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Therapeutic Effects of Bone Marrow-derived Progenitor Cells in Lipopolysaccharide-induced Acute Respiratory Distress Syndrome

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Abstract

Objective: Endotoxin-induced acute respiratory distress syndrome (ARDS) is characterized by diffuse dysfunction of the microvasculature including increased permeability with oedema formation and apoptosis or necrosis of endothelial- and epithelial cells. Concomitantly, an increased concentration of circulating endothelial progenitor cells (EPC) was found in septic patients, which seem to be involved in pulmonary regeneration. The number of circulating EPC correlated inversely to disease severity and mortality. Since bone marrow-derived endothelial progenitor cells (BMDPC) were found homing to damaged lung tissue, a reparative process seems to be initiated right after the initiation of vessel damage or degeneration. In the present study we investigated the potential of BMDPC as a treatment strategy in lipopolysaccharide (LPS)-induced ARDS.

Methods: Male Wistar rats received lipopolysaccharide (LPS) (25 µg/kg) systemically and directly after LPS injection, the animals were administered 1x106 suspension of CD133+-cells dissolved in 1 ml of sodium chloride 0.9% or only 1 ml sodium chloride 0.9% for the control group. Mortality, macroscopic changes in lung tissue, disease symptoms, blood gas analyses, serum cytokine concentration, wet/dry weight and long-term results were analyzed.

Results: Rats treated with BMDPC showed a significantly improved pulmonary gas exchange, an inhibition of proinflammatory cytokine synthesis, an improved clinical course and a reduced mortality ($p<0.024$) compared to rats treated with LPS alone.

Conclusions: These findings suggest that the application of exogenous BMDPC can reduce the severity of septic organ damage. Cell therapy with BMDPC might therefore become a novel option in ARDS therapy.

Keywords: Bone marrow-derived progenitor cells; Cell therapy; Sepsis; Acute respiratory distress syndrome; Endothelial progenitor cells; Lipopolysaccharide

Abbreviations

ALI: Acute Lung Injury; ARDS: Acute Respiratory Distress Syndrome; BMDPC: Bone Marrow-derived Progenitor Cells; EC: Endothelial Cells; ECFC: Endothelial Colony-Forming Cells; EPC: Endothelial Progenitor Cells; LPS: Lipopolysaccharide; PHC:Proangiogenic Hematopoietic Cells

Introduction

Acute Lung Injury (ALI) and end-stage acute respiratory distress syndrome commonly develop in patients with sepsis, multiple trauma, burn injury and aspiration, and are among the most common causes of death in intensive care units. Despite years of well-conducted clinical trials, no specific medical therapies exist to date [1,2]. During the acute exudative phase (first 24-48 h) of ALI/ARDS the histologic changes of lung tissue are characterized by infiltration of inflammatory cells and destruction of pulmonary endothelium [3,4]. Injury of the alveolar–capillary barrier leads to increased pulmonary vascular permeability, pulmonary edema and hypoxemia. At this stage, Endothelial Cells (EC) can be detached from the vasculature and thus appear in the circulation [3,5,6]. Inadequate formation of focal adhesion contacts (7), proteolysis of the endothelial basal membrane [7-9], apoptosis of EC [10], and the production of anti-angiogenic factors [11] are among the various causes for the release of EC into the circulation. There is growing evidence that simultaneously with these pathological processes a reconstitution of the endothelial layer is initiated. This reconstitution generally involves angiogenesis and adult vasculogenesis. In this regard, endothelial progenitor cells (EPC) have been shown to play a role. An increased concentration of bone marrow-derived EPC (BMDPC) was found in septic patients, which seem to be involved in pulmonary regeneration. The number of

