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Autologous Peripheral Blood Mononuclear Cells as Treatment in Refractory Acute Respiratory Distress Syndrome

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Key Words
Mononuclear cells · Interventional pulmonology · Acute respiratory distress syndrome · Cell therapy

Abstract

Background: Acute respiratory distress syndrome (ARDS) is a devastating disorder. Despite enormous efforts in clinical research, effective treatment options are lacking, and mortality rates remain unacceptably high. ***Objectives:*** A male patient with severe ARDS showed no clinical improvement with conventional therapies. Hence, an emergent experimental intervention was performed. ***Methods:*** We performed intratracheal administration of autologous peripheral blood-derived mononuclear cells (PBMCs) and erythropoietin (EPO). ***Results:*** We found that after 2 days of initial PBMC/EPO application, lung function improved and extracorporeal membrane oxygenation (ECMO) support was reduced. Bronchoscopy and serum inflammatory markers revealed reduced inflammation. Additionally, serum concentration of miR-449a, b, c and miR-34a, a transient upregulation of E-cadherin and associated chromatin marks in PBMCs indicated airway epithelial differentiation. Extracel-

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lular vesicles from PBMCs demonstrated anti-inflammatory capacity in a TNF-α-mediated nuclear factor-κB in vitro assay. Despite improving respiratory function, the patient died of multisystem organ failure on day 38 of ECMO treatment. ***Conclusions:*** This case report provides initial encouraging evidence to use locally instilled PBMC/EPO for treatment of severe refractory ARDS. The observed clinical improvement may partially be due to the anti-inflammatory effects of PBMC/EPO to promote tissue regeneration. Further studies are needed for more in-depth understanding of the underlying mechanisms of in vivo regeneration.

Introduction

Acute respiratory distress syndrome (ARDS) is one of the leading causes of morbidity and mortality in critically ill patients. It is characterized by acute inflammatory lung injury leading to parenchymal damage, decreased lung compliance, pulmonary edema and impaired gas exchange resulting in severe life-threatening hypoxemia. Mortality in severe ARDS ranges from 42 to 48% [1, 2]. Despite enormous efforts in critical care research, there is still no reliable and effective treatment available. Lung-protective and anti-inflammatory strategies appear to be promising approaches [3, 4].

Adult stem cell therapy may be a potential therapeutic alternative due to the characteristics of stem cells. Adult stem cells (a) possess anti-inflammatory properties that may combat inflammatory processes, which usually trigger the majority of morbidity in ARDS; in particular, mesenchymal stem cells (MSCs) have demonstrated anti-inflammatory capacity and immunomodulatory characteristics that do not negatively affect patients' innate immune response; (b) can induce notable organ-specific functional restoration, which may assist in treating multiple organ failure often associated with ARDS; (c) can adjunctively treat bacterial sepsis, the most common cause of ARDS, through cell-specific mechanisms such as enhanced bacterial clearance, phagocytosis and secretion of anti-microbial peptides; (d) can support restoration of damaged alveolar barrier by improving endothelial and epithelial cell functions via differentiation and/or secretion of paracrine factors with regenerative capacity; (e) can localize therapy as stem cells migrate to sites of inflammation via various homing mechanisms, such as SDF-1/CXCR4; (f) are safe (autologous), and lastly (g) can be applied both intravenously and intratracheally to maximize bioavailability.

In recent years, cell therapy was introduced as a potential treatment option in patients with lung diseases [5, 6], such as chronic obstructive pulmonary disease [7, 8], pulmonary hypertension [9, 10] (NCT00469027, NCT00551408, NCT00683722) or lung cancer [11]. MSCs have been evaluated in various preclinical lung injury models with favorable outcomes [10, 12, 13] and have now been transferred to the clinical setting for patients with ARDS [14, 15]. Current clinical trials will probably provide new evidence for their beneficial effects in this particular group of patients (NCT02[illegible]455, NCT01775774).

However, the number of MSCs in the bone marrow cell population of bone marrow-derived cells is rather low (0.002–0.02%) [16], and in vitro culture, including its associated disadvantages, is required to obtain a decent number for cell administration. In contrast, MSCs, mononuclear cells as a mixed cell population may be an ideal alternative due to their ease of isolation and sufficient number of cells for direct administration.

Rojas et al. [17] showed beneficial effects of using bone marrow-derived mononuclear cells in an ARDS model in large animals. We hypothesize that the mononuclear cell fraction isolated from peripheral blood, known as peripheral blood-derived mononuclear cells (PBMCs) may have similar beneficial effects in ARDS to the previously described bone marrow-derived subpopulation.

We also postulate that erythropoietin (EPO) may provide a therapeutic benefit in ARDS due to its anti-inflammatory properties, its ability to positively modulate immune responses [18, 19] and influence cell apoptosis [20–22]. Upregulation of anti-apoptotic genes, such as Janus tyrosine kinase-2, STAT5 (signal transducer and activator of transcription 5), Bcl-2, phosphatidylinositol 3, protein kinase B, mitogen-activated protein kinase and nuclear factor-κB (NFκB), after systemic EPO administration for 14 days has been described, and no immediate negative side effects were clinically observed [23].

This clinical case report of a young male with burn-induced refractory ARDS provides early evidence for safe and effective therapeutic use of locally administered autologous PBMCs and EPO.

Material and Methods

The treatment of the patient did not require ethical approval, as it was not performed as a research or a clinical trial but as a last treatment option in a desperate clinical situation. A multidisciplinary meeting of physicians and caregivers took place, and the emergent use of experimental PBMC/EPO therapy was proposed

and agreed upon. Given the urgency of the situation, the patient's family was presented with this possible life-saving option and verbal consent was obtained. Ethical approval for blood and tissue analysis, patient data analysis and the future publication of results were obtained after the patient's death from the local ethics committee under serial number DNR: 2012/2187-31/1 (Stockholm, Sweden). Additional approval for biobanking and logging patient-specific data in a protected registry were also obtained from the responsible authorities at the Karolinska University Hospital. After this, written informed consent for analysis, interpretation and publication of data was obtained from the next of kin.

Extracorporeal Membrane Oxygenation

The cannulation technique, transportation procedures, ventilation strategy (followed ARDSnet protocol if applicable) and calculation of oxygen transfer via extracorporeal membrane oxygenation (ECMO) are described in detail elsewhere [24]. A 29-Fr/50-cm Biomedicus cannula (Medtronic, Minneapolis, Minn., USA) was used for right atrial drainage via the internal jugular vein, and oxygenated blood was returned to the patient through a 19-Fr/18-cm cannula placed in the right femoral vein or a 21-Fr/18-cm cannula in the right femoral artery during venoarterial (V-A) ECMO. A Levitronix® console (Levitronix, Zürich, Switzerland) with a CentriMaq centrifugal pump (Thoratec, Huntingdon, UK) was used for extracorporeal circulation.

Treatment

Approximately 300 × 10^6 PBMCs diluted in 20 ml NaCl with 10% autologous serum were administrated intratracheally via bronchoscopy on ECMO days 9, 10, 16, 21 and 23, and 30,000 IU EPO every other day, from ECMO day 9 to ECMO day 20. The dosage was based on the patient's hematocrit. Blood samples were collected daily upon admission to our ECMO unit.

