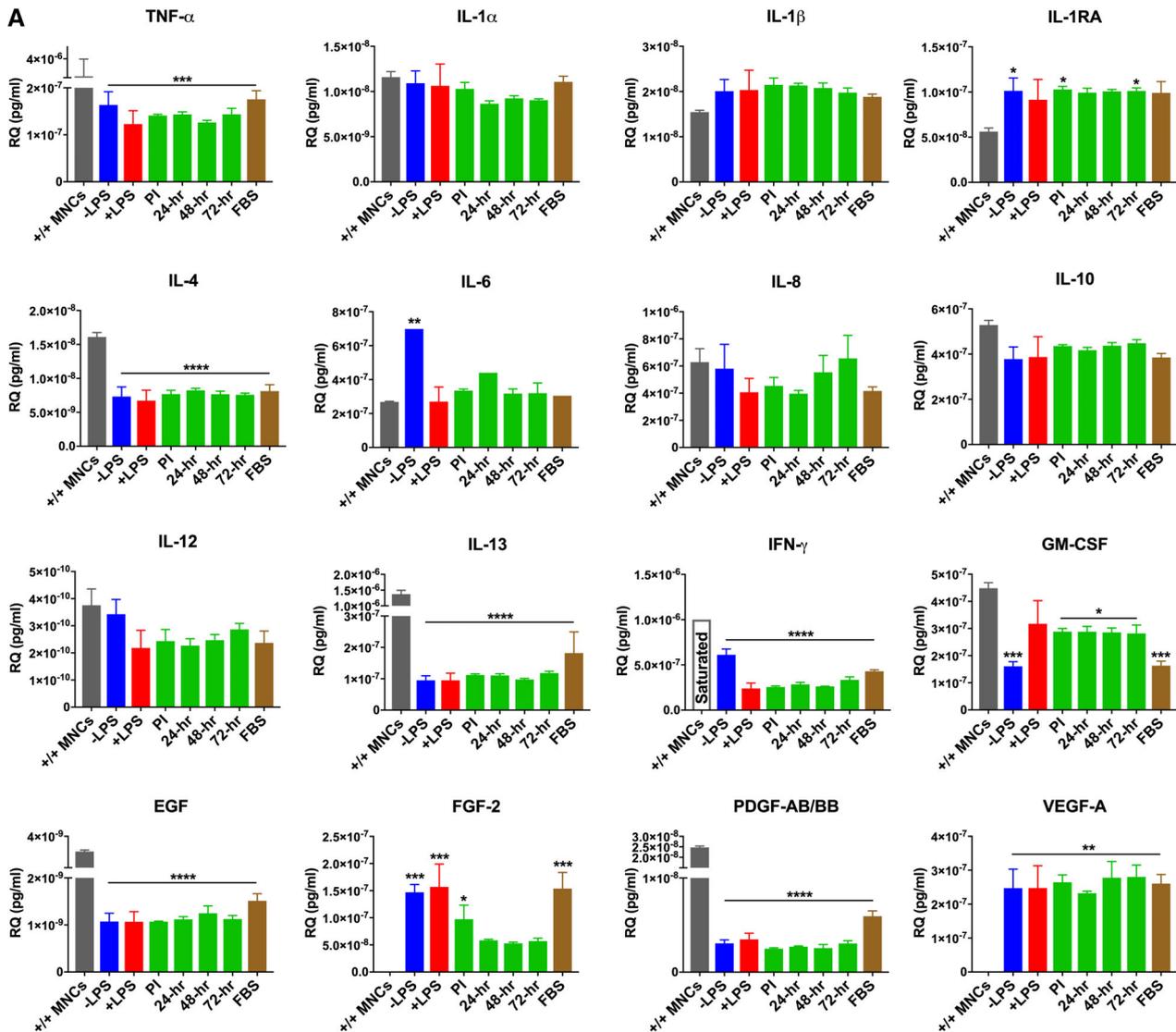
Figure 7. (*continued on next page*)

nearly all cytokines were significantly diminished, including: IFN-γ (*p* < .05), TNF-α (*p* < .05), IL-1α (*p* < .05), IL-2 (*p* < .01), IL-4 (*p* < .0001), IL-8 (*p* < .01), IL-10 (*p* < .001 at 72 hours), IL-12 (*p* < .05), and IL-18 (*p* < .001).