circulating BMDPCcorrelated inversely to disease severity and mortality [12]. While EPC initially were thought to be recruited and incorporated into sites of active neovascularization during e.g. tissue ischemia, vascular trauma, tumor growth and inflammation [13], more recent work suggests different populations of endothelial progenitor cells with distinct functions. In the last several years it has become evident that the population of CD34+ AC133+ KDR+ cells do not form capillary-like structures with lumina in vitro nor in human blood vessels in vivo upon implantation in a collagen/fibronectin scaffold [14-16]. While these cells may be recruited to denuded vessels in ischemic sites, they do not directly become persistent vascular endothelial cells or display de novo in vivo vasculogenic potential, but rather display potent paracrine properties regulating new vessel formation via angiogenesis [15,17]. These cells are referred to as Proangiogenic Hematopoietic Cells (PHC) [18-20]. Other populations of EPC are identified using colony forming assays. One type of cell colony has been identified emerging from plated peripheral blood mononuclear cells in 14-21 days, when adult blood samples are plated, or in 6 days when umbilical cord blood samples are used [14]. The cells of these colonies tightly adhere to the substratum with a typical cobblestone appearance and are referred to as endothelial colony forming cells (ECFC) (20). These cells not only display the ability to form vessels, but these vessels also connect to the host vasculature in immunodeficient mice and become part of the systemic circulation of the host animal [12,21].

A vast amount of clinical and animal studies have been conducted either to quantify the level of EPC/PHC and its association to clinical outcome, or to investigate the potential of mobilization or administration of EPC/ECFC in lung regeneration in different lung disorders. However, it is still unclear whether progenitor cells can beneficially influence regeneration of ARDS and which cell population should be used. In the present study, we investigated whether exogenically administered bone marrow-derived CD133+progenitor cells could beneficially improve pulmonary function and survival in an isolated endotoxin-induced ARDS rat model.

Materials and Methods

Animal experiments

This study was approved by the Institutional Review Board for the care of animal subjects (University of Heidelberg, Mannheim, Germany & the Regional Council of Karlsruhe, Germany). All animals received humane care in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the stipulations of the German Animal Protection Law in its current version.

Specific pathogen free male Wistar rats were used for this study. In anesthesia, the femoral artery was cannulated with a polyethylene catheter (PE-50, neoLab Heidelberg, Germany) for multiple arterial blood collection. Subsequently, a catheter system (AeroProbe, Trudell Medical International, Ontario, Canada) was placed in the trachea for nebulization of lipopolysaccharide (LPS) or saline solution. The operating system (LABneb, Trudell Medical International, Ontario, Canada) exerts a pressure of 60 psi and applies a short puff synchronized with the respiration. The catheter system was removed after application of the respective LPS (serotype: E. Coli O55:B5, Sigma, Deisenhofen, Germany) dose [of 25 μg/kg of body weight].

Following the experimental procedure, the animals were observed with regard to activity, discoloration of the eye rim by secretion of the Harderian gland, grooming as well as weight changes by the examiner of the group and independently thereof by the animal keepers. These parameters and blood were collected twelve, twenty-four, forty and seventy-two hours after LPS- or LPS plus EPC-administration. At the final stage after seventy-two hours, the animals were euthanized under deep anaesthetic, and the lungs were exposed for removal. The right-hand middle lobe was weighed directly after the final stage and after twenty-four hours in the cabinet drier to determine wet/dry-ratio, a correlate for quantification of pulmonary edema. Remaining pulmonary lobes and organs were conserved in paraffin. We will refer to the different animal groups in the rest of our manuscript as follows: control group=animals receiving only sodium chloride (NaCl); non-treatment group=animals with LPS-induced ARDS, which were not administered BMDPC; treatment group=animals with LPS-induced ARDS, which were administered BMDPC. For long-term experiments over a period of ten days, we omitted the placement of a femoral catheter; the other steps of the LPS- and BMDPC-administration were carried out as described before. The progression parameters were recorded every day; the final stage on the tenth day after LPS-induced pulmonary damage was performed as reported earlier. We will refer to these two different animal groups in the rest of our manuscript as follows: long-term non-treatment group=animals with LPS-induced ARDS, which were not administered BMDPC and followed over 10 day; long-term treatment group=animals with LPS-induced ARDS, which were administered BMDPC and followed over 10 day.