PBMC Isolation

We drew 120 ml of heparinized patient peripheral blood from a central venous catheter under sterile conditions. Samples were processed in a completely enclosed, FDA-approved Sepax S-1 system (Sepax, BioSafe America Inc.). PBMC isolation procedure was completed following the manufacturer's instructions. Cell viability and PBMC numbers were determined by automated trypan blue dye exclusion (Sigma Aldrich, Sweden). The PBMC fraction was reconstituted with NaCl and 10% autologous serum.

Analysis of Cell Subsets and Phenotyping by Flow Cytometry

Phenotypic characterization of the administrated PBMC subsets was analyzed by flow cytometry for the following fluorochrome-conjugated mAbs against the following surface antigens: CD11b (ICRF44), CD14 (M5E2), CD206 (19.2), CD3 (SK7), CD31 (WM59), CD34 (581), CD44 (G44-26), CD45 (HI30), CD56 (HCD56), CD79a (HM47), CD83 (HB15e), CD86 (FUN-2), CD90 (5E10; BD Biosciences, San Jose, Calif., USA); CD45 (2D1; Biolegend, San Diego, Calif., USA); CD14 (Tü9), HLA-DR (Tü36; Invitrogen, UK). Data acquisition was performed using LSRFortessa (BD) equipped with a high-throughput system. The data were analyzed with FlowJo (TreeStar Inc.) software. SSC/FSC gates around single cell $CD90^{low}CD79^+CD44^+$ $CD73^+CD45^-CD34^-CD31^-CD11b^-HLA\text{-}DR^-$ populations were used to identify MSC populations. To identify PBMC populations, gating on SSC/FSC and single cells was performed first. $CD3^-$ $CD56^-$ identified $CD83^+CD14^-$ cells as dendritic cells, $CD83^+$ $CD14^{low}$ as nondendritic (intermediate) and $CD83^-CD14^+$ as monocytes. $CD3^-CD56^+$, $CD3^+CD56^+$ and $CD3^+CD56^-$ cells were identified as NK, NK-T and T cells, respectively (see fig. 2a).

Micro-RNA Isolation

Micro-RNA (miRNA) isolation from serum was processed as previously described [25]. 400 µl of patient serum was spiked with miScript miRNA mimic SV40 (Qiagen 2 µM, 1/100 µl serum) for sample normalization; 800 µl phenol (Qiazol) and 200 µl chloroform were added and mixed vigorously for 15 s followed by incubation at room temperature for 10 min. The samples were centrifuged for 30 min at 12,000 *g* until complete phase separation. The aqueous phase (with total RNA) was precipitated with 100 µl 100% isopropanol and 2 µl glycogen (Fermentas, St. Leon-Rot, Germany) overnight at –20°C. Samples were centrifuged at 4°C for 15 min (12,000 *g*) and obtained pellets washed once with 70% ethanol. The precipitated RNA was suspended in 30 µl RNase-free water (Ambion, Austin, Tex., USA). RNA quantity and quality were assessed with a NanoDrop spectrophotometer (NanoDrop, Wilmington, Del., USA) and a smallRNA assay for Agilent's Bioanalyzer (Agilent Technologies, Böblingen, Germany).

Chromatin Immunoprecipitation

We fixed 5 × 10^5 BM cells in 1% formaldehyde for 10 min. Chromatin immunoprecipitation (ChIP) was performed using the Low Cell# ChIP kit (Diagenode, Liège, Belgium) using chromatin from 4,000 to 8,000 cells per reaction. For the immunoprecipitation, antibody against H3K27me3 (9756; Cell Signaling Technology, Danvers, Mass., USA) was used. Control IgG was supplied in the kit code. The ChIP was evaluated by qPCR using 'platinum SYBR green qPCRSuperMix-UDG' (Invitrogen, Carlsbad, Calif., USA). Results were normalized to 1%.

Human Adipose-Derived MSCs

Human adipose-derived MSCs (hASCs; PCS/500/011, ATCC, USA), passage 6, were cultured in MesenPro RS™ (Invitrogen, UK) medium supplemented with growth supplement and 1% penicillin/streptomycin (Invitrogen).

Human Foreskin Fibroblasts

Human foreskin fibroblasts (HFs; CRL-2429; ATCC, USA), passage 8, were cultured in Iscove's modification of Dulbecco's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen).

Extracellular Vesicle Influence on TNF-α Effect in NFκB-luc Cells

Extracellular Vesicle Isolation

Media conditioned for 48 h were centrifuged at 300 *g* for 5 min. The supernatant was filtered through a 0.2-µm filter. The extracellular vesicles (EVs) were pelleted by spinning the media at 110,000 *g* for 70 min. The pellet was resuspended in PBS and frozen until further use.

Nanoparticle Tracking Analysis

Nanoparticle tracking analysis (NTA) was performed with a NanoSight NS500 equipped with NTA 2.3 analytical software. A camera level of 16 and automatic function for all postacquisition settings were used except for the detection threshold which was

fixed at 6. Using the *script control* function, five 30- or 60-second videos (online suppl. videos; for all online suppl. material, see www.karger.com/doi/10.1159/000441799) were recorded. Measurements were analyzed using the *batch process* function.

Western Blot

EV samples were mixed with sample buffer and heated at 65°C for 5 min. Samples were loaded on a NuPAGE® Novex® 4–12% Bis-Tris Gel and ran at 120 V. Proteins were transferred to an iBlot nitrocellulose membrane (Invitrogen) for 7 min with the iBlot system. The membranes were blocked with Odyssey blocking buffer diluted 1:1 in PBS for 60 min at room temperature. The membrane was incubated with primary antibody solution (anti-CD9; 1:1,000, Abcam, Cambridge, UK) overnight at 4°C. Membranes were washed before adding the secondary antibody solution (anti-rabbit IgG DyLight-800, 1:15,000) and incubated for 1 h at room temperature. Membranes were washed again and scanned on the LI-COR Odyssey CLx infrared imaging system.

Real-Time Quantitative PCR

RNA from isolated PBMCs was extracted using RNeasy Mini-Kit. Contaminating DNA was removed using the RNase-free DNase kit (all from QIAGEN) and cDNA synthesized using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time PCR was performed using Fast SYBR Green Master Mix (EPO-R; Applied Biosystems) or TaqMan® Gene Expression Assays together with TaqMan Gene Expression Master Mix (CDH1, SNAIL1; Applied Biosystems) and run on the 7500 Real Time PCR System. mRNA levels were quantified relative to GAPDH, which was used as endogenous control.

NFκB Activity

HEK293T cells were transfected (Lipofectamin 2000) with a luciferase reporter plasmid of NFκB activity (pNFκB-luc). 24 h after transfection, 75,000 pNFκB-luc cells were seeded. 3 h after transfection, cells were treated with hTNF-α (5 ng/ml) to activate NFκB) and 4.5×10^{10} HF or PBMC EVs were added. Luminescence was analyzed 24 h after EV treatment using Glomax imaging system (Glomax, Promega) and protein was quantified (Bio-Rad *DC* protein Assay). The luciferase values were normalized to the protein values. Results were quantified as signal increase over hTNF-α-only treated wells.

In vitro Determination of Interleukin-6, -10 and TNF-α

PBMCs were stimulated with LPS and evaluated for interleukin (IL)-6, IL-10 and TNF-α production and compared to MSCs. Cells (3×10^5/ml) were seeded on cell plates and incubated at various concentrations of LPS (0.5–10 μg/ml) for 20 h at 37°C. Supernatant was collected and analyzed by immunometric assay on an Immulite 1 analyzer (Siemens Healthcare Diagnostics, Los Angeles, Calif., USA) according to the manufacturer's instructions.