Coculture of Preconditioned MSCs and MSC-CM with MNCs

Cytokine secretion from preconditioned MSCs and their CM were also evaluated in a coculture system to elucidate whether immunomodulation is triggered by direct contact or paracrine activity is sufficient to modulate MNCs. Similar to direct secretion and in vivo serum levels, IL-1RA secretion from the injured treated MSCs was significantly elevated. Secretion levels of GM-CSF, IL-2, IL-4, and IL-10 from injured treated MSCs were significantly increased throughout all time points. This is in contrast to direct secretion by MSCs where these levels were significantly suppressed. Additionally, in contrast to direct secretion, levels of TNF-α and IL-6 were significantly suppressed by injured treated MSCs (Fig. 4B).

Unlike the preconditioned MSCs, the preconditioned MSC-CM were evaluated alone in comparison to control (+/+ MNCs) to

elucidate the therapeutic function of the injured treated group. The MSC-CM significantly suppressed the secretion of the pro-inflammatory cytokines IL-1α (*p* < .0001), IL-1β (*p* < .0001), IL-12 (*p* < .05), and TNF-α (*p* < .05), although concomitantly promoting the secretion of IL-1RA (*p* < .0001), IL-2 (*p* < .0001), and IL-6 (*p* < .0001), most prominently at the 72-hour time point (Fig. 4C).