Isolation and administration of bone marrow-derived progenitor cells

In order to isolate bone marrow-derived progenitor cells (BMDPC), the femoral bone marrow of about eight-weeks-old Wistar rats has been used; the rats have first been narcotized with Isoflurane and then euthanized by cervical dislocation. The cell cylinder was filtered through a 40 μm filter, and the mononuclear cells were isolated by density centrifugation. The vitality test of the cell suspension was carried out with trypan blue staining.

BMDPC were isolated from the bone marrow cell suspension by positive selection for CD133 by microbeads associated with monoclonal anti-CD133-antibodies (Miltenyi Biotech). For the CD133-microbead column 2 x 108 bone marrow mononuclear cells were used, and the magnetic column was applied according to the manufacturer’s instructions.

Directly after LPS injection, the animals were administered under anaesthesia via the tail vein either a 1 x 106 suspension of CD133+-cells dissolved in 1 ml of sodium chloride 0.9% or only 1 ml sodium chloride 0.9% for the control group.

Enzyme-linked immune-sorbent assay (ELISA)

The concentrations of TNF-α, IL-1 and IL-6 were assessed in plasma using enzyme-linked immunosorbent assay kits (R&D Systems, Wiesbaden-Nordenstadt, Germany) in triplicate samples. Enzyme-linked immunosorbent assays were performed according to the manufacturer’s instructions. The sensitivity of the assays is indicated to be approximately 15 pg/ml.

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Statistical methods

All data are presented as mean ± SEM. The statistical comparisons were carried out by means of the Wilcoxon rank sum test, one-way ANOVA or Mann-Whitney U test. The survival rates determined according to Kaplan-Meier were compared with the log rank test. The corrections for multiple comparisons were ensured by the Holm-Sidak test. We considered $p<0.05$ to be statistically significant. All analyses were performed using the SAS system (version 8.2, SAS Institute, Cary, NC).

Results

Animal groups

The results of an extensive dose-response analysis revealed reproducible lung damage with distinct clinical symptoms at a LPS-concentration of 25 µg/kg of body weight. To confirm the onset of experimentally induced ARDS, TNF-α -concentration in serum was determined apart from monitoring the clinical symptoms. The number of animals in the individual groups was as follows: control group: n=9, treatment group: n=17 (of which four died prematurely), non-treatment group: n=17 (of which nine died prematurely), long-term non-treatment group: n=6, long-term treatment group: n=6.

Mortality

To address the main question, whether exogenously administered BMDPCs can favourably influence the overall mortality in this ARDS model, we calculated the Kaplan-Meier survival curves of the animals recruited in our study. Our results show a significantly reduced probability of survival of the animals in the non-treatment group compared to the treatment group ($p<0.024$) (Figure 1).

Macroscopic changes and disease symptoms

Distinct differences of the lungs in the two study groups could be revealed macroscopically seventy-two hours after LPS- or LPS plus BMDPC-administration. In the non-treatment group, the lungs of the rats showed large confluent or smaller disseminated infiltrations, to some extent livid discolorations and hemorrhages, all of which were much less prevalent in the lungs of the animals in the treatment group (Figure 2).

For the evaluation of the disease course, we chose the following four signs: reddish lacrimation, condition of the fur, activity and food intake. These four symptoms were classified over a period of three days according to different degrees of severity (1=very good to 4=very bad). The ophthalmological signs with reddening and flow of reddish secretion due to LPS-nebulisation were clearly observable in the treatment and non-treatment group already twelve hours after application and continued to be invariably distinct from controls over the entire observation period (Figure 3A). However, markable differences between the study groups were revealed in the other three signs: a dishevelled, dull and rough fur developed within the first one to two days in the non-treatment group only (Figure 3B). These animals revealed distinctly more signs, a reduced food intake (data not shown) and activity (Figure 3C).

