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Direktor Prof. Dr.med. Dr.med.habil. Bernhard Zwissler

Inhaled Interleukin-10 before and after induction of experimental endotoxemia in the rat. Effects on the inflammatory response.

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Nguyen, Ngoc Thach

aus Hanoi, Vietnam

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Dekan : Prof. Dr. J. Pfeilschifter

Referent : Prof. Dr. B. Zwißler

Koreferent: Priv. - Doz. Dr. H. Mühl

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To my wife Hà Thị Thu Hiền and daughter Nguyễn Hà Quỳnh Anh

and to the memories of my father Prof. Nguyễn Hữu Mô and my mother Thạch Thị Lợi.

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ABBREVIATIONS

ARDS	Acute respiratory distress syndrome
ALI	Acute lung injury
AM	Alveolar macrophage
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BE	Base excess in plasma
ELISA	Enzyme-linked immunosorbent assay
FiO_2	Fraction of inspired oxygen
hi-aft group	The group of inhalation of 5.0µg IL-10 after LPS injection
hi-bef group	The group of inhalation of 5.0µg IL-10 before LPS injection
HCO_3^-	Plasma bicarbonate concentration
HR	Heart rate
IQR	Interquartile range
IFN-γ	Interferon-gamma
IL-1ß	Interleukin-1 beta
IL-10	Interleukin-10
IL-1ra	IL-1-receptor antagonist
IL-6	Interleukin-6
IL-8	Interleukin-8
LPS	Lipopolysaccharide
I:E	Ratio of inspiration time to expiration time
iNOS	Inducible nitric oxide synthase
kg	Kilogram
lo-aft group	The group of inhalation of 0.5µg IL-10 after LPS injection

lo-bef group	The group of inhalation of 0.5µg IL-10 before LPS injection
MAP	Mean arterial blood pressure
MMAD	Mass median aerodynamic diameter
Mea	Measurement-time point during the experimental protocol
mEq	Milliequivalent
mEq/l	Milliequivalent per litre
mRNA	Messenger-RNA
NK	Natural killer cell
$PaCO_2$	Arterial carbon dioxide tension
PaO_2	Arterial oxygen tension
PBS	Phosphate buffered saline
PEEP	Positive end expiratory pressure
pH	The logarithm of the reciprocal of hydrogen-ion concentration in gram atoms per litre
P_{max}	Peak airway pressure
rhIL-10	Recombinant human IL-10
Semi-IQR	Semi interquartile range
TNF-α	Tumour necrosis factor-alpha
vs.	Versus

1. INTRODUCTION

1.1. Definition and Epidemiology of Acute Respiratory Distress Syndrome (ARDS)

The acute respiratory distress syndrome (ARDS) is a clinical syndrome of acute lung inflammatory injury which was first described in 1967 by Ashbaugh with the following symptoms: acute respiratory failure refractory to oxygen therapy, decreased lung compliance, and diffuse bilateral alveolar infiltration on the chest x-ray [3].

In 1994, the American-European Consensus Conference defined criteria for the diagnosis of ARDS and acute lung injury (ALI), a less severe form of acute respiratory failure. The common criteria for ALI and ARDS include acute onset, bilateral infiltrates on chest x-ray, and no clinical evidence of increased left atrial pressure with a pulmonary artery wedge pressure ≤ 18 mmHg [7]. While ALI is defined with a quotient of arterial oxygen tension (PaO_2) and fraction of inspired oxygen (FiO_2) ratio between 200 and 300 mmHg, ARDS is defined by a more severe oxygenation failure with the PaO_2/FiO_2 ratio < 200 mmHg [7].

Based on that definition, the incidence of ARDS is reported from 23 to 59 cases per 100000 citizens in Europe and the United States, respectively [48] [62].

1.2. Etiology and Pathophysiology of ARDS

1.2.1. Etiology of ARDS

ARDS arises either as a complication of a direct insult to the lung (pneumonia or aspiration of gastric contents) or indirectly, when systemic inflammation reaches the lung which can be the case during clinical sepsis, severe trauma, or following massive transfusion. The leading risk factor for the development of ALI and ARDS is the sepsis syndrome, which is responsible for up to 40% of ARDS cases [1] [35]. Although several progresses have been made in supportive care, the mortality arising from ARDS

still ranges high from 35 to 40 percent [62]. Thus, alternative therapeutical strategies in ARDS-treatment are in the focus of clinical and experimental research to possibly improve the prognosis of ARDS.

1.2.2. Pathophysiology of ARDS

ARDS is characterized by widespread injury of structures of the alveolar capillary membrane, which causes a loss of epithelial integrity and disruption of junctions between alveolar and endothelial cells. This results in increased pulmonary capillary permeability and flooding of the alveolar spaces with protein-rich fluid containing large number of immunocompetent cell populations among them neutrophils and alveolar macrophages [78].

In the acute phase of ARDS, activated alveolar macrophages secret proinflammatory cytokines that locally stimulate chemotaxis and activation of neutrophils. Activated neutrophils adhere to the injured capillary endothelial cells and marginate through the interstitium into the air space. Subsequently, they liberate other proinflammatory mediators among them oxidants, proteases, and leukotriens thus contributing for the aggravation of lung injury [78].

There is evidence that the intrapulmonary origin of proinflammatory cytokines in ARDS plays an important role for the initiation and perpetuation of the inflammatory processes within the lung. Alveolar macrophages from ARDS-patients express proinflammatory cytokines, emphasizing their intrapulmonary origin [40]. Proinflammatory cytokine levels have been found increased in bronchoalveolar lavage fluid (BALF) of patients at risk for and with sustained ARDS as compared to nonseptic control patients [18] [70].

The overwhelmed and sustained proinflammatory condition contributes to the poor prognosis in ARDS patients. Meduri et al. in 1995 reported that, at the onset of ARDS, the nonsurvivors had significantly higher levels of proinflammatory cytokines like TNF-α, IL-1ß, and IL-6 in plasma and in bronchoalveolar lavage fluid. In contrast, in survivors initial lower levels of these proinflammatory cytokines have been found [51] [52].

Based on the results of the aforementioned studies [51] [52], the question arises if anti-inflammatory therapies aiming the overwhelming inflammation may help to improve the outcome in ARDS patients.

1.3. Anti-inflammatory Therapy and ARDS

Several clinical studies which directed toward neutralizing proinflammatory cytokines in ARDS induced by sepsis (like the administration of interleukin-1 receptor antagonist and soluble tumor necrosis factor receptor) did not show any significant improvement in the outcome from ARDS patients [23] [24].

1.3.1. Interleukin-10 and ARDS

There is evidence that low concentration of interleukin-10 (IL-10), an anti-inflammatory cytokine, is related closely to the incidence of ARDS [2] and the mortality rate of ARDS patients [17]. In addition, kinetics of IL-10 synthesis during the acute phase of ARDS showed that IL-10 appeared later (7h after activation of monocytes by lipopolysaccharide) in lower concentration than proinflammatory cytokines (4h after activation of monocytes by lipopolysaccharide) [13] [50].