Phase III: In Vitro—Human MSCs
Step 1: MSC Secretion in LPS Milieu

To further elucidate on MSC MOA as well as illustrate that the "reconditioning" phenomenon is not species dependent, we repeated this assay with human bone marrow MSCs cultured in an inflammatory environment in vitro. In the first step, MSCs were added to MNCs exposed to LPS. Analysis of bioactive factors demonstrated significant increases in inflammatory mediators due to the LPS stimulus. Specifically, TNF-α (*p* < .0001), IL-1α (*p* < .0001), IL-1β (*p* < .0001), IL1-RA (*p* < .0001), IL-6 (*p* < .01), IL-10 (*p* < .0001), and IL-12 (*p* < .001) were all significantly increased in all groups compared with controls (−LPS). Additionally, the "injured treated" groups (PI, 24 hours, 48 hours, and
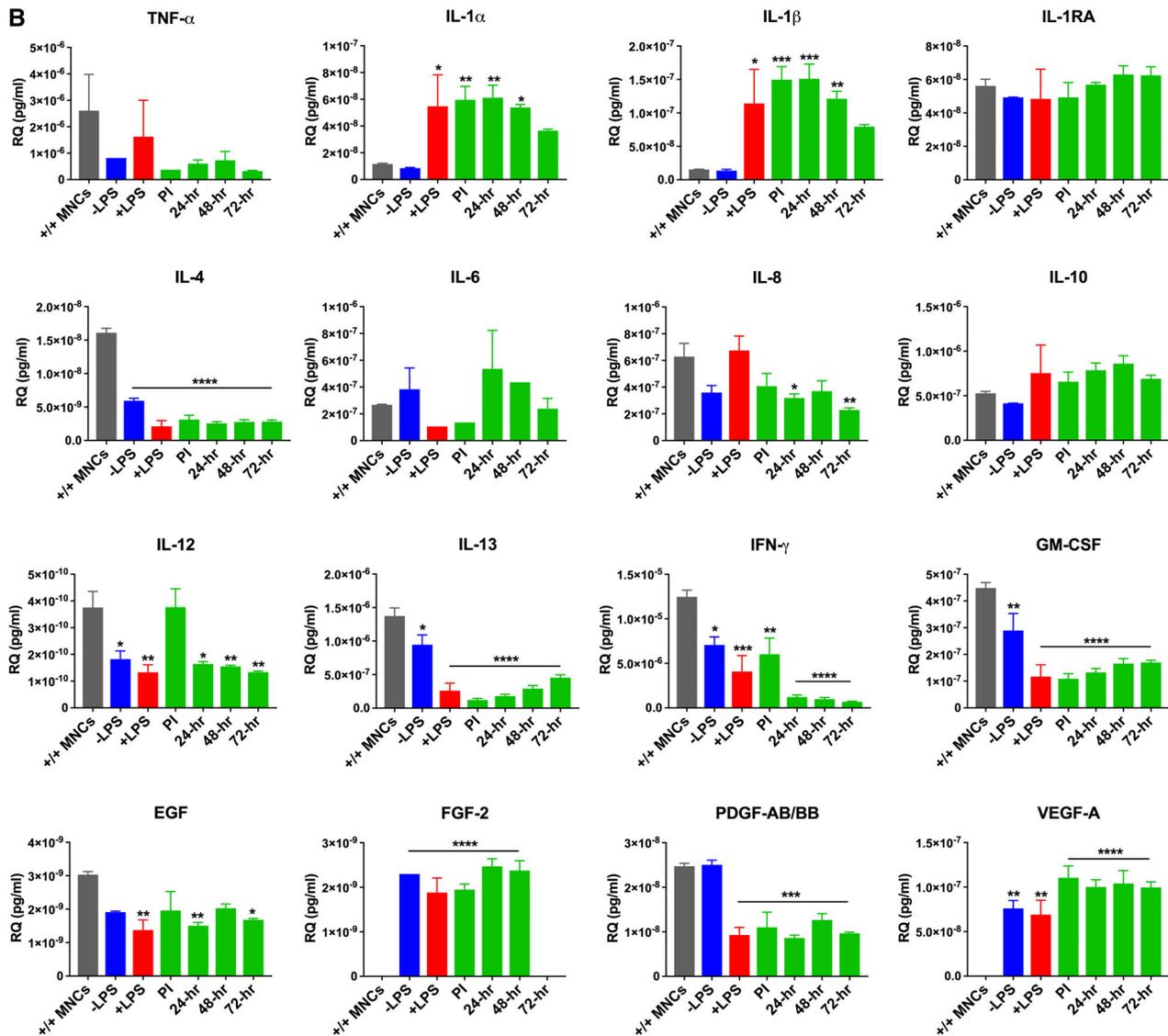
Figure 7. *(continued)*

72 hours) further immune modulated the inflammatory response. Specifically, the inflammatory mediators TNF-α and IL-1α were significantly repressed, whereas the anti-inflammatory cytokines IL-1RA, IL-4, IL-6, IL-10, and IL-13 were significantly increased compared with the injured untreated (+LPS) group. Moreover, the injured treated group exhibited significant secretion of IFN-γ ($p < .0001$), GM-CSF ($p < .0001$), FGF-2 ($p < .01$), and VEGF-A ($p < .0001$) beginning at the 24-hour time point. PDGF-AB/BB significantly peaked at PI ($p < .0001$) but sharply dropped within 24 hours, whereas EGF exhibited a gradual decrease with time progression. Similar to phases I and II, CM from the 72-hour time point had significantly elevated levels of IL-6 compared with all other groups (Fig. 5).