Statistical Analysis

Analysis, graph preparations and statistical comparisons were performed on GraphPad Prism 5.0a and SPSS 22 software for Macintosh (Chicago, Ill., USA).

Results

Clinical Course Description

In November 2011, a 23-year-old male patient was admitted to an intensive care unit after a burn-induced airway trauma caused by inhalation of hydrated lime. He developed refractory hypoxemic respiratory failure. On day 8, venovenous (V-V) ECMO was initiated, and he was transferred to the ECMO Department of the Karolinska University Hospital, Stockholm, Sweden. The patient's clinical parameters prior to ECMO initiation are shown in table 1. On ECMO day 7, the circuit was changed from V-V to V-A ECMO due to right heart decompensation. He showed initial signs of clinical improvement, and due to the lack of further conventional treatment options we attempted experimental intratracheal administration of autologous PBMCs and EPO on ECMO day 9. Although notable improvement was observed immediately after treatment, the patient ultimately succumbed to multi-system organ failure possibly secondary to disseminated fungal infection and intra-abdominal sepsis.

Instillation and EPO Administration

Experimental instillation of PBMC/EPO into the airways was initiated on the 9th day of ECMO. $300 \pm 50 \times 10^6$ autologous mononuclear cells were isolated from patient's peripheral blood (PBMCs) and administrated directly into the trachea via bronchoscopy on ECMO days 9, 10, 16, 21 and 23. EPO (30,000 IU) were administrated three times per day, every other day via bronchoscopy for 18 total doses.

Bronchoscopy

Pretreatment bronchoscopy revealed pan-bronchial lesions and necrotic mucous membranes with bronchial constriction and severe inflammatory infiltration in both lungs (fig. 1a). Inflammatory infiltration was improved 3 days after the first administration of PBMC/EPO. On day 5 of treatment, bronchoscopy images demonstrated further regression of inflammation and more healthy appearing bronchi (fig. 1b).

Clinical Parameters

Twenty-four hours after PBMC/EPO administration, an improvement in ventilatory parameters was observed (table 1; fig. 1c–e). In parallel to respiratory recovery, V-A ECMO support could be de-escalated to V-V ECMO support again and subsequently reduced (fig. 1e), indicating an improvement in gas exchange by the lungs. Infiltrates

Table 1. Patient characteristics at ECMO start and before PBMC/EPO therapy

	ECMO start	ECMO treatment				
		day 9	day 10	day 16	day 21	day 23
Weight, kg	130	129	126	120.7	109	107.5
Age, years	23	–	–	–	–	–
Vent mode	PSV	PCV	PCV	PCV	PCV	PCV
PIP, cm H_2O	38	32	30	33	32	30
PEEP, cm H_2O	18	8	8	9	8	8
MAP, cm H_2O	missing	13	13	15	14	
Respiration rate	40	13	12	23	15	
Tidal volume, ml	300	87	221	380	378	387
Minute ventilation, ml/min	12	1	2.6	7.5	5.4	12.2
FiO_2	1	0.5	0.5	0.5	0.5	0.4
PaO_2, kPa	7	missing	missing	10.6		5.4
$PaCO_2$, kPa	12	missing	missing	6		6.7
SaO_2, %	84	79	75	95	85	69
PvO_2, kPa		4.4	5.3	7	7	5.4
SvO_2, %		59	69		63	66
pH	7.42	7.34	7.35		7.30	7.32
P/F ratio	52.6	–	–	159	99	101
Murray	3.75	3				
C-reactive protein, mg/dl	446	264	247		181	165
Procalcitonin, ng/ml	6.5	34	28	14	3.9	3.6
White blood cell count, $10^3/mm^3$	23	25.5	20.1	17.5	10.4	8.6
ECMO mode	–	VV-A	V-A	V-V	V-V	V-V
ECMO flow, l/min	–	6.8		3.4	3.4	2.7
Compliance, ml/cm H_2O	15	2.7	9.0	18.6	12.5	17.59

PIP = Peak inspiratory pressure; PEEP = positive end expiratory pressure; FiO_2 = fraction of oxygen applicated by mechanical ventilation; P/F ratio = ratio of PaO_2 and FiO_2 to describe the degree of hypoxemia; Murray = lung injury score.

on chest X-ray rapidly improved within a day of PBMC/EPO treatment initiation (fig. 1f–k).

In order to further explain the mechanisms behind the patient's clinical improvement after PBMC/EPO administrations, isolated PBMCs and the patient's serum samples were evaluated.

PBMC Characterization by Flow Cytometry

Flow cytometric phenotyping of the administrated PBMCs revealed an overall decrease in white blood cells (table 1; fig. 2b) and changes in the composition of its cellular subpopulations (fig. 2a) on different treatment days (fig. 2c–e). Specifically, T cell fraction ($CD3^+CD56^-$) increased from 12.10% (day 0) to 29.20% (day 14; fig. 2c), whereas the ratio of NK-T cells ($CD3^+CD56^+$) remained stable. The percentage of pro- and anti-inflammatory cytokines secreting monocytes (IL-6, IL-10 and TNF) [26] gradually increased over the treatment period with the highest on days 7, 12 and 14. A delayed increase in dendritic cell population was observed; nondendritic (intermediate) antigen-presenting cells which have the potential to secrete anti-inflammatory cytokine IL-10 (myeloid-derived suppressor cells, MDSCs; $CD14^{low}CD83^+$) [27, 28] increased until day 12 and returned to the original level on day 14 (fig. 2d). Cell surface expressions of CD11b, CD11c, CD86, HLA-DR, CD40 and CD206 at different days of treatment were also determined in myeloid cell subpopulations (see online suppl. fig. S1). Cell surface markers associated with immunosuppressive M2 macrophages and MDSC such as CD206 and CD11b gradually increased throughout the treatment and administered PBMCs, especially in $CD14^-CD83^+HLA\text{-}DR^-$ cells.

MSCs which could differentiate into lung epithelial cells [29] may play a role in modulating immune response to lung injury [30–32], and were just a negligible part of the PBMCs (fig. 2e) that were administered. Interestingly, an increase in a subpopulation bearing an MDSC phenotype at day 7 was observed.