Based on these findings, the supplementation of IL-10 may be a promising approach for ARDS patients. Thus far, there is only one clinical report concerning the application of IL-10 during ARDS [6]. Bernard et al. in 1999 reported that lower proinflammatory

cytokine levels and a trend towards reduced duration of mechanical ventilation in ARDS patients who received intravenous injection of IL-10 [6].

Interleukin-10 is mainly produced by T lymphocytes, B lymphocytes, monocytes, and macrophages [14] [55] [63] [76]. Functionally, IL-10 inhibits the synthesis of proinflammatory cytokines (like TNF-α, IL-1ß, IL-6, and IL-8) [20] [47] and the formation of reactive nitrogen species in activated macrophages or monocytes in vitro [11]. Interleukin-10 also suppresses the synthesis of interferon gamma (IFN-γ) by helper T cells [21] and natural killer cells (NK) [34].

Moreover, IL-10 enhances the release of anti-inflammatory mediators such as interleukin-1 receptor antagonist (IL-1ra) [72] and soluble tumor necrosis factor receptor [15] by activated monocytes. Based on its anti-inflammatory properties, it was hypothesized that IL-10 administration may be useful in experimental sepsis [33]. Ge'rard et al. in 1993 reported that tumor necrosis factor (TNF) serum levels of mice that received intraperitonealy IL-10 (1000U) 30min before intravenous injection of 100µg lipopolysaccharide (LPS) were significantly reduced and all animals survived [28].

Related to supplementation of IL-10 in models of experimental endotoxemia, the route of administration is of special interest, specifically for the therapy of pulmonary inflammation. Usually, IL-10 has been administered intravenously [41] [42] [57]. Regarding the therapy of pulmonary inflammation, Inoue in 1999 demonstrated that, when LPS (12mg/kg) and IL-10 (20 or 40µg/kg) were simultaneously intravenously injected, TNF-α production and neutrophil activation were inhibited thus leading to a significant reduction of inflammatory change in lung tissue [36].

Taking into account the pathogenesis of ARDS which is closely related to cytokines synthesized in the lungs, IL-10 administration via the inhalational route in the treatment

for ARDS may be more attractive. Local administered IL-10 preferably would act within lungs which is the site of inflammation as well as production of proinflammatory mediators. At the same time, local administered IL-10 may limit the systemic side-effects of IL-10. In a previous study, our group could demonstrate that the levels of the proinflammatory cytokines TNF-α, IL-1ß, IL-6, and IFN-γ in plasma and bronchoalveolar lavage fluid were significantly reduced by inhaled IL-10 in endotoxemic rats [31].

In that study, IL-10 was administered prior to induction of experimental endotoxemia (LPS 5mg/kg), at a median dose of 0.19µg per animal [31]. The question arises if inhaled IL-10 is also effective when it is inhaled during already existing endotoxemia and, further, if its effects are dose-dependent, specifically, if equipotent effect could be achieved with a much less dosage of IL-10. The present study was performed to clarify these questions.

1.4. Objective

In the present study, we aimed to determine if inhaled IL-10 either prior to or following to the intravenous injection of LPS (5mg/kg) at two doses (5.0µg or 0.5µg) will:

Reduce the concentrations of proinflammatory cytokine TNF-α, IL-1ß, IL-6, and IFN-γ in plasma and bronchoalveolar lavage fluid (BALF) from endotoxemic animals.

Reduce the concentration of nitrite release from ex vivo cultured alveolar macrophages from endotoxemic animals.

2. MATERIALS and METHODS

2.1. Animals

The study was approved by the local board for the care and use of animal subjects, Darmstadt, Germany (decision number VI63-19c20/15-F91/31). Experiments were performed in 48 male Sprague-Dawley rats (Harlan Winkelmann, Borchen, Germany). Mean body weight of rats was 500 ± 33 g (mean ± standard deviation). Care of animals followed the "Guide for the Care and Use of Laboratory Animals" (National Academic Press, Washington D.C, 1996) enforced by local regulations.

Experiments were conducted as a controlled, prospective, and randomized study and performed in the research centre of the faculty of medicine of the Johann-Wolfgang Goethe Frankfurt University, Frankfurt am Main, Germany.

2.2. Anaesthesia and Surgical Preparation

Induction of anaesthesia in rats was performed by intraperitoneal injection of pentobarbital 50mg/kg (Narcoren®, Merial, Halbergmoss, Germany) and fentanyl 0.05mg/kg (Janssen-Cilag, Neuss, Germany). After sufficient depth of anaesthesia, rats were weighted and placed supine on a heating pad to keep body temperature between 37.0^{0}C and 38.0^{0}C throughout the experiment. Body temperature was continuously monitored with a rectal probe (Siemens Sirecust 404, Siemens, Erlangen, Germany). After tracheotomy, a 13-gauge cannula (Abbott, Wiesbaden, Germany) modified with a connector for paediatric endotracheal tube was inserted into the trachea. The tracheal leak was sealed by an external ligature encompassing the intubated trachea. Thereafter, polythene catheters with inner and outer diameter of 0.58mm and 0.96mm, respectively, (SIMS Portex, Hythe, UK) were inserted into the right femoral vein and artery. Physiologic saline (NaCl 0.9%, Delta Select, Pfullingen, Germany) was intravenously

infused with the rate of 12ml/kg/h. Pentobarbital and fentanyl were intravenously infused with the rate of 5–10mg/kg/h and 2.5–5µg/kg/h, respectively. Heart rate and arterial blood pressure were continuously monitored by an electromechanical pressure transducer (Statham physiological pressure transducer P23x1-1, Viggo-Spectramed, Oxnard, CA, USA). Parameters were continuously displayed on a monitor (Siemens Sirecust 404, Siemens, Erlangen, Germany).

When mean arterial blood pressure decreased below 80mmHg, a 10% hetastarch solution (HES, 200/0.5, 10%, B.Braun Melsungen, Melsungen, Germany) and natrium chloride 0.9% (B.Braun Melsungen, Melsungen, Germany) in 1:1 mixture was administered as bolus injections. When hypotension did not improve following two boluses, norepinephrine (Arterenol®, Aventis, Frankfurt, Germany) was infused at 0.02 – 1mg/kg/h. Blood gas analysis was performed hourly (ABL 500 Radiometer, Frankfurt, Germany). If metabolic acidosis occurred ($pH < 7.30$, $BE < -5mEq/l$, no respiratory components), a natriumbicarbonat solution 8.4% ($NaHCO_3$ 8.4%, B.Braun Melsungen, Melsungen, Germany) was intravenously injected. The dose of natriumbicarbonat solution 8.4% was calculated according to the following formula:

$$NaHCO_{3\,[mEq]} = BE_{[mEq/l]} \times 0.3 \times \text{body weight}_{[kg]}$$

BE: Base excess in plasma; mEq: Milliequivalent; mEq/l: Milliequivalent per litre; kg: Kilogram.