Step 2: Preconditioning MSCs in LPS Serum

Similar to phase II of the porcine study, MSCs were preconditioned in the CM from step 1. Analysis of the resulting CM following incubation of 48 hours demonstrated significant differences in secreted factors between the three

main groups. Specifically, reconditioning with the serum-like MSC-CM significantly suppressed the secretion of TNF-α ($p < .001$), whereas inducing the secretion of the anti-inflammatory cytokines IL-1RA ($p < .0001$), IL-4 ($p < .05$), IL-6 ($p < .0001$), IL-10 ($p < .0001$), and IL-13 ($p < .01$). Additionally, levels of IL-1β ($p < .0001$), IFN-γ ($p < .0001$), and GM-CSF ($p < .0001$) were significantly higher in the injured treated groups. Unlike step 1, no apparent differences were noted in growth factor secretion, other than significant secretion of PDGF-AB/BB ($p < .01$; Fig. 6A).

Apart from their paracrine activity, the preconditioned MSCs revealed significant differences in gene expression. Specifically, in injured treated MSCs, HMOX ($p < .05$) and TF ($p < .001$) were significantly upregulated, whereas the proapoptotic genes BAX ($p < .0001$), BCL-2 ($p < .001$), and CYCS ($p < .05$) were downregulated compared with control (−LPS). Additionally, significant changes can be seen in comparison to MSCs cultured in standard FBS, particularly in HMOX, CAT, TF, and VEGF expression (Fig. 6B).
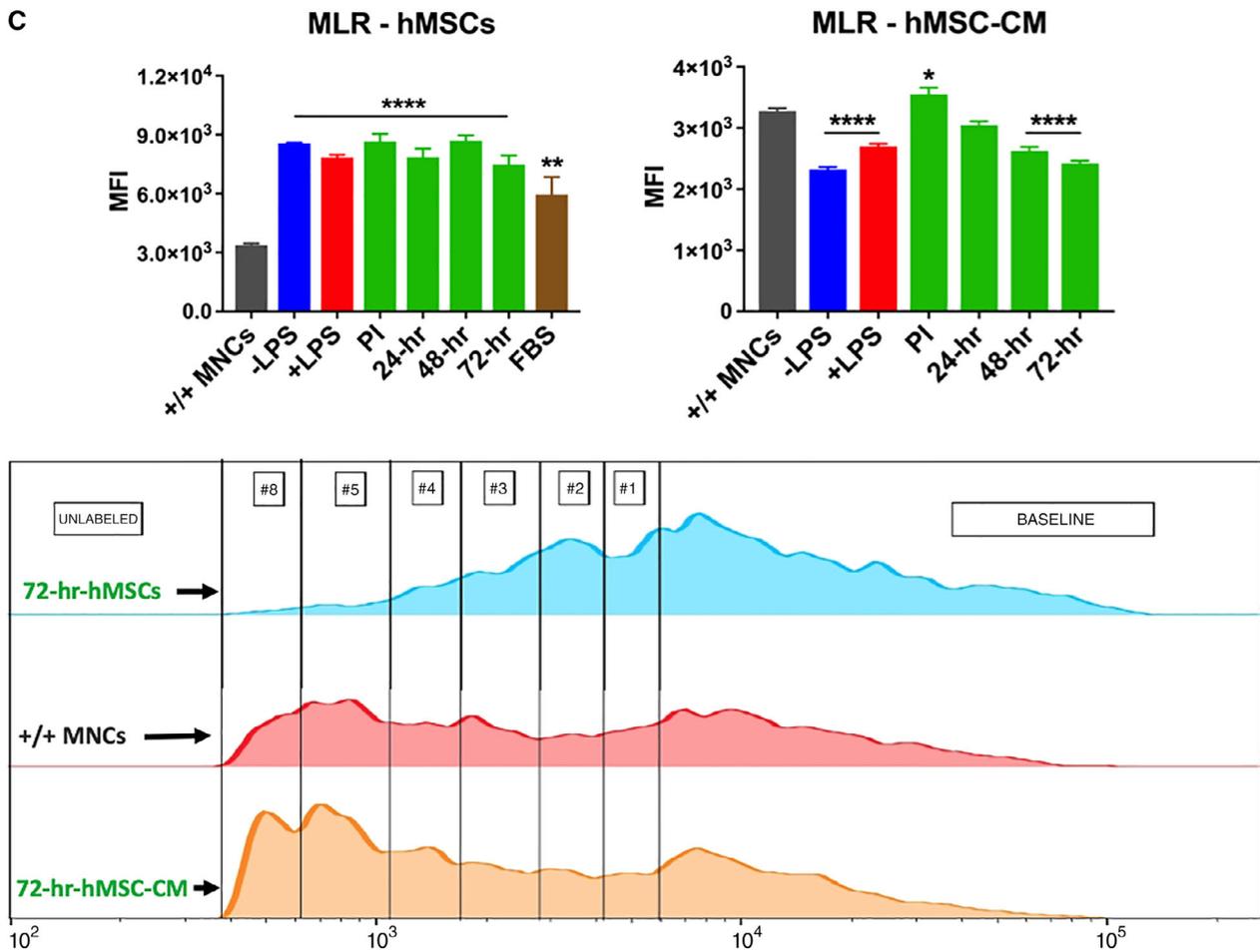
Figure 7. *(continued)*

