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. Wiklander i  Joel Z. Nordin i  Michael Chrobok i  Christoph Roderburg i  Sebastian Sjöqvist a  Björn Anderstam k  Antonio Beltrán Rodríguez a  Johannes C. Haag a  Ylva Gustafsson a  Katharina G. Roddewig a  Petra Jones l  Matthew J.A. Wood m  Tom Luedde l  Ana I. Teixeira g  Ola Hermanson h  Ola Winqvist l  Håkan Kalzén c, d  Samir El Andaloussi i, m  Evren Alici f  Paolo Macchiarini a

a Division of Ear, Nose and Throat, Advanced Center for Translational Regenerative Medicine, Department for Clinical Science, Intervention and Technology, Karolinska Institutet, Huddinge, Stockholm, Sweden; b Department of Thoracic Surgery, Thoraxklinik, Heidelberg University, Heidelberg, Germany; c Karolinska University Hospital, ECMO Centre Karolinska, Solna, Stockholm, and d Department of Physiology and Pharmacology, Karolinska Institutet, Solna, Stockholm, Sweden; e Center for Diseases of Aging, Vaccine and Gene Therapy Institute of Florida, Port St. Lucie, Fla., USA; f Cell and Gene Therapy Group, Center for Hematology and Regenerative Medicine (HERM), Department of Medicine, Karolinska Institutet, Huddinge, Stockholm, and Departments of g Cell and Molecular Biology and h Neuroscience, Karolinska Institutet, Solna, Stockholm, and i Unit for Molecular Cell Biology and Gene Therapy Science, Karolinska Institutet, Karolinska University Hospital, Huddinge, Stockholm, Sweden; j Department of Medicine III, University Hospital RWTH Aachen, Aachen, Germany; k Division of Renal Medicine, Department of Clinical Science, Karolinska Institutet, Karolinska University Hospital, Huddinge, Stockholm, and l Unit of Translational Immunology, Department of Medicine, Karolinska Institutet, Solna, Stockholm, Sweden; m Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, UK

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-
ular vesicles from PBMCs demonstrated anti-inflammatory capacity in a TNF-α-mediated nuclear factor-kB 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.

© 2015 S. Karger AG, Basel

**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 impact patients’ innate immune response; (b) can influence 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 cell barrier by improving endothelial and epithelial cell functions via differentiation and/or secretion of paracrine factors with regenerative capacity; (e) can locally target 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 in patients with ARDS [14, 15]. Current clinical trials may probably provide new evidence for their beneficial effects in this particular group of patients (NCT02444455, NCT01775774).

However, the number of MSCs in the overall 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 to 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 application.

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-kB (NFκB), after systemic EPO administration 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.

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.

**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.

© 2015 S. Karger AG, Basel

**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 impact patients’ innate immune response; (b) can influence 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 cell barrier by improving endothelial and epithelial cell functions via differentiation and/or secretion of paracrine factors with regenerative capacity; (e) can locally target 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 in patients with ARDS [14, 15]. Current clinical trials may probably provide new evidence for their beneficial effects in this particular group of patients (NCT02444455, NCT01775774).

However, the number of MSCs in the overall 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 to 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 application.

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-kB (NFκB), after systemic EPO administration 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.

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.
CD56–CD34+CD14−CD11b−HLA-DR− populations were identified as dendritic cells, CD83+CD14+ as non-dendritic (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 500 μl 100% isopropanol and 2 μl glycogen (Fermentas, St. Leonroth, 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 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 × 10^6 PBMCs in 1% formaldehyde for 10 min. Chromatin immunoprecipitation (ChIP) was performed using the LowCell® ChIP kit (Diagenode, Liège, Belgium) using chromatin from 3,000 to 8,000 cells per reaction. For the immunoprecipitation, an antibody against H3K27me3 (9756; Cell Signaling Technology, Danvers, Mass., USA) was used. Control IgG was supplied by Diagenode. The ChIP was evaluated by qPCR using ‘platinum SYBR Green qPCR SuperMix-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

Respiration 2015;90:481–492

DOI: 10.1159/000441799
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 Master Mix Assays together with TaqMan Gene Expression Master Mix (CDH1, SNAI1; 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. 48 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 fold 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) were seeded on 48-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**

Data 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 hypoxic 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 4, the circuit was changed from V-V to V-A ECMO due to right heart decompensation. He showed no signs of clinical improvement, and due to the lack of further conventional treatment options we attempted experimental intrathoracic administration of autologous PBMCs and EPO on ECMO day 9. Although a notable improvement was observed immediately after treatment, the patient ultimately succumbed to multisystem organ failure possibly secondary to disseminated fungal infection and intra-abdominal sepsis.

**Cell Instillation and EPO Administration**

Experimental instillation of PBMC/EPO into the airway was initiated on the 9th day of ECMO. 300 ± 50 × 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...
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; CD14lowCD83+) [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 administrated 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.
miRNA Serum Levels

Serum levels of specific miRNAs may reflect epithelial cell 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]. Here, 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 SNAI1, a suppressor of CDH1 (fig. 3b, c). In the...

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-e Ventilation parameters. 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 VO₂ ECMO during ECMO treatment, where VO₂ ECMO 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).
Fig. 2. Characterization of administered PBMCs. Analysis of MSC and PBMC 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⁻)CD90dimCD79adim 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.
4 days preceding the patient’s death, this expression pattern was reversed as 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 at the time points beyond 8 days after initiation of cell/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
reports described 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 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 $4.5 \times 10^{10}$ particles (according to NTA) from HF and PBMCs to the NFκB-luc-positive

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 graphs displaying relative luminescent signal from reporter cells with NFκB-driven luciferase expression treated with hTNF-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.
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 cell therapy, the application of MSCs is both time and resource consuming. Clinical cell therapy requires in vitro culture, expansion and storage. Without in vitro consuming. Clinical cell therapy requires in vitro culture, expansion and storage. Without in vitro consumption, the application of MSCs is both time and resource consuming. 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, 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 responses upon stimuli.

On a 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 repression 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 Conflicts of Interest

All authors declare no competing financial interests.

References

4. Weiss DJ, Casaburi R, Flannery R, LeRoux-Berthelot S, Girard A, Rojas M: Adult stem cells for chronic 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.

Cell Therapy in ARDS

References