Completion of surgical preparation was followed by a stabilization period of 15 minutes.

2.3. Mechanical Ventilation

Rats were mechanically ventilated using pressure controlled ventilation by an infant ventilator (Stephanie®, Stephan, Gackenbach, Germany). Respiratory settings were: peak airway pressure (P_{max}) 1.6kPa; positive end-expiratory pressure (PEEP) 0.4kPa; respiratory rate 30/min; fraction of inspired oxygen (FiO_2) 0.21, ratio of inspiration time to expiration time (I:E) 1:2.

2.4. Nebulisation

For the period of nebulisation, rats were connected to a specifically designed nebuliser system, which was constructed and characterized by our group in a previous study, that was performed at the University Hospital of Munich [32]. Briefly, the system includes a ventilator (Siemens Servo® 900 B, Siemens, Elema, Solna, Sweden) with a nebuliser unit (Siemens Nebuliser 945®, Siemens, Elema, Solna, Sweden) and a nebuliser chamber (Micro Cirrus®, Intersurgical, St.Augstin, Germany).

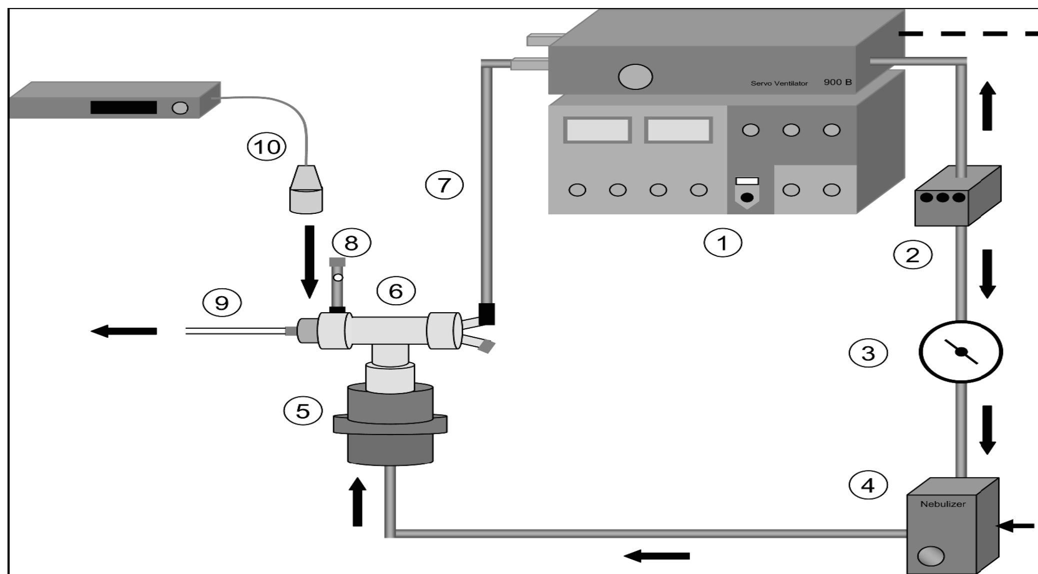

Figure 1. Schematic illustration of the nebuliser system. 1, Servo Ventilator (Siemens Servo 900 B); 2, gas supply; 3, supply pressure-reducing valve; 4, nebuliser unit (Siemens nebuliser 945); 5, nebuliser chamber (Micro Cirrus); 6, T-piece; 7, expiratory

line; 8, valve for control of peak airway pressure; 9, endotracheal tube; 10, monitor for peak airway pressure.

For the period of aerosolisation, the nebuliser settings were: peak airway pressure (P_{max}) 1.5kPa, respiratory rate 40/min, FiO_2 0.5. The nebuliser system generates and delivers aerosol during inspiration. Evaluation of resulting droplet size of the nebuliser system, effectiveness in aerosol delivery and animal compliance was previously published [32]. The generated particles have a mass median aerodynamic diameter of 2µm which is effective for alveolar deposition in intubated ventilated rats. Particle deposition fraction was 3.8%(1.3%) of the delivered dose (median [interquartile range]) [32].

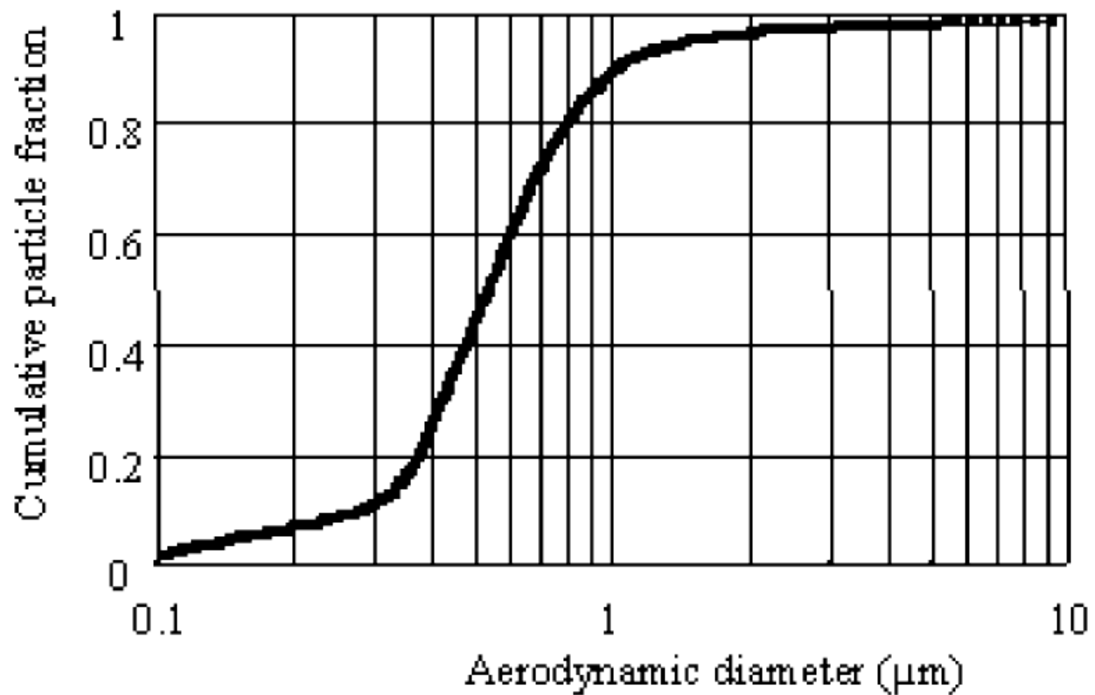

Figure 2. Description of aerosol. Cumulative particle fraction of the dried ^{99m}Tc-labeled salt particles as a function of their aerodynamic diameter measured by the aerosol centrifuge.