Step 3: Evaluation of Immunomodulatory Function

In this step, the therapeutic function of MSCs and their CM was evaluated in a coculture with MNCs to inform on immunomodulation via direct or indirect mechanism, respectively. Compared with controls (+/+ MNCs), MSCs from all groups suppressed the secretion of TNF-α ($p < .001$), IL-4 ($p < .0001$), IL-13 ($p < .0001$), IFN-γ ($p < .0001$), GM-CSF ($p < .05$), EGF ($p < .0001$), PDGF-AB/BB ($p < .0001$), whereas concomitantly enhancing the secretion of the anti-inflammatory IL-1RA ($p < .05$) and the growth factors FGF-2 ($p < .05$) and VEGF ($p < .01$; Fig. 7A). The preconditioned-CM demonstrated similar effects, albeit at a different potency. More specifically, addition of MSC-CM suppressed the secretion of IL-4 ($p < .0001$), IL-12 ($p < .05$), IFN-γ ($p < .05$), GM-CSF ($p < .01$), EGF ($p < .05$), and PDGF-AB/BB ($p < .001$), whereas concomitantly enhancing the secretion of IL-1α ($p < .05$), IL-1β ($p < .05$), FGF-2 ($p < .0001$), and VEGF ($p < .01$). Unlike MSCs, the CM did not inhibit TNF-α production or enhanced the secretion of IL-1RA; however, levels of IL-1α, IL-1β, FGF-2, and VEGF were significantly higher than in the MSC cocultures (Fig. 7B).

In addition to immunomodulation via paracrine activity, MSCs are known to arrest T-cell proliferation. A mixed lymphocyte reaction-like assay demonstrated that MSCs were able to significantly suppress the proliferation of mixed immune cells

($p < .0001$). The MSC-CM demonstrated opposite effects; that is, apart from the "CM-PI" group, their addition significantly increased the proliferation of the mixed immune cells (Fig. 7C).

