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

Philipp Jungebluth^{a,b} Bernhard Holzgraefe^{c,d} Mei Ling Lim^a Adil Doganay Duru^{e,f}
Vanessa Lundin^g Nina Heldring^h Oscar P.B. Wiklanderⁱ Joel Z. Nordinⁱ Michael C. Cook^f
Christoph Roderburg^j Sebastian Sjöqvist^a Björn Anderstam^k Antonio Beltrán Rosendo^l
Johannes C. Haag^a Ylva Gustafsson^a Katharina G. Roddewig^a Petra Jones^l
Matthew J.A. Wood^m Tom Luedde^j Ana I. Teixeira^g Ola Hermanson^h Ola Winberg^l
Håkan Kalzén^{c,d} Samir El Andaloussi^{i,m} Evren Alici^f Paolo Macchiarini^a

^aDivision of Ear, Nose and Throat, Advanced Center for Translational Regenerative Medicine, Department for Clinical Science, Intervention and Technology, Karolinska Institutet, Huddinge, Stockholm, Sweden; ^bDepartment of Thoracic Surgery, Thoraxklinik, Heidelberg University, Heidelberg, Germany; ^cKarolinska University Hospital, ECMO Centre Karolinska, Solna, Stockholm, and ^dDepartment of Physiology and Pharmacology, Karolinska Institutet, Solna, Stockholm, Sweden; ^eCenter for Diseases of Aging, Vaccine and Gene Therapy, Institute of Florida, Port St. Lucie, Fla., USA; ^fCell and Gene Therapy Group, Center for Hematology and Regenerative Medicine (HERM), Department of Medicine, Karolinska Institutet, Huddinge, Stockholm, Sweden; ^gDepartment of Cell and Molecular Biology and ^hNeuroscience, Karolinska Institutet, Solna, Stockholm, and ⁱUnit for Molecular Cell Biology and Gene Therapy Science, Department of Laboratory Medicine, Karolinska Institutet, Huddinge, Stockholm, Sweden; ^jDepartment of Medicine III, University Hospital RWTH Aachen, Aachen, Germany; ^kDivision of Renal Medicine, Department of Clinical Science, Karolinska Institutet, Karolinska University Hospital, Huddinge, Stockholm, and ^lUnit of Translational Immunology, Department of Medicine, Karolinska Institutet, Solna, Stockholm, Sweden; ^mDepartment of Physiology, Anatomy and Genetics, University of Oxford, Oxford, UK

Key Words

Mononuclear cell intervention · Pulmonology · Acute respiratory distress syndrome · Cell therapy

Abstract

Introduction: 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 per-

formed 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-

P. Jungebluth and B. Holzgraefe contributed equally to this work.

lular vesicles from PBMCs demonstrated anti-inflammatory capacity in a TNF- α -mediated nuclear factor- κ B 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.

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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 a 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 endothelium by improving endothelial and epithelial cell functions via differentiation and/or secretion of paracrine factors with regenerative capacity; (e) can locally act as 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 probably provide new evidence for their beneficial effects in this particular group of patients (NCT02114455, NCT01775774).

However, the number of MSCs in a general population of bone marrow-derived cells is rather low (0.002–0.02%) [16], and in vitro expansion, 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- κ B (NF κ B), 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 administered 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 Separator system (Sepax, BioSafe America Inc.). PBMC isolation procedure was completed following the manufacturer's instructions. Cell viability and PBMC numbers were determined by 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 antibodies against the following surface antigens: CD11b (ICRF44), CD11c (1176), CD206 (19.2), CD3 (SK7), CD31 (WM59), CD33 (13C3), CD44 (G44-26), CD45 (HI30), CD56 (HCD56), CD7 (A712), CD79a (HM47), CD83 (HB15e), CD86 (FC11 (F1111), CD90 (5E10; BD Biosciences, San Jose, Calif., USA), CD95 (F1111), CD133 (AC133; BD Biosciences, San Jose, Calif., USA); CD14 (Ti119), HLA-DR (Tü36; Invitrogen, UK). Data acquisition was performed using LSRII (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⁺CD3⁺CD45⁺CD34⁺CD31⁺CD11b⁺HLA-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 200 µl 100% isopropanol and 2 µl glycogen (Fermentas, ThermoFisher, 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 resuspended in 20 µl RNase-free water (Ambion, Austin, Tex., USA). RNA quantity and quality were assessed with a NanoDrop spectrophotometer (NanoDrop, Wilmington, Del., USA) and a small RNA assay for Agilent's Bioanalyzer (Agilent Technologies, Böblingen, Germany).

Chromatin Immunoprecipitation

We fixed 5 × 10⁵ PBMCs in 1% formaldehyde for 10 min. Chromatin immunoprecipitation (ChIP) was performed using the EZ-Mag ChIP kit (Millipore, Billerica, Mass., USA) using chromatin from 100,000 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 by Millipore. 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 in 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. Luciferase 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 significant increase over hTNF-α-only treated wells.

In vitro Determination of Interleukin-6, IL-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 96 well 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 1000 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 Windows (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 at the Karolinska University Hospital, Stockholm, Sweden. The patient's clinical parameters prior to ECMO initiation are shown in table 1. On ECMO day 1 the circuit was changed from V-V to V-A ECMO due to right heart decompensation. He showed indications of clinical improvement, and due to the lack of other conventional treatment options we attempted experimental intratracheal administration of autologous PBMCs and EPO on ECMO day 9. Although a 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.