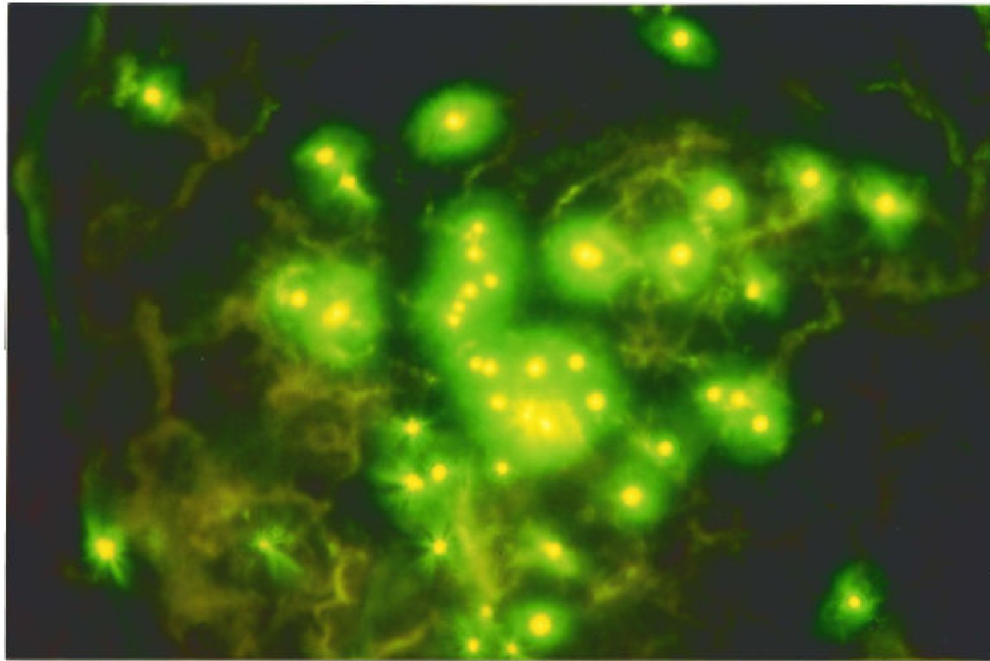

Figure 3. Detection of deposited intrapulmonary fluorescent microspheres. Fluorescence microscopy of liquid nitrogen-fixed pulmonary tissue of the right middle lobe of a rat exposed to aerosolized microspheres for 40min (magnification 200x ; gauge: 20µm)

2.5. Experimental Protocol of the Present Study

After surgical preparation and a stabilization period of 15 minutes, animals were randomly assigned to six groups (n = 8, each).

2.5.1. Preparation of LPS

LPS was prepared from *Escherichia coli* 055:B5 (Sigma-Aldrich, Deisenhofen, Germany), diluted in phosphate buffered saline (PBS) (Serva, Heidelberg, Germany).

2.5.2. Preparation of Interleukin-10

Recombinant murine interleukin-10 (R&D Systems, Wiesbaden, Germany) as a sterile powder, purity > 98%, specific activity 5 x 10^5U/mg, was reconstituted in PBS to two final concentrations (5.0µg/ml or 0.5µg/ml) and stored in aliquots of 1ml at -80^0C.

2.5.3. Protocol of Experiments

(1) *Sham* group: Animals received 1ml nebulised PBS (the solvent of IL-10 used in the other groups) within 40 minutes before 1ml intravenous injection of saline (placebo), which was the solvent of LPS used in the other groups.

(2) *LPS-only* group: Animals received intravenous injection of LPS 5.0mg/kg and no further intervention.

(3) *hi-bef* group: Animals received nebulised IL-10 at 5.0µg (1ml, 5.0µg/ml, corresponding to 0.19µg/rat) within 40 minutes before intravenous injection of LPS 5.0mg/kg.

(4) *lo-bef* group: Animals received nebulised IL-10 at 0.5µg (1ml, 0.5µg/ml, corresponding to 0.019µg/rat) within 40 minutes before intravenous injection of LPS 5.0mg/kg.

(5) *hi-aft* group: Animals received nebulised IL-10 at 5.0µg (1ml, 5.0µg/ml, corresponding to 0.19µg/rat) within 40 minutes after intravenous injection of LPS 5.0mg/kg.

(6) *lo-aft* group: Animals received nebulised IL-10 at 0.5µg (1ml, 0.5µg/ml, corresponding to 0.019µg/rat) within 40 minutes after intravenous injection of LPS 5.0mg/kg.

Haemodynamic and respiratory parameters were continuously monitored with hourly performed blood gas analyses as described above. At the end of the observation period (after 4 hours of endotoxemia or saline injection, respectively), animals were sacrificed by exsanguination via the femoral artery catheter. A thoracotomy was performed and lungs were immobilized for bronchoalveolar lavage.

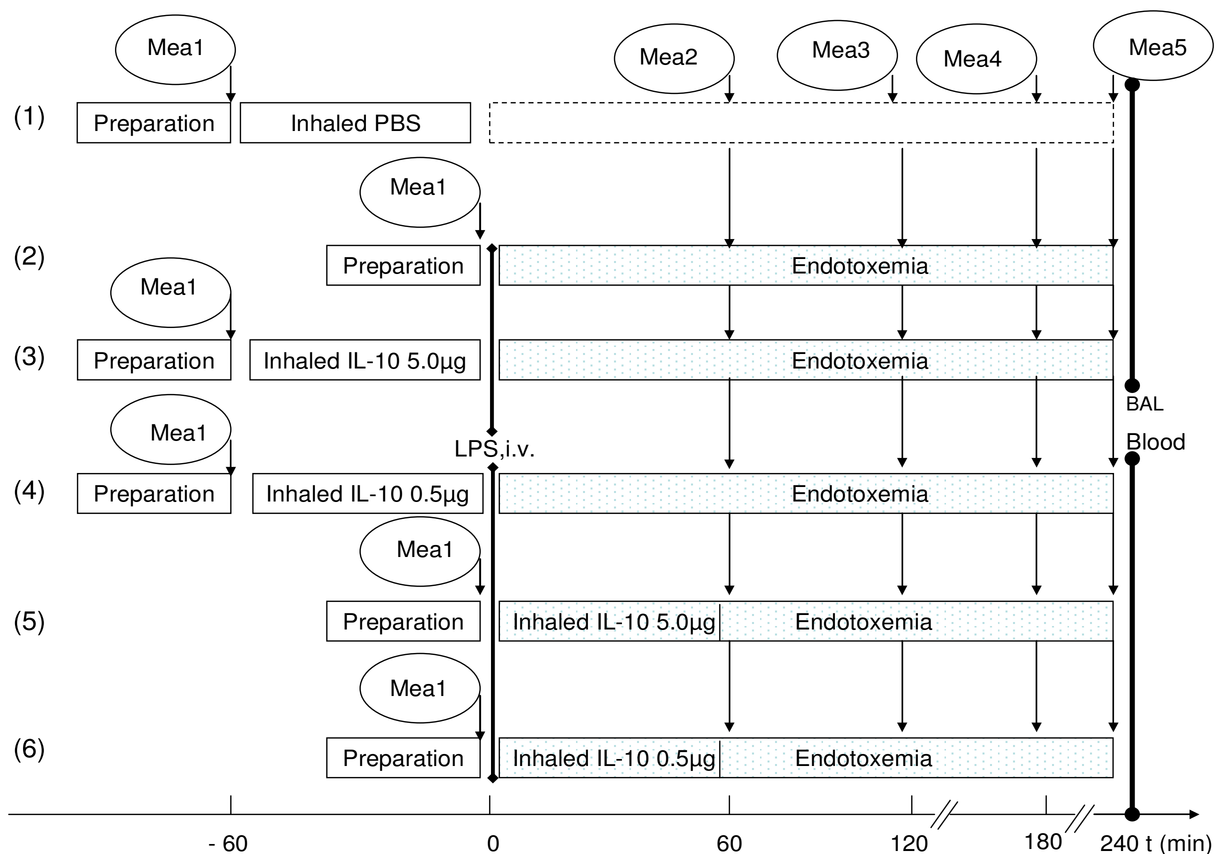

Figure 4. Schematic illustration of the characterization of six groups.