Mechanism of Action

To elucidate MSC MOA, levels of PGE_2 were also evaluated. In the porcine samples, levels of PGE_2 were measured in the CM following preconditioning (phase II), prior to the MNC cocultures. Higher levels of PGE_2 were seen in the injured treated group with significantly higher levels at the 72-hour time point ($p < .05$). PGE_2 levels were also measured in the human CM. Throughout all steps, PGE_2 levels were significantly higher in the injured treated groups compared with the rest of the groups. In step 1, PGE_2 levels were significantly higher beginning at 24 hours ($p < .0001$). In step 2, all LPS-exposed groups demonstrated significantly higher levels of PGE_2 compared with the rest of the groups. In step 3, the CM from the MSC cocultures demonstrated robust secretion of PGE_2 compared with controls (+/+ MNCs). In addition, compared with FBS, PGE_2 levels were significantly higher in all LPS-exposed groups ($p < .0001$). Similarly, significant PGE_2 levels were observed in the CM co-cultures, again significantly more in the LPS-exposed groups compared with controls ($p < .0001$; Fig. 7D).
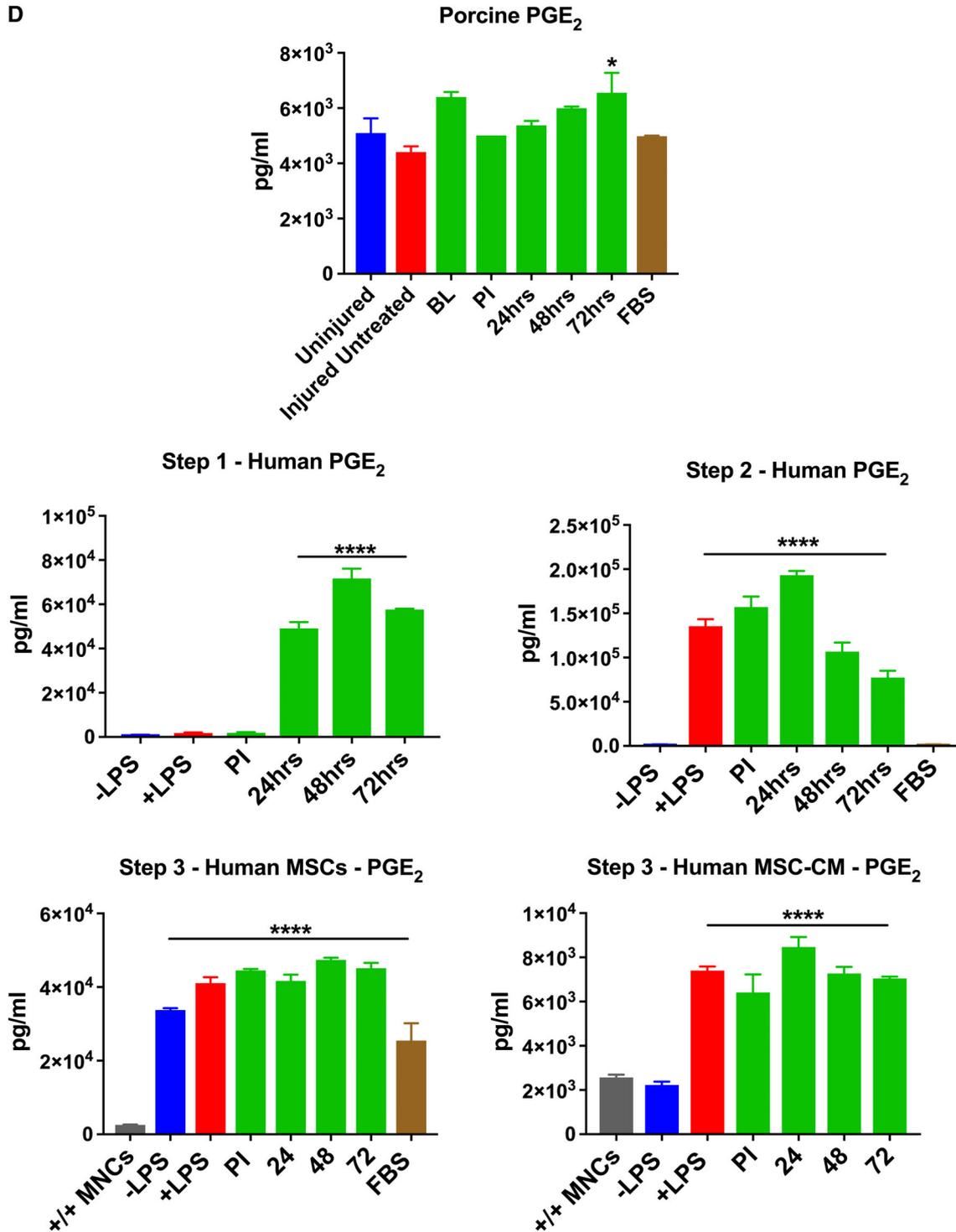
Figure 7. *(continued)* Mechanism of immunomodulation by preconditioned MSCs and MSC-CM in an MNC coculture. **(A):** Preconditioned MSCs cocultured with MNCs suppressed the secretion of proinflammatory cytokines, whereas enhancing the secretion of anti-inflammatory cytokines and key growth factors, such as VEGF and FGF-2. **(B):** MSC-CM exhibited similar effects although at a reduced potency exemplified by the inability to suppress TNF-α and promote the secretion of IL-1RA. **(C):** Top panels: MSCs immunosuppressed T-cell proliferation, whereas MSC-CM did not show the same effect; bottom panel: graphical representation of the mean fluorescence intensity of stained cells, illustrating high levels in nonproliferating cells (MSC-treated) and low levels in proliferating cells (controls and MSC-CM samples). **(D):** Elevated levels of PGE_2 were seen in the injured treated porcine MSCs samples as compared with controls and standard FBS. This mechanism remained consistent in human CM with significant increases of PGE_2 in all steps of the injured treated MSCs and their CM. *, $p < .05$; **, $p < .01$; ***, $p < .001$; ****, $p < .0001$. Abbreviations: CM, conditioned media; EGF, epidermal growth factor; FBS, fetal bovine serum; FGF-2, fibroblast growth factor-2; GM-CSF, granulocyte-stimulating factor; hMSCs, human mesenchymal stem cells; IFN-γ, interferon-γ; IL, interleukin; IL-1RA, interleukin-1 receptor antagonist; LPS, lipopolysaccharide; MNC, mononuclear cell; PDGF-AB/BB, platelet-derived growth factor; PGE_2, prostaglandin E2; PI, postinjury; RQ, relative quotient; TNF-α, tumor necrosis factor-α; VEGF-A, vascular endothelial growth factor.