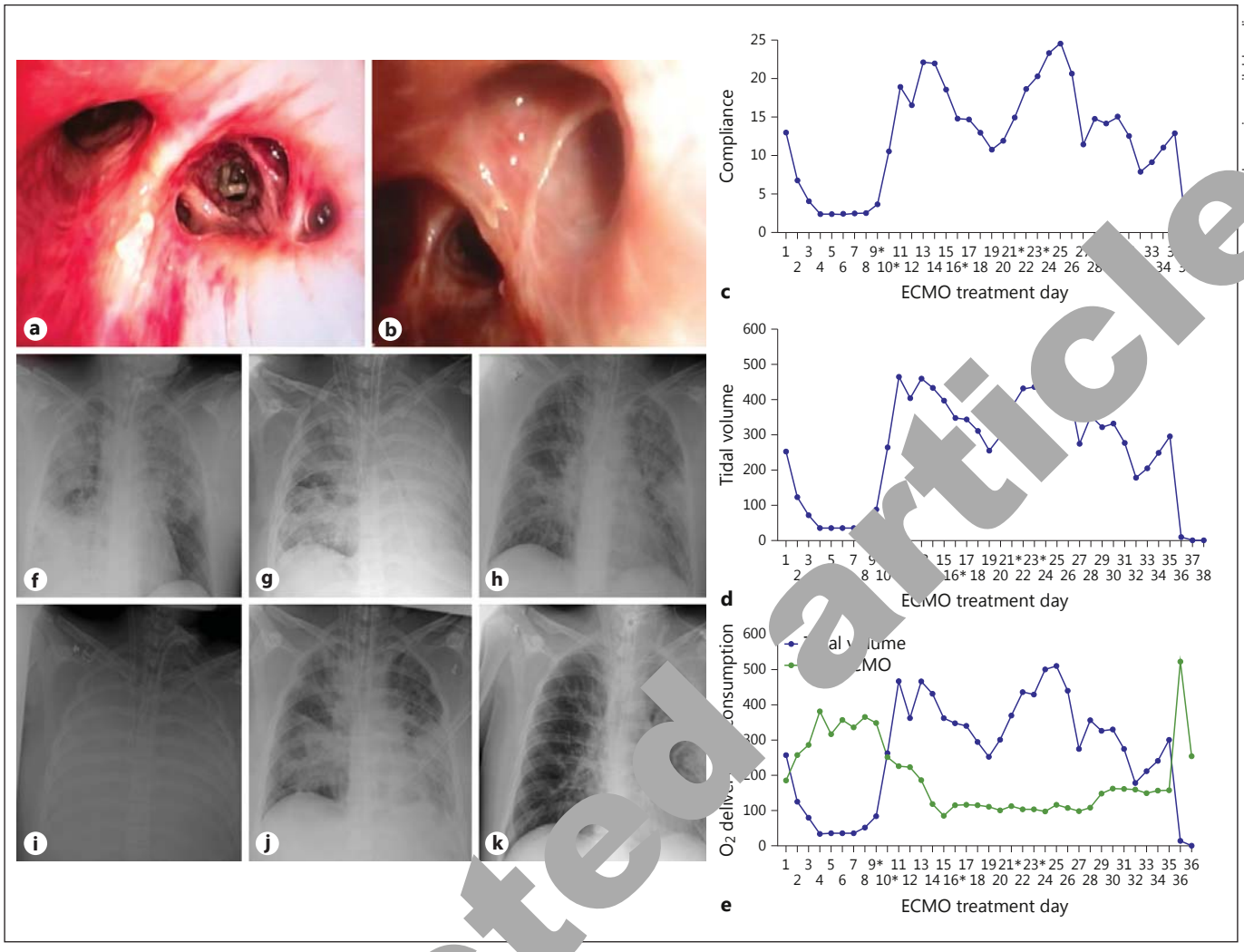

Fig. 1. a, **b** Bronchoscopic view. **a** Image sho[illegible]g bron[illegible]hial system with pan-inflammatory reaction after seve[illegible]urn i[illegible]y on day 9 of ECMO treatment. **b** Regenerated bronch[illegible] on day 5 after initiation of PBMC/EPO treat[illegible]e Ventilation parameters. **c** Lung compliance change[illegible]ur[illegible]MO support. Asterisk indicates instillation of P[illegible]MC/E[illegible]O. **d** Tidal volume changes during ECMO support. A[illegible]isk i[illegible]s instillation of PBMC/EPO. **e** ECMO da[illegible]a. Tida[illegible]me and VO_2 ECMO during ECMO treatment, wher[illegible] EC[illegible] represents oxygen transfer by ECMO. Blood samples were taken before and after the oxygenator for calculation. Asterisk indicates instillation of PBMC/EPO. For calculation, see Holzgraefe et al. [24]. **f–k** X-ray. **f** Chest X-ray on day 1 of ECMO support. **g** Day 3 of ECMO support. **h** First day after PBMC/EPO administration (day 10 of ECMO support). **i** Second day after PBMC/EPO administration (day 11 of ECMO support). **j** Seven days after PBMC/EPO administration (day 16 of ECMO support). **k** Sixteen days after PBMC/EPO administration (day 25 of ECMO support).

miRN[illegible]se[illegible]n Levels

Seru[illegible]is specific miRNAs may reflect epithelial [illegible]ffer[illegible]n. In this context, the expression of miR-34[illegible]d miR-449 may serve as biomarkers for the promo-[illegible]minal differentiation of airway epithelium [33]. [illegible], periodic upregulation of miR-34 and miR-449 was n[illegible]d 2 days after initial PBMC/EPO treatment. This upregulation was noted throughout 12 days of treatment but declined on days 13 and 14 (fig. 3a). Interestingly, serum level of miR-16, which is usually elevated in sepsis [34], was reduced after initial treatment and sustained at baseline levels throughout the study period (fig. 3a).

Gene Expression in PBMCs

Gene expression profiles from the isolated PBMCs were investigated, revealing an upregulation of the epithelial-associated CDH1 with a corresponding downregulation of SNAI1, a suppressor of CDH1 (fig. 3b, c). In the