Mea1, Mea2, Mea3, Mea4, and Mea5: are the first, second, third, fourth, and fifth time point of measurement of heart rate, arterial blood pressure, and arterial blood gas analysis after preparation (Mea1) and one, two, three, and four hours following the injection of LPS or saline (Mea2-5), respectively.

2.6. Bronchoalveolar Lavage, Isolation, and Culture of Alveolar Macrophages

Lungs were rinsed with sterile PBS through the tracheal cannula with ten aliquots of 10ml sterile PBS. Every rinse with a 10ml aliquot was performed within 30 seconds, and then bronchoalveolar lavage fluid was gently aspirated within 30 seconds. Recovered fluid of the first aliquot was collected in a 50ml conical tube and that of the nine remaining aliquots was pooled in other 50ml conical tubes.

The 50ml conical tubes containing bronchoalveolar lavage (BAL) were centrifuged at 1500rpm for 10min. The supernatant of the first aliquot of BAL was stored at -80^0C for further analysis. The cell pellet of the first aliquot was pooled with the cell pellet of the remaining nine aliquots of BAL, then washed twice with PBS and resuspended in RPMI 1640 culture medium supplemented with 100U/ml penicillin, 100µg/ml streptomycin and 10% fetal bovine serum (Gibco-BRL, Eggenstein, Germany). Total number of cells was assessed with a haemocytometer (Coulter, Krefeld, Germany). After staining with May-Gruenwald-Giemsa, cellular populations were identified by using air-dried cytocentrifuge smears (500rpm x 5min) and the fraction of mononuclear cells was determined by morphologic criteria. Cell viability was determined by trypan blue method (1% trypan blue solution, Fluka, Neu Ulm, Germany). Ten micro litres of trypan blue solution was added to 20µl of cell suspension, and then the number of unstained cells was counted under a microscope. Viable cells do not take up the trypan blue stain. The percentage of viable cells was calculated by dividing the number of unstained cells by the total number of cells, multiplied by 100. Cells were plated in 24-well polystyrene plates (Nuclon Delta Surface, Nunc, Roskilde, Denmark) at 0.2×10^6 vital cells/well. After cultivation for 2h (37°C, 5% CO_2, 21% O_2), cells were washed and non-adherent cells were removed. Adherent cells were regarded as alveolar

macrophages. Alveolar macrophages were cultured with 0.5ml of the aforementioned culture-medium for 24h except for alveolar macrophages of two wells stimulated with LPS (5µl/well; 10µg/ml) just prior to the culture.

2.7. Processing of Plasma Samples

The blood samples were centrifuged at 5000rpm for 5min. The resulting plasma was collected and frozen at -80^{0}C until further analysis.

2.8. Nitrite Assay

After 24 hours of the culture of alveolar macrophages, culture supernatant was collected and centrifuged at 2000rpm for 10min. Nitric oxide release from alveolar macrophages was determined by evaluating the accumulation of their oxidation product nitrite in cell-free supernatants with the Griess reaction [16]. Fifty micro litres of the supernatant were mixed with 50µl of Griess reagent (Merck, Darmstadt, Germany) (1% sulphanilamide, 0.1% N-1-naphtylethylenediamine, and 2.5% phosphoric acid). After ten-minute incubation at room temperature, the absorbance of the reaction product was measured at 550nm with a micro plate reader (Salzburger Labortechnik, Salzburg, Austria). The nitrite concentration in culture supernatants was determined by comparison with a nitrite standard curve.

2.9. Enzyme Linked Immunosorbent Assay

Concentrations of TNF-α, IL-1ß, IL-6, IFN-γ, and IL-10 in BALF and plasma were determined by enzyme linked immunosorbent assay (ELISA; R&D Systems, Wiesbaden, Germany). These assays were implemented by the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody (capture antibody) specific for a determined cytokine was pre-coated onto a micro plate. Standards, controls, and samples were pipetted into the wells and any determined cytokine present was bound by

the immobilized antibody. After washing, any unbound substances were removed. An enzyme-linked polyclonal antibody (detection antibody) specific for each cytokine was added to the wells. After incubation and washing substrate solution was added to the wells. The enzyme reaction yielded a blue product that turned yellow when Stop solution was added. Colour intensity was measured in proportion to the amount of cytokine bound in the initial step. Cytokine concentration in pg/ml was then determined on the basis of the standard curve.

2.10. Statistics

Data were expressed as median (semi-interquartile range, semi-IQR). Statistical analysis was performed with the SigmaStat® software package (version 2.0, SPSS-Jandel Scientific, Erkrath, Germany).

Haemodynamic and arterial blood gas parameters at time points Mea1 and Mea5 within each group were compared by the Signed Rank Test.

Haemodynamic and arterial blood gas parameters of groups receiving IL-10 aerosol were compared to those of the *LPS-only* group at the time points Mea1 and Mea5, respectively, by the multisample test – the Rank Sum Test with Bonferroni Correction.

Similarly, nitric oxide release from cultured alveolar macrophages, total numbers of alveolar macrophages and the number of vital alveolar macrophages in BAL, cytokine concentrations in BALF and in plasma of groups receiving IL-10 aerosol were compared to those of the *LPS-only* group by the multisample test - the Rank Sum Test with Bonferroni Correction.

Differences in the aforementioned parameters compared by the multisample test- the Rank Sum Test with Bonferroni Correction and by the Signed Rank Test were considered significant at $p < 0.0125$ and $p < 0.05$, respectively.

3. RESULTS

3.1. Haemodynamics

Haemodynamic parameters in this study including mean arterial blood pressure (MAP) and heart rate (HR) were measured after preparation, then hourly until termination of experimental protocol after 4h at induction of experimental endotoxemia or saline injection. Results of haemodynamic parameters are shown in Figure 5 and 6.

There were no statistically significant differences in mean arterial blood pressure and heart rate when groups receiving IL-10 aerosol were compared to the *LPS-only* group at Mea1 and Mea5, respectively. Similarly, there were no statistically significant differences in the aforementioned parameters at Mea1 versus Mea5 within each group.