DISCUSSION

The key findings of this study are (a) MSC treatment appears to suppress proinflammatory cytokine levels, whereas promoting secretion of anti-inflammatory mediators following administration in vivo; (b) ex vivo experiments reflect trends seen in vivo, in which the IL-6 and IL-1RA relationship appears to play a prominent role; and (c) MSCs reconditioned in their own serum exhibit augmented immunotherapeutic function, which appears to be mediated in part by TLR-4, VEGF, and PGE$_2$.

Given emerging promise of MSC therapy for ARDS [9, 17], we sought to characterize the ARDS microenvironment and evaluate MSC function in a subset of animal models from a larger study, in which ARDS was induced by smoke inhalation and severe burns [16, 17]. MSC infusion to human patients has been shown to improve resolution of respiratory and multiorgan failure through reducing pulmonary and systemic markers of inflammation [18]. One of the caveats to MSC treatment for ARDS is the fate of the cells following exposure to a hostile microenvironment. Therefore, replenishment of preconditioned MSCs with enhanced adaptive potential and therapeutic capabilities may lead to enhanced efficacy in vivo [19, 20].

In this study, we demonstrated that MSC treatment dampens the ARDS microenvironment; and likewise, the ARDS microenvironment alters MSC function. Serum from animals with ARDS treated with MSCs exhibited significantly enhanced levels of IL-1RA and IL-6, which was matched by elevated secretions of these cytokines in vitro. Production of IL-1RA is central to MSC function and appears to be triggered by the positive feedback of IL-6 as a late-phase anti-inflammatory mediator, whereas concomitantly decreasing TNF-α production [21, 22]. IL-6 acts as a proinflammatory cytokine in the short term and is suppressed before increasing at 72 hours after injury to enhance IL-1RA levels. The relationship between these two cytokines appears to be central to the therapeutic effect of MSCs, as suggested by others [23–26].