Blood gas analyses

Arterial blood gas analyses were carried out at time points 12, 24, 40 and 72 hours after LPS- or LPS plus BMDPC-administration. The application of BMDPC caused a significant improvement of the oxygenation (Figure 4). The PaO2 in the treatment group was significantly increased for a total of two days compared to the non-treatment group, reaching values close to those of the control group (Figure 4A). After three days, mean PaO2 values were still better in the treatment group, but did not meet statistical significance. The PaCO2 values revealed a discrete hyperventilation of all animals treated with LPS independent of the BMDPC administration (data not shown). However, oxygen saturation was significantly better at time points 24 and 40 hours in the treatment group compared to the non-treatment group (Figure 4B).

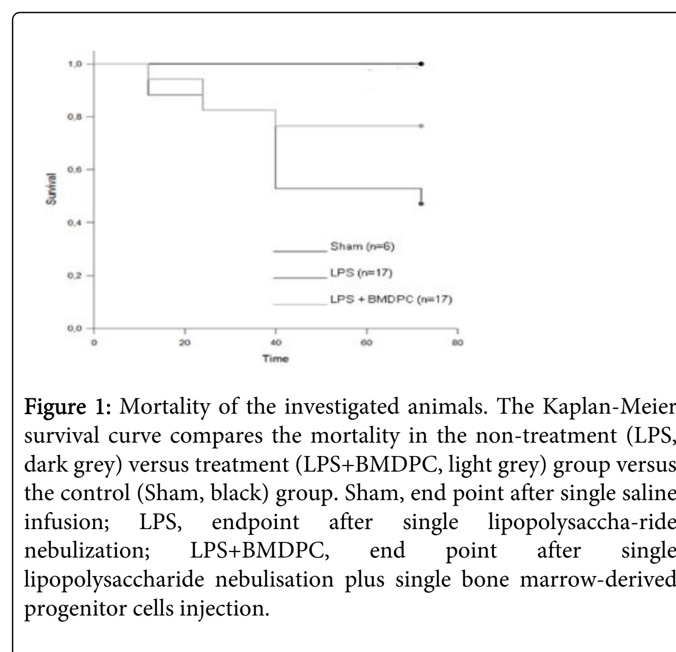

Figure 1: Mortality of the investigated animals. The Kaplan-Meier survival curve compares the mortality in the non-treatment (LPS, dark grey) versus treatment (LPS+BMDPC, light grey) group versus the control (Sham, black) group. Sham, end point after single saline infusion; LPS, endpoint after single lipopolysaccha-ride nebulization; LPS+BMDPC, end point after single lipopolysaccharide nebulisation plus single bone marrow-derived progenitor cells injection.

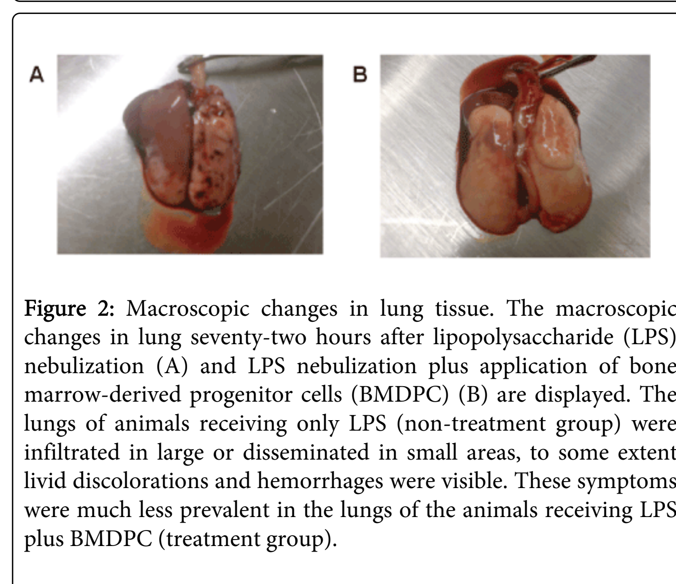

Figure 2: Macroscopic changes in lung tissue. The macroscopic changes in lung seventy-two hours after lipopolysaccharide (LPS) nebulization (A) and LPS nebulization plus application of bone marrow-derived progenitor cells (BMDPC) (B) are displayed. The lungs of animals receiving only LPS (non-treatment group) were infiltrated in large or disseminated in small areas, to some extent livid discolorations and hemorrhages were visible. These symptoms were much less prevalent in the lungs of the animals receiving LPS plus BMDPC (treatment group).