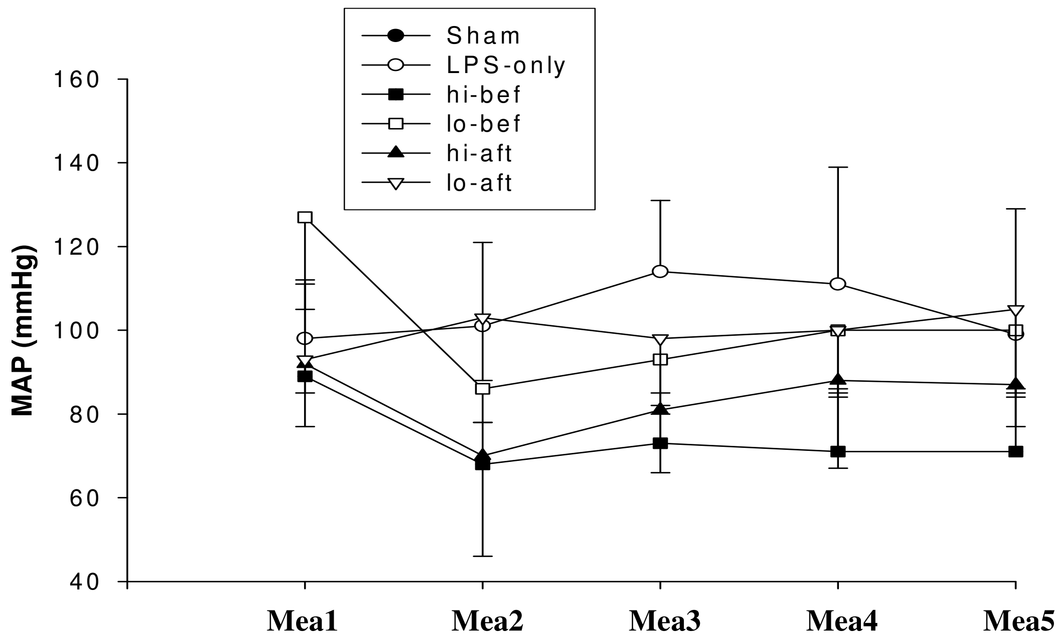

Figure 5. MAP at the time points after preparation and then hourly until termination of the experimental protocol after 4h of endotoxemia or saline injection. Data as median (semi-IQR).

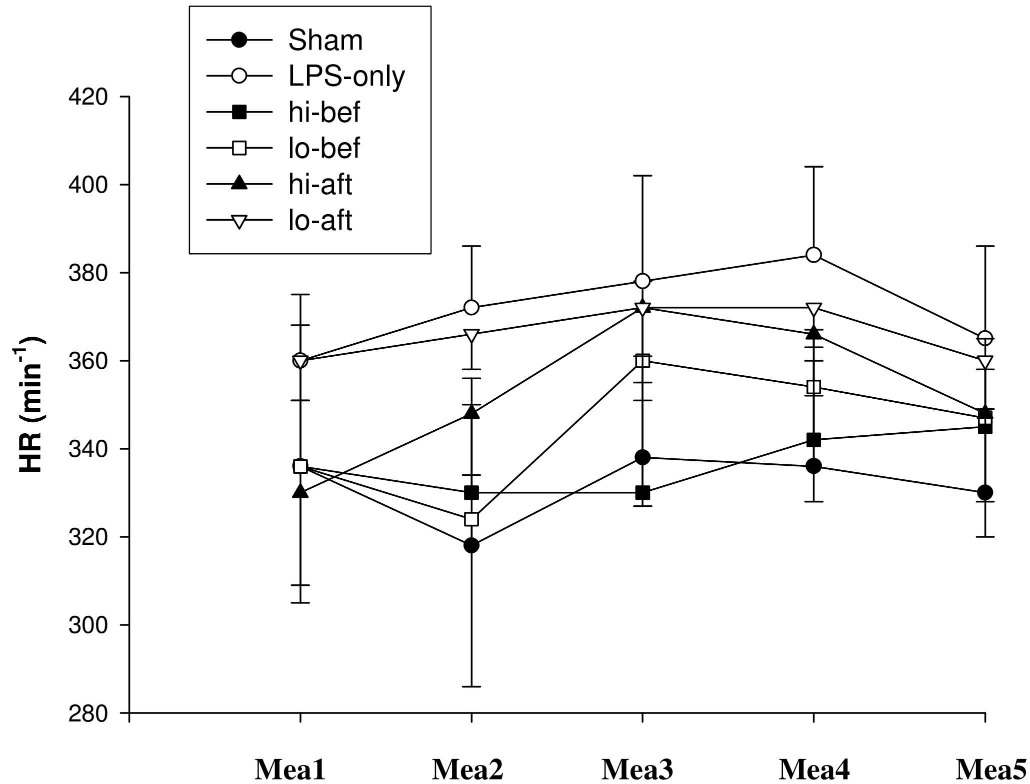

Figure 6. HR at the time points after preparation and then hourly until termination of the experimental protocol after 4h of endotoxemia or saline injection. Data as median (semi-IQR).

3.2. Arterial Blood Gas Analysis

Results of arterial blood gas analysis are shown in Figures 7 to 11. There were no statistically significant differences in PaO_2, $PaCO_2$, pH, HCO_3^- concentration, and BE when groups receiving IL-10 aerosol were compared to the *LPS-only* group at Mea1 and Mea5, respectively. Similarly, there were no statistically significant differences in the aforementioned parameters at Mea1 versus Mea5 within each group.