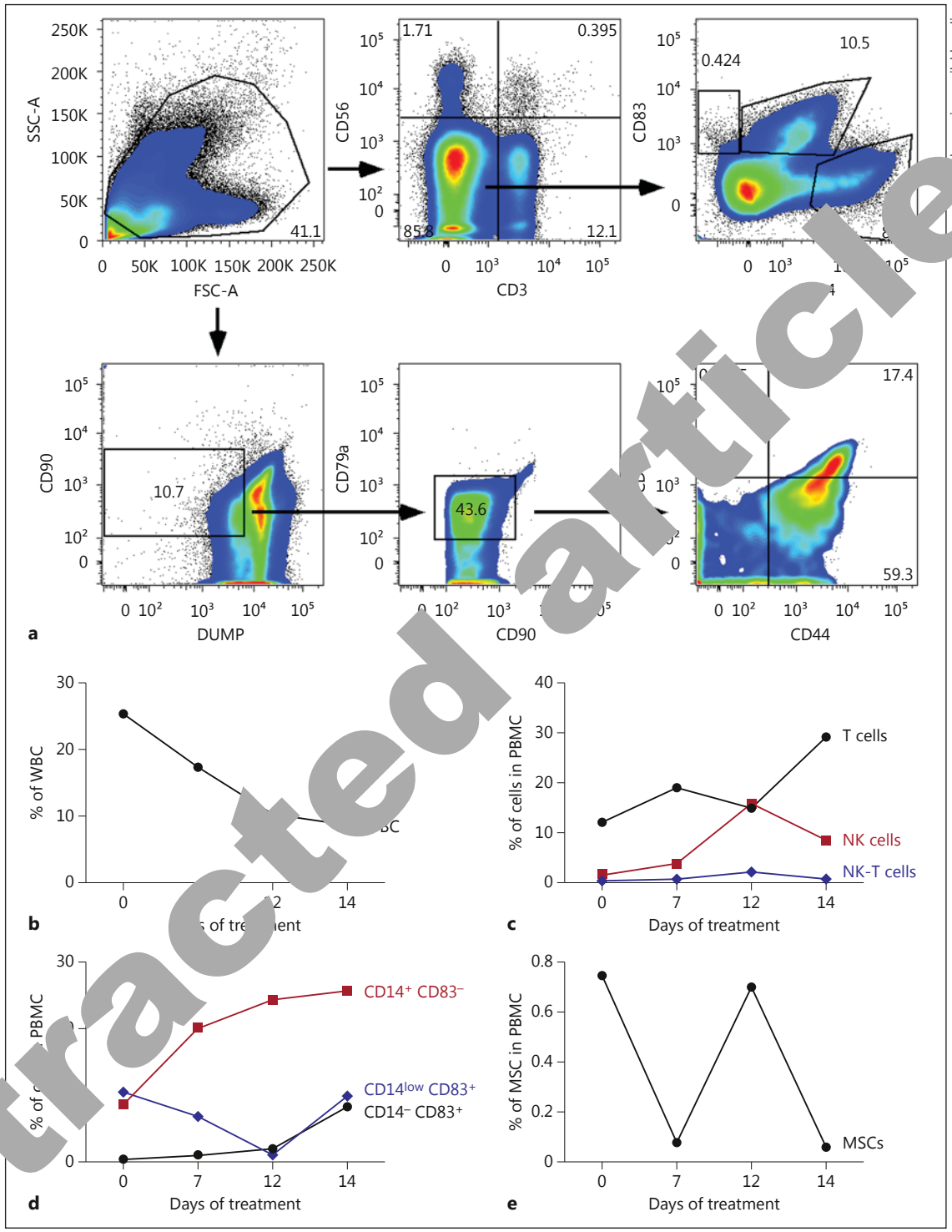

Fig. Characterization of administered PBMCs. Analysis of MSC subsets through flow cytometry. **a** Gating strategy for cytometry analysis. Upper part: Gating was performed on single and FSC/SSC as shown in the right panel. Further gating for T cells ($CD3^+CD56^-$), NK-T cells ($CD3^+CD56^+$) and NK cell ($CD3^-CD56^+$) is shown in the middle panel. Additional CD83 and CD14 staining was performed on $CD3^-CD56^-$ cells to visualize subsets of APC cells. Lower part: Gating strategy for identification of MSCs [$CD31^-CD45^-CD34^-CD11b^-HLA\text{-}DR^-(DUMP^-)$ $CD90^{dim}CD79a^{dim}$ $CD73^+CD44^+$]. **b** White blood cell (WBC) percentage of the patient at the cell treatment days (data retrieved from table 1). **c** Percentage of T cells, NK cells and NK-T cells in PBMCs. **d** Percentage of monocytes (red), intermediate APCs (blue) and dendritic cells (black) from the days of cell treatment. **e** Percentage of MSCs at given time points.

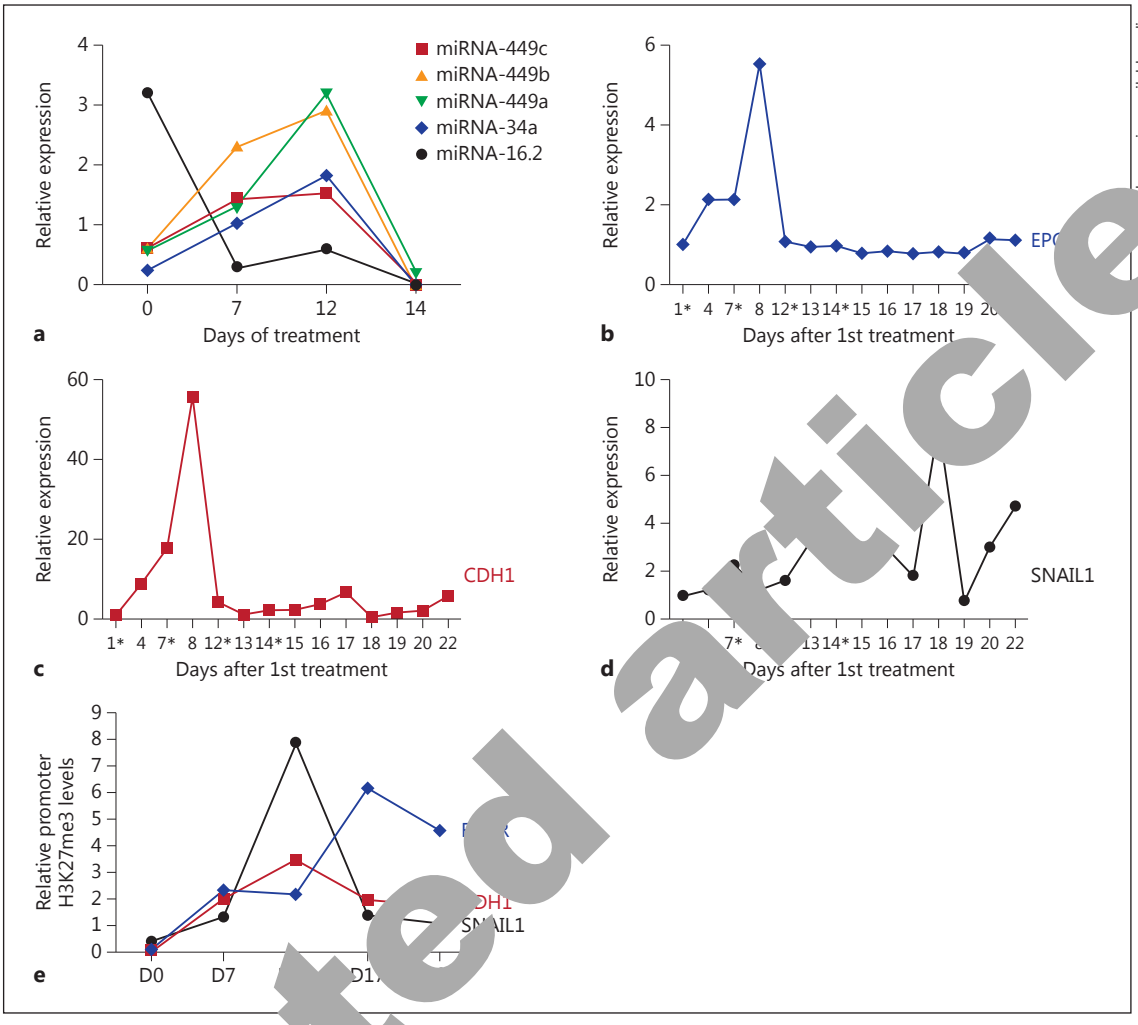

Fig. 3. miRNA and gene expression. **a** miRNA serum level expression after initiating the PBMC/EPO treatment. Gene expression levels of EPO (**b**), CDH1 (**c**) and SNAIL1 (**d**). **e** Relative promoter H3K27me3 (tri-methylated lysine 27 on histone H3) levels of CDH1, EPO and SNAIL1. Asterisks indicate the PBMC/EPO instillation time points after first appl

4 days preceding the patient's death, this expression pattern was reversed. The patient succumbed to multisystem organ failure. Additionally, the EPO receptor demonstrated a gene expression pattern similar to CDH1 after PBMC/EPO treatment initiation (fig. 3d).

Promoter Histone Mark Profile

The dramatic downregulation of CDH1 and EPO receptor time points beyond 8 days after initiation of PBMC/EPO treatment was in accordance with the condensation of the repressive H3K27me3 (tri-methylated lysine 27 on histone H3) mark in the promoter regions of the genes between day 7 and day 14. In parallel, a decrease in this repressive histone mark after day 17 correlated with an increase in SNAI1 expression. While the increased methylation of H3K27 observed is likely regulated by the Polycomb repressor complex, the dramatic increase in CDH1 and EPO receptor gene expression between day 7 and 8 may be due to additional epigenetic regulation by histone modifying proteins [35] activated by EPO administration as well as PBMC therapy (fig. 3e).