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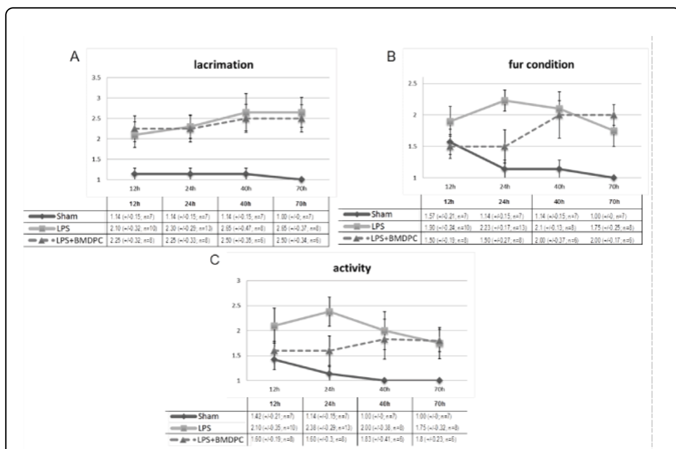

Figure 3: Clinical symptoms of the animals. The clinical symptoms of the animals in the control group (Sham, black line), in the non-treatment (LPS, gray-line) and in the treatment group (LPS plus BMDPC, dashed line) are displayed. The points were awarded as follows: reddish lacrimation (1=normal to 4=very red), condition of the fur (1=normal to 4=dishevelled, dull and rough) and activity (1=normal to 4=adynamic). The reddening of the eyes and flow of reddish secretion were clearly prevalent in the treatment and non-treatment group due to lipopolysaccharide (LPS)-nebulization already twelve hours after application and continued to be invariably distinct over the entire period. But a dishevelled, dull and rough fur developed within the first one to two days only in the non-treatment group. These animals revealed distinctly more clinical symptoms and reduced activity. BMDPC, bone marrow-derived progenitor cells.

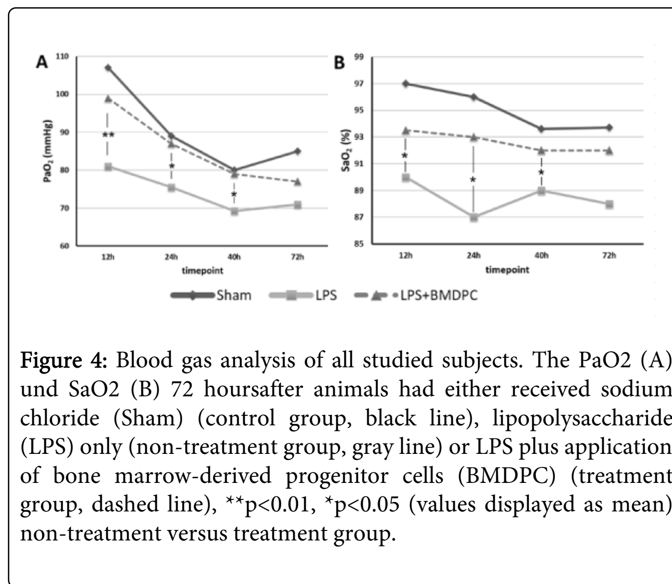

Figure 4: Blood gas analysis of all studied subjects. The PaO2 (A) und SaO2 (B) 72 hoursafter animals had either received sodium chloride (Sham) (control group, black line), lipopolysaccharide (LPS) only (non-treatment group, gray line) or LPS plus application of bone marrow-derived progenitor cells (BMDPC) (treatment group, dashed line), **p<0.01, *p<0.05 (values displayed as mean) non-treatment versus treatment group.