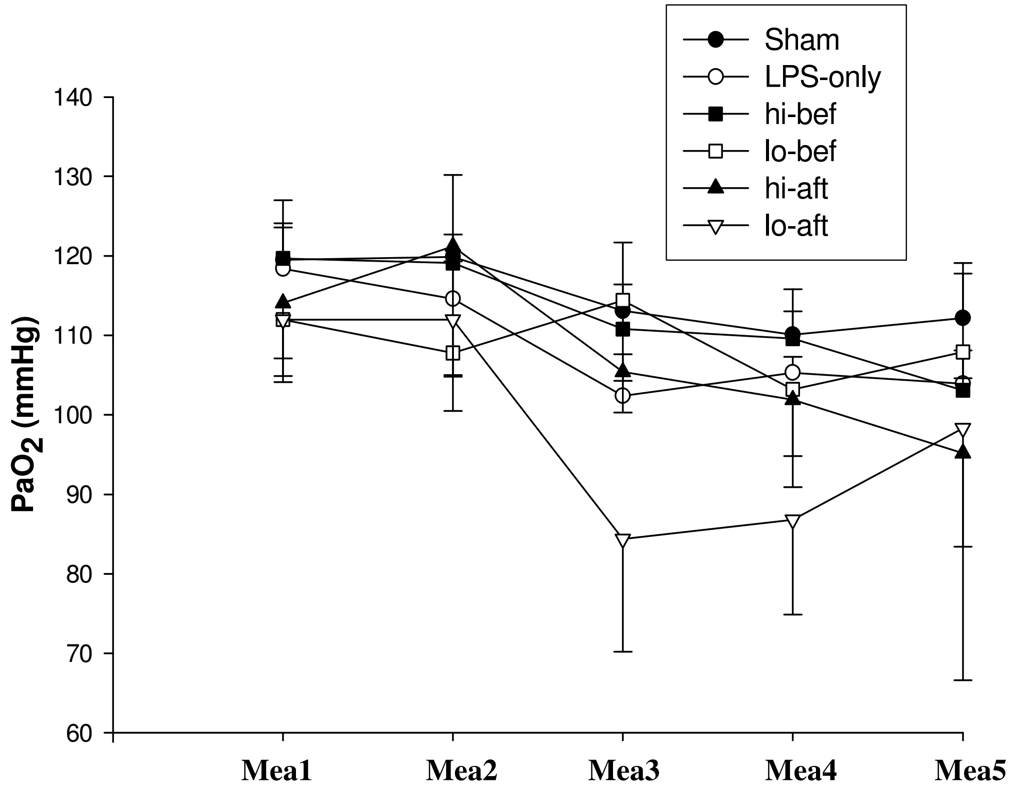

Figure 7. PaO_2 at the time points after preparation and then hourly until termination of the experimental protocol after 4h of endotoxemia or saline injection. Data as median (semi-IQR).

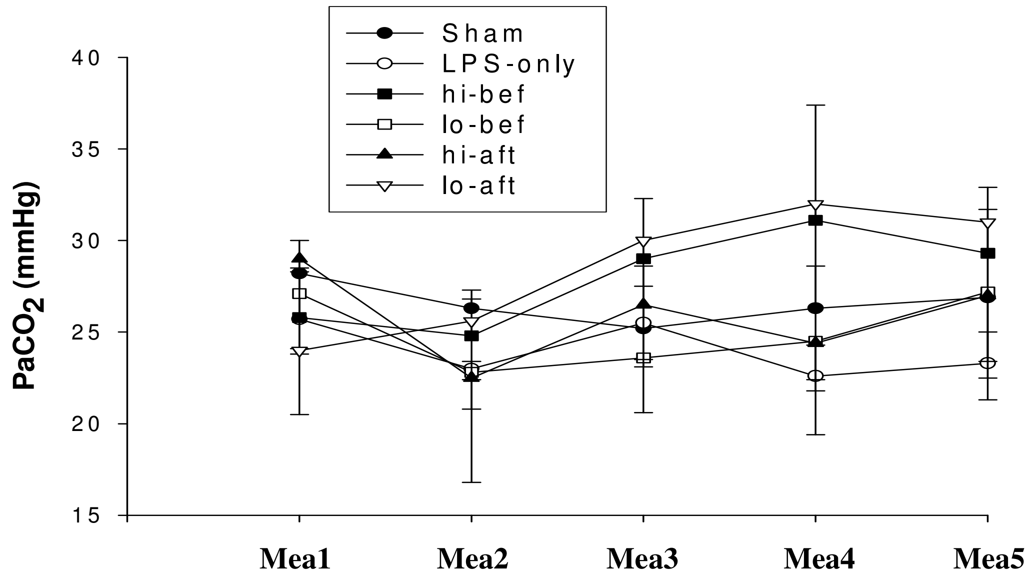

Figure 8. $PaCO_2$ at the time points after preparation and then hourly until termination of the experimental protocol after 4h of endotoxemia or saline injection. Data as median (semi-IQR).

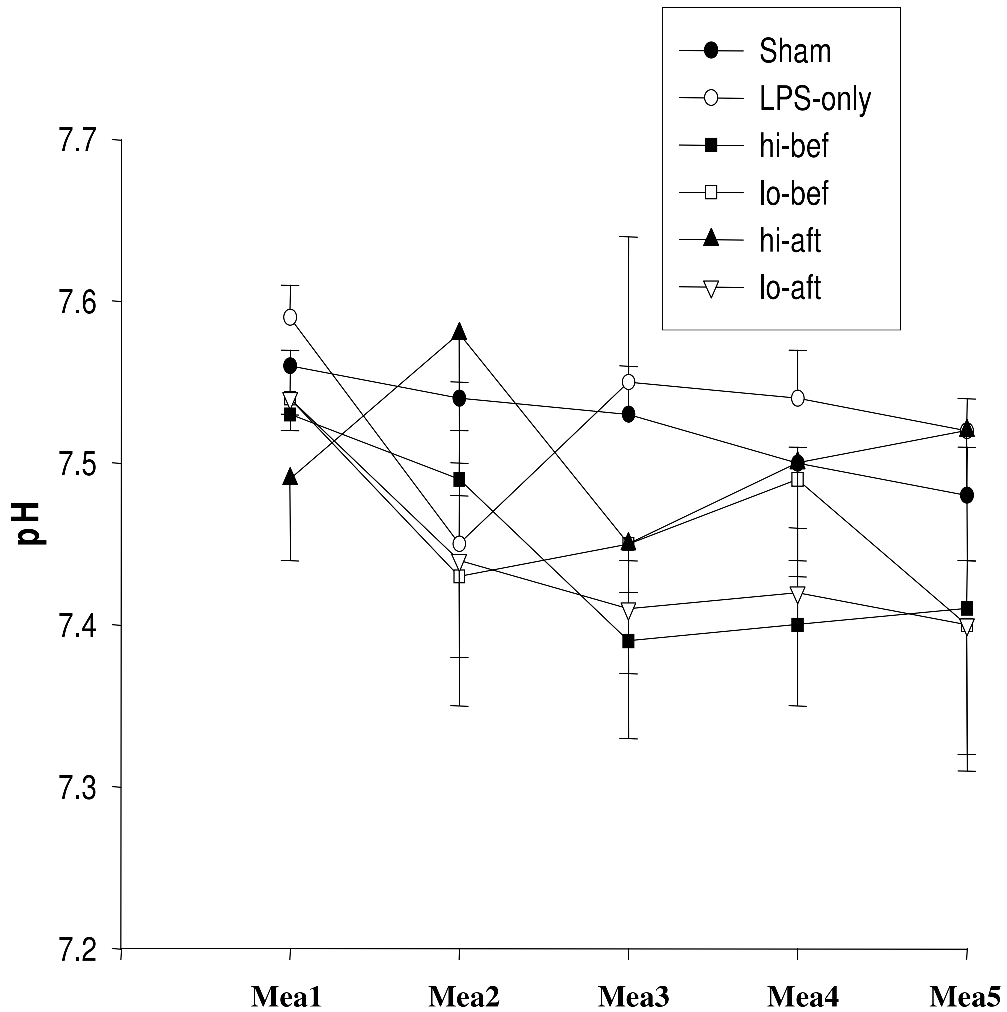

Figure 9. pH at the time points after preparation and then hourly until termination of the experimental protocol after 4h of endotoxemia or saline injection. Data as median (semi-IQR).