EVs Derived from PBMCs

In order to explain the underlying mechanisms of the beneficial clinical effects of PBMC/EPO treatment, the anti-inflammatory capacity of the cells was tested. Recent

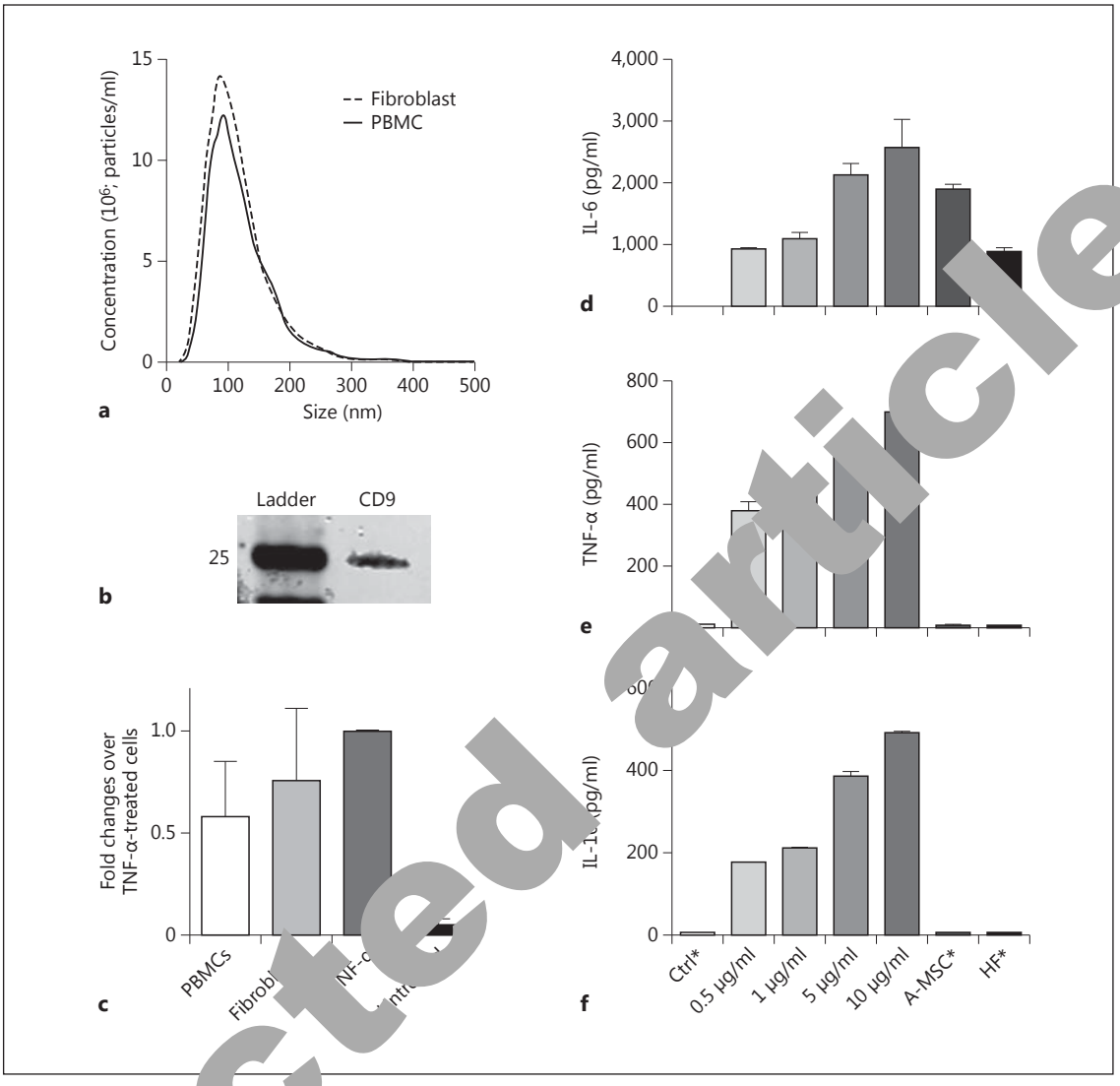

Fig. 4. ECVs and cellular effects. **a** NTA pr[illegible]s. Representative NTA profile of EVs derived [illegible] and fibroblasts, displaying similar mode size of [illegible]nm. **b** CD9 detected by Western blot of PBMC-EVs. **c** P[illegible]s displaying relative luminescent signal from re[illegible]er c[illegible]h NFκB-driven luciferase expression treated w[illegible] TNF-α and EVs. EVs derived from PBMCs, fibroblasts, TNF-α only (TNF-α control) or PBS (untreated). **d–f** LPS stimulation on PBMCs. **d** IL-6. **e** TNF-α. **f** IL-10 displayed the same effect as the previous two cytokines, having an elevation according to the LPS dose; no response was observed with the HFs and hASCs. Asterisks indicate significant differences compared to stimulated PBMCs.

reports d[illegible]d the importance of paracrine signaling in tissue [illegible]ive cell therapies. In particular, cell-se[illegible] EV[illegible]been shown to play an important role as a p[illegible]rine axis of intercellular communication, especial[illegible]n to tissue regeneration and immune modula[illegible] [36–38]. Thus, investigation of the potential role of E[illegible] derived from PBMCs was undertaken. The anti-inflammatory effect of EVs was evaluated in an in vitro TNF-α reporter assay, where the readout is luciferase expression driven by an NFκB promoter. Upon TNF-α challenge of the reporter cells, NFκB activity increases with a concomitant elevation of luciferase expression. NTA showed a typical EV-like curve with the mode size of the particles of around 100 nm and the Western blot of PBMC EVs was positive for the EV marker CD9, which corroborates that the particles purified were indeed EVs (fig. 4a, b). After adding 4.5×10^{10} particles (according to NTA) from HF and PBMCs to the NFκB-luc-positive

cells, PBMC EVs downregulated luciferase activity by nearly 30%; in contrast, HF EVs only had a modest effect on reporter gene expression (fig. 4c).

IL Release from Patient's PBMCs

In response to stimulation with LPS, the patient's PBMCs exhibited an elevation in three different cytokines (IL-6, TNF-α and IL-10) in a dose-dependent manner. All three cytokines (fig. 4d–f) had significant changes as compared to the control, with IL-6, which exerts both pro- and anti-inflammatory activities, displaying the highest levels. TNF-α exhibited an inflammatory response secondary to LPS stimulation, which was undetected in control, hASCs and HFs. The anti-inflammatory marker IL-10 exhibited higher levels with an increased dose of LPS, suggesting a compensatory mechanism by PBMCs. These results indicate the presence of an anti-inflammatory signal from the patient's PBMCs, which may contribute to the clinical anti-inflammatory responses.

Discussion

Clinically to date, adult stem cells such as MSCs derived from bone marrow or adipose tissue have been safely utilized in human patients [7, 39, 40]. The primary [illegible] of MSCs involves harnessing their anti-inflammatory [illegible] tential in acute and chronic adverse immu[illegible]ac and other inflammatory conditions. Althou[illegible]SCs remain a major target for investigation in clinical [illegible]erapy, the application of MSCs is both [illegible]e and resource consuming. Clinical cell therapy requ[illegible]s in v[illegible]o culture, expansion and storage. Without in v[illegible] expansion, the natural proportion of auto[illegible]g[illegible]MSCs in the peripheral blood and bone marro[illegible]ar[illegible]in[illegible]quate for clinical applications. It is theref[illegible]be[illegible]d to explore alternative cell sources co[illegible]able to MSCs such as naïve PBMCs.