Cytokine concentrations

To confirm the initiation of a pro-inflammatory cascade by LPS, the serum concentrations of TNF-α, IL-1 and IL-6 were measured. After the first hour, TNF-α concentration reached its maximum value; subsequently, the concentration decreased continuously to the initial value (data not shown). The maximum increase of IL-1 concentration was measured twelve hours after LPS-nebulisation. At this point significant differences could already be measured for the two study groups: In the treatment group, a distinctly lower IL-1 synthesis was noted during the entire study period (Figure 5A). A similar result was noted for IL-6 synthesis in the treatment group (Figure 5B).

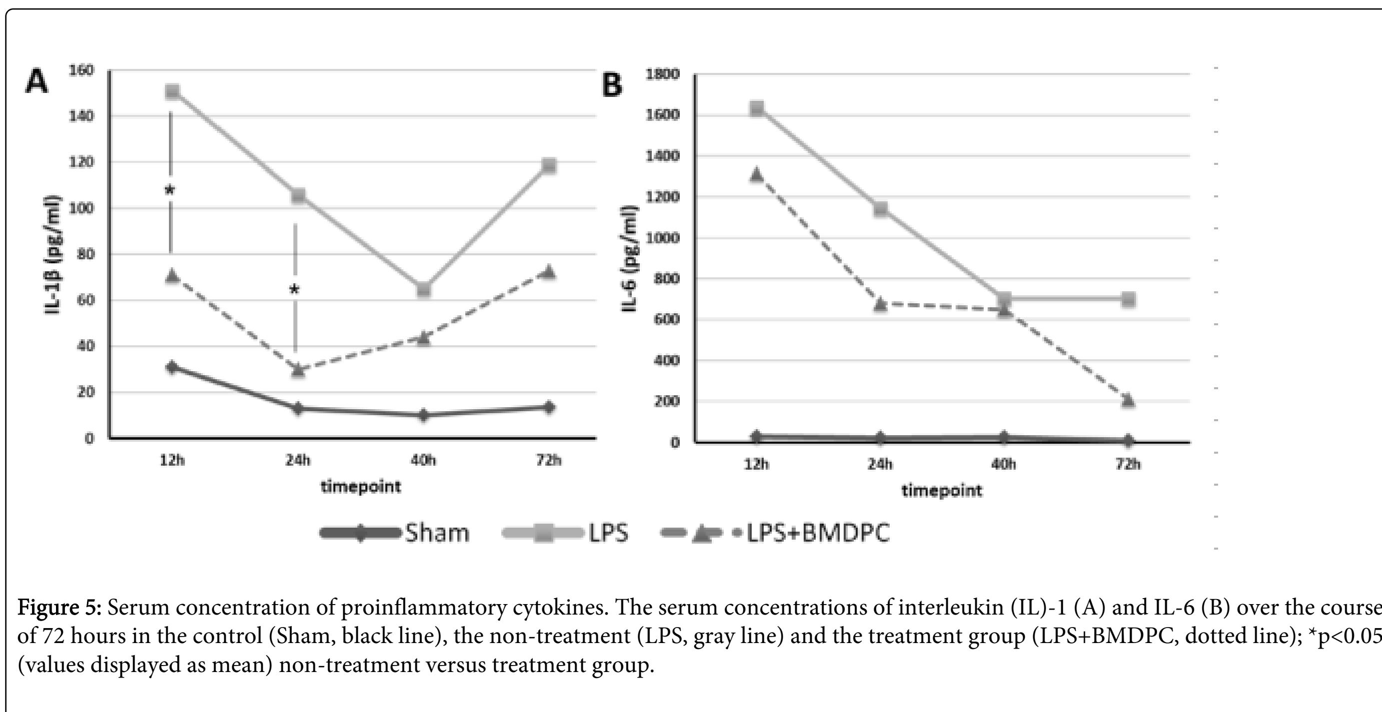

Figure 5: Serum concentration of proinflammatory cytokines. The serum concentrations of interleukin (IL)-1 (A) and IL-6 (B) over the course of 72 hours in the control (Sham, black line), the non-treatment (LPS, gray line) and the treatment group (LPS+BMDPC, dotted line); *p<0.05 (values displayed as mean) non-treatment versus treatment group.