Experimental Instillation and EPO Administration

Experimental instillation of PBMC/EPO into the airway 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 administered directly into the trachea via bronchoscopy on ECMO days 9, 10, 16, 21 and 23. EPO (30,000 IU) were administered 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 ₂ O	38	32	30	33	32	30
PEEP, cm H ₂ O	18	8	8	9	8	2
MAP, cm H ₂ O	missing	13	13	15	14	–
Respiration rate	40	13	12	23	15	5
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	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	85	63	66
pH	7.42	7.34	7.35	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	VV-A	V-V	V-V	V-V
ECMO flow, l/min	–	6.8	–	3.4	3.4	2.7
Compliance, ml/cm H ₂ O	15	2.7	9.6	18.6	12.5	17.59

PIP = Peak inspiratory pressure; PEEP = positive end expiratory pressure; FiO₂ = fraction of oxygen applied by mechanical ventilation; P/F ratio = ratio of PaO₂ and FiO₂ 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 mechanism 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. 2a) and changes in the composition of its cellular subpopulations (fig. 2a) on different treatment days (fig. 2b). 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 den-

dritic 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-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 showing bronchial system with pan-inflammatory reaction after severe burn injury on day 9 of ECMO treatment. **b** Regenerated bronchial system on day 5 after initiation of PBMC/EPO treatment. **c** Lung compliance changes during ECMO support. Asterisk indicates instillation of PBMC/EPO. **d** Tidal volume changes during ECMO support. Asterisk indicates instillation of PBMC/EPO. **e** ECMO data. Tidal volume and O_2 delivery/consumption during ECMO treatment, where O_2 delivery/consumption 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).

miRNA Serum Levels

Serum levels of specific miRNAs may reflect epithelial differentiation. In this context, the expression of miR-34 and miR-449 may serve as biomarkers for the promotion of terminal differentiation of airway epithelium [33]. The periodic upregulation of miR-34 and miR-449 was noted 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 SNAIL, a suppressor of CDH1 (fig. 3b, c). In the

Figure 1 Characterization of administered PBMCs. Analysis of MSC subsets through flow cytometry. **a** Gating strategy for flow cytometry analysis. Upper part: Gating was performed on single cells 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-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. **b** Gene expression level of EPO. **c** CDH1. **d** SNAIL1. **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 application.

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 H3K27me3 Mark Profile

The dramatic downregulation of CDH1 and EPO receptor at the 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 SNAIL1 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 profile of EVs. Representative NTA profile of EVs derived from PBMCs and fibroblasts, displaying similar mode size of around 100 nm. **b** CD9 detected by Western blot of PBMC-EVs. **c** Bar graph displaying relative luminescent signal from reporter cells with NF κ B-driven luciferase expression treated with 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 demonstrated the importance of paracrine signaling in tissue regenerative cell therapies. In particular, cell-secreted EVs have been shown to play an important role as a paracrine axis of intercellular communication, especially in relation to tissue regeneration and immune modulation [36–38]. Thus, investigation of the potential role of EVs 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 ex-

pression driven by an NF κ B promoter. Upon TNF- α challenge of the reporter cells, NF κ B 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 NF κ B-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 use of MSCs involves harnessing their anti-inflammatory potential in acute and chronic adverse immune reactions and other inflammatory conditions. Although MSCs remain a major target for investigation in clinical therapy, the application of MSCs is both time and resource consuming. Clinical cell therapy requires *in vitro* culture, expansion and storage. Without *in vitro* expansion, the natural proportion of autologous MSCs in the peripheral blood and bone marrow are inadequate for clinical applications. It is therefore beneficial to explore alternative cell sources comparable to MSCs such as naïve PBMCs.

Naïve PBMCs may be considered in the future as a suitable alternative for specific applications that require immediate anti-inflammatory therapies. To address this, an FDA-approved cell-processing device, SepaxII (BioSpherix), was used for immediate cell separation after obtaining peripheral blood from patient.

In the current ARDS case, no clinical improvement was made after 9 days of maximal conventional therapy including 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 cannot exclude their possible role in the clinical improvement. A dramatic decrease in NK cell compartment may suggest their contribution to control acute inflammatory responses and their assistance in chemotaxis of anti-inflammatory cells. Alternatively, this finding may simply correspond to the onset of septicemia. Timing of peak NK cell levels favors the former, rather than the latter hypothesis. Moreover, an increased expression of surface markers associated with macrophages and MDSC, such as CD11b, CD206 and an increase in CD14^{low}CD83⁺ population in PBMCs together with the continuous and drastic increase in monocyte mobilization may suggest that these cells play an important role in the resolution of inflammation. PBMCs possess autocrine/paracrine effects as cell-secreted EVs from patient's PBMCs demonstrated anti-inflammatory capacity when compared to human fibroblasts and also released dose-dependent IL-6 upon stimuli.

At the molecular level, upregulation of serum miR-34 and miR-449 may play a role in the promotion of terminal airway 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,

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 Conflicts of Interest

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