Naïve PBM[illegible]y be considered in the future as a suitable alterative [illegible]ecific applications that require immedia[illegible]an[illegible]nflammatory therapies. To address this, an FDA [illegible]ov[illegible] cell-processing device, SepaxII (Bio-[illegible] was [illegible]or immediate cell separation after obtaining [illegible]e peripheral blood from patient.

In [illegible]current ARDS case, no clinical improvement [illegible]made after 9 days of maximal conventional therapy in[illegible]ding V-V and V-A ECMO support. An experimental therapy using PBMC/EPO intratracheal administration demonstrated an immediate, albeit temporary, clinical improvement. Most notable was the conversion of profoundly inflamed bronchial mucosa to more normal-appearing tissue and a simultaneous improvement in lung function.

Detailed flow cytometric analysis on the administered PBMCs revealed MSC subpopulation to be negligible (<1%). However, with the current data, we canno[illegible]ex[illegible]clude their possible role in the clinical im[illegible]ovem[illegible]t. A dramatic decrease in NK cell compartment[illegible]y su[illegible] their contribution to control acute inflamm[illegible]y responses and their assistance in chemota[illegible]anti-[illegible]ammatory cells. Alternatively, this findi[illegible]may [illegible]mply correspond to the onset of septi[illegible]mia. T[illegible]in[illegible]g of peak NK cell levels favors the former, [illegible]er than the latter hypothesis. Moreover, an i[illegible]ed [illegible]ression of surface markers associated with [illegible]acrophages and MDSC, such as CD11b, CD206 [illegible]d an [illegible]ase in $CD14^{low}CD83^{+}$ population in PBMC[illegible]ether with the continuous and drastic increase i[illegible]mo[illegible]e mobilization may suggest that these cell[illegible]pla[illegible]important role in the resolution of inflammatio[illegible]. P[illegible]M[illegible] possess autocrine/paracrine effects as cell-sec[illegible]EVs from patient's PBMCs demonstrated anti-inflammatory capacity when compared to hu[illegible]fibroblasts and also released dose-dependent IL [illegible]pon[illegible]pon stimuli.

At [illegible]molecular level, upregulation of serum miR-34 and miR-449 may play a role in the promotion of terminal [illegible]rway epithelium differentiation. PBMCs displayed a transient upregulation of the epithelial cell marker CDH1 and repression of SNAIL1, a CDH1 suppressor, as well as upregulation of the EPOR gene. Analysis of chromatin modifications revealed partially corollary changes in histone methylation as well as unexpected changes in methylation pattern. EPO, administered externally, may bind EPOR and activate the antiapoptotic pathway as well as affect histone methylation [20, 22].

In comparison to more commonly used intravenously administered, systemic cell-based therapies, this case represents the first clinical application of our previously described localized intratracheal cell therapy administration [13]. Localized administration prevents unpredictable systemic distribution of PBMC/EPO, particularly in the setting of supportive devices such as ECMO and/or hemodialysis, which cause undesirable dilution and possibly adhesion and destruction of cells in the extracorporeal systems. Therefore, targeted therapy under these circumstances is preferable for directing maximal therapeutic effects to damaged tissue. However, with the currently available data, the clinical results from treatment remain corollary and require further investigation. Additionally,

no data are presented here or in the current literature to suggest the safest and most therapeutic cell number for localized or systemic application. Mathematical modeling may further support the optimization of cell therapy's regenerative and healing effects.

This patient, who suffered from burn-induced refractory ARDS and ultimately succumbed to multisystem organ failure possibly secondary to disseminated fungal infection and intra-abdominal sepsis, demonstrated a direct response after targeted cell therapy as evidenced by dramatically improved clinical parameters. Despite the death of this patient, notable clinical improvement shortly after initiating treatment provides new support for the future use of autologous PBMCs in critically ill patients with severe ARDS. Whether PBMCs have a direct beneficial effect on fungal infection cannot be answered at this stage but must be further investigated. The partial and notable recovery of lung function and directly visible improvement of the bronchial mucosa may be attributed to the anti-inflammatory capacity of locally administered autologous PBMCs and EPO. Evidence provided here suggests this may be mediated via secreted EVs, upregulation of anti-inflammatory cytokines and previously described effects of EPO. More functional investigations are now warranted to further describe the mechanisms of tissue healing and regenerative processes of the damaged and inflamed respiratory mucosa.

Financial Disclosure and Conflict of Interest

All authors declare no conflicts of financial interests.

References

1 ARDS Definition Task Force; Ranieri VM, Rubenfeld GD, Thompson BT, Ferguson ND, Caldwell E, Fan E, Camporota L, Slutsky AS: Acute respiratory distress syndrome: the Berlin Definition. JAMA 2012;307:2526–2533.

2 Sharp C, Millar AB, Medford AR: Advances in understanding of the pathogenesis of acute respiratory distress syndrome. Respiration 2015;89:420–434.

3 Hayes M, Curley G, Ansari B, Laffey JG: Clinical review: stem cell therapies for acute lung injury/acute respiratory distress syndrome – hope or hype? Crit Care 2012;16:205.

4 Cárdenes N, Cáceres E, Romagnoli M, Rojas M: Mesenchymal stem cells: a promising therapy for the acute respiratory distress syndrome. Respiration 2013;85:267–278.

5 Tzouvelekis A, Ntolios P, Bouros D: Stem cell treatment for chronic lung diseases. Respiration 2013;85:179–192.

6 Huleihel L, Levine M, Rojas M: Therapeutic potential of cell-based therapy in lung diseases. Expert Opin Biol Ther 2013;13:1431–1440.

7 Weiss DJ, Casaburi R, Flannery R, LeRoux-Williams M, Tashkin DP: A placebo-controlled, randomized trial of mesenchymal stem cells in COPD. Chest 2013;143:1590–1598.

8 Weiss DJ: Concise review: current status of stem cells and regenerative medicine in lung biology and diseases. Stem Cells 2014;32:16–25.

9 Kwapiszewska G, Kosanovic D, Schermuly RT, Tian X, Kolb M: Vascular repair and regeneration as therapeutic target for pulmonary arterial hypertension. Respiration 2013;85:355–364.

10 Rojas M, Mora AL, Rojas M: Adult stem cells for chronic lung diseases. Respirology 2013;18:1041–1046.

11 Kolluri KK, Laurent GJ, Janes SM: Mesenchymal stem cells as vectors for lung cancer therapy. Respiration 2013;85:442–451.

12 Jungebluth P, Macchiarini P: Stem cell-based therapy and regenerative approaches to diseases of the respiratory system. Med Bull 2011;99:169–187.

13 Jungebluth P, Luedde M, Luedde T, Vucur M, Peiner M, Schreiber C, von Richthofen C, Bader A, Haag J, Darsow KH, Bartel SJ, Lerut A, Calani D, Steinhoff G, Macchiarini P: Mesenchymal stem cells restore lung function by recruiting resident and non-resident stem cell proteins. Cell Transplant 2011;20:1561–1572.

14 Wilson JG, Liu KD, Zhuo H, Caballero L, McMillan M, Fang X, Cosgrove K, Vojnik R, Calfee CS, Lee JW, Rogers AJ, Levitt J, Wiener-Kronish J, Sabjwa EK, Leavitt A, McKenna D, Thompson BT, Matthay MA: Mesenchymal stem (stromal) cells for treatment of ARDS: a phase 1 clinical trial. Lancet Respir Med 2015;3:24–32.