Wet/dry weight

To quantify the degree of pulmonary edema, we determined the wet/dry weight of the lungs at the final time point. As expected, the comparison of wet/dry weight between treatment/non-treatment groups and control group revealed a distinct difference, but the treatment group did not differ significantly with respect to the lung weight and the wet/dry ratio compared to the non-treatment group (data not shown).

Long-term results

To assess long-term observation data, we have evaluated 6 animals with LPS-induced ARDS, which were not administered BMDPC (long-term non-treatment group), and 6 animals with LPS-induced ARDS, which were administered BMDPC (long-term treatment group) over a period of ten days. With respect to the clinical symptoms and the course of cytokine syntheses there were no longer significant differences between the two study groups after the initial observation period of three days (data not shown).

Discussion

Today it is undisputed within the field that angiogenesis and vasculogenesis progenitor cells are recruited from the bone marrow and migrate to the location of revascularisation, initiating proangiogenic effects via paracrine signalling or in rather a small number differentiating directly to mature endothelial or other cell types [22]. Increasing significance is also attached to these progenitor cells with respect to endothelial cell regeneration after acute and chronic inflammation-induced tissue damage [23]. In previous studies, we and others have demonstrated that progenitor cells are increasingly mobilized from the bone marrow into the circulation during sepsis [12], ARDS [24,25] and other inflammatory lung diseases [25]. The concentration of progenitor cells in the circulation also correlates with the clinical outcome of the patients. Therefore, it was the aim of this study to examine whether the exogenic application of BMDPC can induce clinically relevant effects on the pulmonary function in experimental LPS-induced ARDS.

We were able to demonstrate an improved macroscopic appearance of the lung, an improved functional gas exchange, a reduced synthesis of pro-inflammatory cytokines and a significantly lower mortality in animals which were treated with BMDPC after LPS nebulization. Thus, the disease course was significantly improved, in particular during the early phase of ARDS. In our vulnerable in vivo model, LPS was nebulized only once to observe the acute phase of ARDS for seventy-two hours. For long-time observations, multiple or permanent applications of LPS will certainly have to be considered and evaluated. In this study, the animals receiving LPS only, who survived the acute stadium after seventy-two hours, remained stable and reconstituted fully.

Our observations confirm previous in vivo results, which had been published in 2008 on a rabbit lung model with oil-acid induced ARDS [26]. Animals undergoing an autologous transplantation of progenitor cells showed significant reduction of iNOS expression in the pulmonary artery and of leukocyte infiltration as well as haemorrhages in the pulmonary tissue. In contrast to the results of our study, oedema formation in the lung was also reduced significantly. Recently, the same group has observed a significantly improved oxygenation and gas exchange by intravenous progenitor cell application in rabbits with endotoxin-induced ALI [27]. In a different study, Gao et al. have demonstrated in a LPS-induced ARDS rabbit model that systemic application of progenitor cells inhibited the expression of adhesion molecules and the synthesis of pro-inflammatory cytokines (TNF-α or IL-1b) while significantly increasing the synthesis of anti-inflammatory cytokines (IL-10) [28]. In our study, we too observed a significantly reduced IL-1 and IL-6 synthesis following administration of BMDPC. Interestingly, Gao et al. were also able to show a significant reduction of the inflammation-induced apoptosis of endothelial and epithelial cells by the application of progenitor cells [28]. Based on these observations, it seems that BMDPC, besides neovascularisation, not only stimulate vessel regeneration and repair but also inhibit local inflammation [29,30].