In phase II, following exposure to ARDS serum, there is a decreased capacity to self-renew (as evident via reduced clonogenic capacity), as well as reduced proliferative and metabolic activity. This is in line with our previous findings demonstrating the negative effects of ARDS on MSC characteristics [11]. Yet, preconditioning with injured treated serum allows MSCs to proliferate at a level comparable to controls. Thus, MSCs preconditioned in serum that was previously exposed to MSCs better retained normal functioning, which may potentially translate to augmented therapeutic benefit in the clinic [27].

As it is difficult to completely ascertain the role of cytokine expression in a complex in vivo ARDS model, we have examined cytokine secretion both ex vivo using the porcine serum and in vitro using human CM. upon exposure to ARDS serum, cytokine levels changed dynamically and significantly across time points and cohorts. Exposure to serum from injured treated swine induced a significant secretion of IL-1RA and IL-6 at the 72-hour time point. Preconditioning with injured treated serum also suppressed the secretion of various proinflammatory cytokines (i.e., TNF-α, IFN-γ, IL-1β, IL-8, and IL-12) throughout ARDS progression. Conversely, there is a rebound in these cytokines for MSCs preconditioned with 72-hour injured treated serum. This phenomenon may be attributed to the transient nature of systemic MSC action as the last infusion was performed at 48 hours. Therefore, by the 72-hour time point, some of the short-lasting effects of MSCs may have diminished.

In regards to gene expression, our results propose that MSCs reconditioned with MSC-treated media trigger activation of innate immunity through upregulation of TLR-4. Additionally, preconditioned MSCs exhibit downregulation of antiapoptotic genes and upregulation of the antioxidant gene HMOX and the angiogenic genes VEGF and TF. We then measured secretions following coculture with MNCs to elucidate the mechanism by which MSCs regulate other immune cells ex vivo. The upregulation of IL-1RA following coculture remains intact for MSCs exposed to injured treated serum, similar to direct secretion. The anti-inflammatory IL-10 is also secreted in significantly larger amounts, a response not seen without MNC addition, which indicates that unlike IL-1RA, IL-10 secretion is mediated via other immune cells, such as macrophages [28]. Intriguingly, following coculture of the preconditioned MSCs and their CM with MNCs, various cytokines show inverse trends in the secretion of various cytokines as compared with direct secretion from MSCs. These findings further highlight the fundamental immunomodulatory function of MSCs in the setting of ARDS.

Finally, phase III revealed that MSCs—in humans and porcine alike—work through a combination of direct and indirect mechanisms to modulate the microenvironment of ARDS. As shown through MNC cocultures, IL-1RA secretion and TNF-α suppression are controlled directly, whereas growth factors VEGF and FGF-2 are mainly enhanced indirectly. Other proinflammatories are inhibited and anti-inflammatories increased via both mechanisms, demonstrating a synergistic collaboration between the pathways. Through direct interactions, MSCs also work to arrest T-cell proliferation via PGE$_2$, which is significantly enhanced following preconditioning. Therefore, our results demonstrate that MSCs' therapeutic MOA involves a combination of paracrine mechanism, and most likely autocrine signaling evident via the preconditioning approach, to immunomodulate the inflammatory environment in ARDS (Supporting Information Figure S2).

CONCLUSION

MSC treatment appears to suppress the inflammatory ARDS milieu in vivo. Preconditioning MSCs with ARDS serum appears to negatively impact their function. Conversely, cells incubated in injured treated serum exhibit enhanced characteristics along with increased synergistic secretion of the anti-inflammatory mediators IL-6 and IL-1RA as well as VEGF and PGE$_2$. Aside from direct secretion, MSCs preconditioned with injured treated serum also modulate the anti-inflammatory milieu via other immune cells. Therefore, activation of allogeneic MSCs, through incubation in an environment previously exposed to MSCs, may induce stronger immunomodulatory effects in patients compared with infusion of nonactivated MSCs. These findings are most applicable to ARDS diagnoses, although generalization to other conditions is likely.

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AUTHOR CONTRIBUTIONS

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

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