15 Liu KD, Wilson JG, Zhuo H, Caballero L, McMillan ML, Fang X, Cosgrove K, Calfee CS, Lee JW, Kangelaris KN, Gotts JE, Rogers AJ, Levitt JE, Wiener-Kronish JP, Delucchi KL, Leavitt AD, McKenna DH, Thompson BT Matthay MA: Design and implementation of the START (STem cells for ARDS Treatment) trial, a phase 1/2 trial of human mesenchymal stem/stromal cells for the treatment of moderate-severe acute respiratory distress syndrome. Ann Intensive Care 2014;4:22.

16 Alvarez-Viejo M, Menendez-Menendez Y, Blanco-Gelaz MA, Ferrero-Gutierrez A, Fernandez-Rodriguez MA, Gala J, Otero-Hernandez J: Quantifying mesenchymal stem cells in the mononuclear cell fraction of bone marrow samples obtained for cell therapy. Transplant Proc 2013;45:434–439.

17 Rojas M, Parker RE, Thorn N, Corredor C, Iyer SS, Bueno M, Mroz L, Cardenes N, Mora AL, Stecenko AA, Brigham KL: Infusion of freshly isolated autologous bone marrow derived mononuclear cells prevents endotoxin-induced lung injury in an ex-vivo perfused swine model. Stem Cell Res Ther 2013;4:26.

18 Schramm R, Kirsch S, Schäfers H-J, Langer F, Scheuer C, Nickels R, Harder Y, Menger MD: Erythropoietin inhibits post-ischemic leukocyte adhesion but does not affect rejection in murine cardiac allografts. J Heart Lung Transplant 2010;29:1185–1192.

19 Sølling C: Organ-protective and immunomodulatory effects of erythropoietin – an update on recent clinical trials. Basic Clin Pharmacol Toxicol 2012;110:113–121.

20 Brines M, Cerami A: Erythropoietin-mediated tissue protection: reducing collateral damage from the primary injury response. J Intern Med 2008;264:405–432.

21 Brines M: The therapeutic potential of erythropoiesis-stimulating agents for tissue protection: a tale of two receptors. Blood Purif 2010;29:86–92.

22 Bader A, Macchiarini P: Moving towards in situ tracheal regeneration: the bionic tissue engineered transplantation approach. J Cell Mol Med 2010;14:1877–1889.

23 Jungebluth P, Alici E, Baiguera S, Le Blanc K, et al: Tracheobronchial transplantation with a stem-cell-seeded bioartificial nanocomposite: a proof-of-concept study. Lancet 2011;378:1997–2004.

24 Holzgraefe B, Broomé M, Kalzén H, Konrad D, Palmér K, Frenckner B: Extracorporeal membrane oxygenation for pandemic H1N1 2009 respiratory failure. Minerva Anestesiol 2010;76:1043–1051.

25 Roderburg C, Urban G-W, Bettermann K, Vucur M, Zimmermann H, Schmidt S, Janssen J, Koppe C, Knolle P, Castoldi M, Tacke F, Trautwein C, Luedde T: Micro-RNA profiling reveals a role for miR-29 in human and murine liver fibrosis. Hepatology 2011;53:209–218.

26 Schildberger A, Rossmanith E, Eichhorn T, Strassl K, Weber V: Monocytes, peripheral blood mononuclear cells, and THP-1 cells exhibit different cytokine expression patterns following stimulation with lipopolysaccharide. Mediators Inflamm 2013;2013:1–10.

27 Gerlini G, Mariotti G, Chiarugi A, Di Gennaro P, Caporale R, Parenti A, Cavone L, Tun-Kyi A, Prignano F, Saccardi R, Borgognoni L, Pimpinelli N: Induction of CD83+CD14+ nondendritic antigen-presenting cells by exposure of monocytes to IFN-alpha. J Immunol 2008;181:2999–3008.

28 Ganguly D, Paul K, Bagchi J, Rakshit S, Mandal L, Bandyopadhyay G, Bandyopadhyay S: Granulocyte-macrophage colony-stimulating factor drives monocytes to CD14low CD83+ DCSIGN– interleukin-10-producing myeloid cells with differential effects on T-cell subsets. Immunology 2007;121:499–507.

29 Krause DS, Theise ND, Collector MI, Henegariu O, Hwang S, Gardner R, Neutzel S, Sharkis SJ: Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell 2001;105:369–377.

30 Gupta N, Su X, Popov B, Lee JW, Serikov V, Matthay MA: Intrapulmonary delivery of bone marrow-derived mesenchymal stem cells improves survival and attenuates endotoxin-induced acute lung injury in mice. J Immunol 2007;179:1855–1863.

31 Mei SHJ, McCarter SD, Deng Y, Parker CH, Liles WC, Stewart DJ: Prevention of LPS-induced acute lung injury in mice by mesenchymal stem cells overexpressing angiopoietin 1. PLoS Med 2007;4:e269.

32 Mei SHJ, Haitsma JJ, Dos Santos CC, Deng Y, Lai PFH, Slutsky AS, Liles WC, Stewart DJ: Mesenchymal stem cells reduce inflammation while enhancing bacterial clearance and improving survival in sepsis. Am J Respir Crit Care Med 2010;182:1047–1057.

33 Lizé M, Herr C, Klimke A, Bals R, Dobbelstein M: MicroRNA-449a levels increase by several orders of magnitude during mucociliary differentiation of airway epithelia. Cell Cycle 2010;9:4579–4583.

34 Wang H, Zhang P, Chen W, Feng D, Jia Y, Xie L: Evidence for serum miR-15a and miR-16 levels as biomarkers that distinguish sepsis from systemic inflammatory response syndrome in human subjects. Clin Chem Lab Med 2012;50:1423–1428.

35 Perissi V, Jespen K, Glass C, Rosenfeld M: D constructing repression ...lving models ... co-repressor action. Nat ...enet 2010;11:109–123.

36 EL Andaloussi S, Mäger I, Breakefield XO, Wood MJA: Extracellular vesicles: biology and emerging therapeutic opportunities. Nat Rev Drug Discov 2013;12:347–357.

37 Timmers L, Lim SK, Hoefer IE, Arslan F, Lai RC, van Oorschot AAM, Goumans MJ, Strijder C, Sze SK, Choo A, Piek JJ, Doevendans PA, Pasterkamp G, de Kleijn DP: H... man mesenchymal stem cell-condition... dium improves cardiac function f... wi... myocardial infarction. Ste... ll Re... ;6:206–214.

38 Kordelas L, Rebmann V, Ludw... Radtke S, Ruesing J, Doeppner ... Epp... Horn PA, Beelen DW, Gieb... MSC-der... d exosomes: a novel tool ... eat the ...y-refractory graft-ver... host d... Le... nia 2014;28:970–973.

39 Le B... nc K, Fr... F, Ball L, Locatelli F, Roelo... wis I, ... E, Sundberg B, Bernard ... Remberg ... M, Dini G, Egeler RM, P... galu... ...be W, Ringdén O: Mesen... mal ste... s for treatment of steroid-re... nt, severe, acute graft-versus-host dis... phase II study. Lancet 2008;371:1579–158...

... Blanc K, Rasmusson I, Sundberg B, ...otherström C, Hassan M, Uzunel M, Ringdén O: Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet 2004;363:1439–1441.