The mechanisms involved in the beneficial effects of BMDPC/EPC are still unclear for the most part. Recruitment and incorporation of BMDPC/EPC from bone marrow into ischemic or injured tissue sites requires a coordinated multistep process including mobilization, chemotaxis, adhesion to the endothelium, transendothelial migration, invasion and in situ differentiation [22]. The transdifferentiation of BMDPC/EPC into mature endothelial cells is rather a rare event. In most cases BMDPC/EPC, after invasion into the subendothelial layer, release growth factors which stimulate angiogenic activity of resting mature endothelial cells [22]. This was also confirmed in a single-lung induced ARDS model after intravenous BMDPC application [31]. BMDPC homed exclusively to the lung with damaged endothelium, but not to the contralateral healthy lung or to any other healthy organ. Nonetheless, the experimental models were not able to confirm an in vivo differentiation of BMDPC into mature endothelial cells. Also, BMDPC did not integrate into the vessel wall in ischemic models, but colonized in subendothelial layers or on the surface of the endothelium to induce their effects in a paracrine manner [32]. These paracrine effects consist of an increased release of growth factors, such as vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF) 1, stromal-derived factor (SDF)-1 as well as chemokines and cytokines. Thus, a proangiogenic environment is established, by which resident endothelial and other cell groups are stimulated to proliferate [33].

Based on angiogenesis studies, first results on the potential significance of the BMDPC surface molecules α4- or α5-integrin involved in the adhesion of the endothelial matrix have been published [34,35]. In an endotoxin-induced lung vascular injury and edema model in mice, 80-90% of the injected BMDPC could be found in the damaged pulmonary tissue within twenty minutes. But the concentration of the BMDPC decreased by 40% over the next twenty-four hours [34]. This phenomenon could explain our observation that BMDPC induce significant effects only in the acute phase up to forty-eight hours in our isolated ARDS model and that the investigated groups did not exhibit any further significant difference thereafter. The above mentioned study [34] also confirmed that adhesion of BMDPC to the tissue is required to induce the BMDPC-triggered reduction of vessel damage, oedema formation and mortality after LPS-induced inflammation. The therapeutic effects of BMDPC were not detectable anymore when the integrins were blocked and by that adhesion of BMDPC diminished [34].

BMDPC/EPC are characterized and isolated by the presence of trans-membrane glycoprotein CD133, which is expressed as surface antigen on human progenitor cells, but also on human hemangioblasts [35-37]. In contrast to the hematopoietic progenitor marker CD34, CD133 is not expressed on mature endothelial cells, and sub-populations of CD34+ cells, which also express CD133, have a high

proliferative capacity and lead to the formation of endothelial colonies in cell culture [35]. Studies in animal models report that circulating CD133+ BMDPC are involved in the neoangiogenesis after tissue ischemia and in the regeneration of the damaged organ [38,39].

The function of CD133, as well as CD34, is not completely clarified. Given its molecular structure, it could likely serve as a growth factor receptor, but a corresponding ligand could not be identified yet. Since the localization of CD133 expression is restricted (?) to cytoplasmatic protrusions, a role in the membrane organization and/or intercellular interaction is postulated [40,41].

An increase of the EPC concentration in blood, as a therapeutical option for severe inflammation with endothelial damage in ARDS/ALI, may be induced in two manners. First, an endogenic mobilization of EPC from the bone marrow can be stimulated by administration of growth factors, such as VEGF, GM-CSF, EPO or SDF-1. By this means, an effect could only be documented in a single cardiological study [42] so far, and results from our own experimental series in our LPS-induced isolated ARDS rat model are still pending. Second, the exogenic application/transplantation of EPC, as carried out in this study, seems to cause significant effects in the inflammation model, but comprises the disadvantage of requiring previous EPC preparation. In addition, differences in the treatment of local vessel damage with "on-site application" as opposed to treatment of generalized microvascular damage with systemic EPC application have to be taken into account. So far, established sources of EPC for therapeutic purposes include bone marrow, umbilical cord blood and peripheral blood.

Conclusions

In endotoxin-induced pulmonary endothelial dysfunction, BMDPC seem to initiate repair mechanisms, which result in a reduction of increased permeability and oedema formation, thus reducing mortality. Moreover, pro-inflammatory processes are inhibited and gas exchange is improved. Future studies will have to analyze the underlying mechanisms involved in this reparative process, so that BMDPC/EPC may become an innovative therapeutic strategy option in ARDS and sepsis.

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