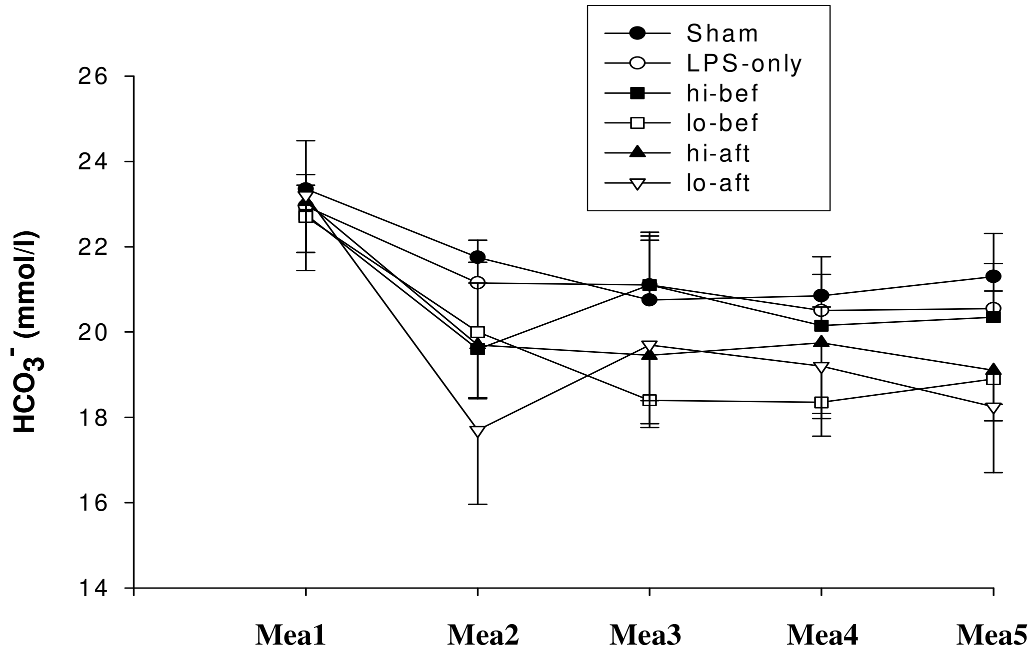

Figure 10. HCO_3^- concentration at the time points after preparation and then hourly until termination of the experimental protocol after 4h of endotoxemia or saline injection. Data as median (semi-IQR).

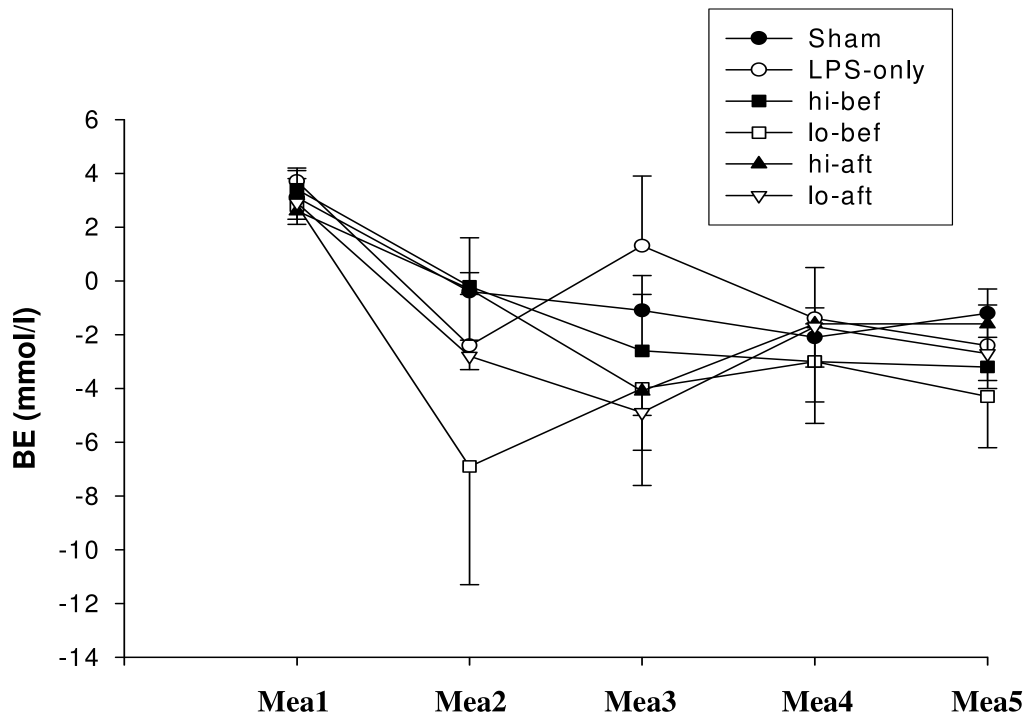

Figure 11. BE at the time points after preparation and then hourly until termination of the experimental protocol after 4h of endotoxemia or saline injection. Data as median (semi-IQR).

3.3. Alveolar Macrophages and Nitrite Release from cultured Alveolar Macrophages

Total number of alveolar macrophages (AMs) and the number of vital AMs in bronchoalveolar lavage (BAL) are presented in table 1.

When compared to the *LPS-only* group, total number of AMs in BAL of the *hi-bef* group was significantly increased by 88% (2.6(0.6)x10^6/ml vs. 4.9(0.9)x10^6/ml) ($p< 0.0125$) and the number of vital AMs in BAL of the *hi-bef* group was significantly increased by 92% (2.5(0.6)x10^6/ml vs. 4.8(0.9)x10^6/ml) ($p< 0.0125$).

There were no statistically significant differences in total number of AMs and the number of vital AMs in BAL, when the *lo-bef*, the *hi-aft*, and the *lo-aft* groups were compared to the *LPS-only* group.

Table 1. Characteristics of alveolar macrophages in the bronchoalveolar lavage.

	Sham group	*LPS-only* group	*hi-bef* group	*lo-bef* group	*hi-aft* group	*lo-aft* group
Total AMs in BAL (x10^6/ml)	3.9 (0.8)	2.6 (0.6)	4.9 (0.9)*	2.9 (0.2)	3.0 (1.0)	2.8 (0.9)
Vital AMs in BAL (x10^6/ml)	3.8 (0.8)	2.5 (0.6)	4.8 (0.9)*	2.8 (0.2)	2.8 (0.9)	2.7 (0.9)

Data as median (semi-IQR); * *hi-bef* group vs. *LPS-only* group ($p< 0.0125$).

Spontaneous release of nitrite from ex-vivo cultured alveolar macrophages within 24h is displayed in Figure 12. When compared to the *LPS-only* group, nitrite release of the *hi-*

bef group was significantly reduced by 69.3% (17.6(18.7)nmol/24h vs. 5.4(4.6)nmol/24h) (p< 0.0125). There were no statistically significant differences in spontaneous release of nitrite, when the *lo-bef*, the *hi-aft*, and the *lo-aft* groups were compared to the *LPS-only* group.

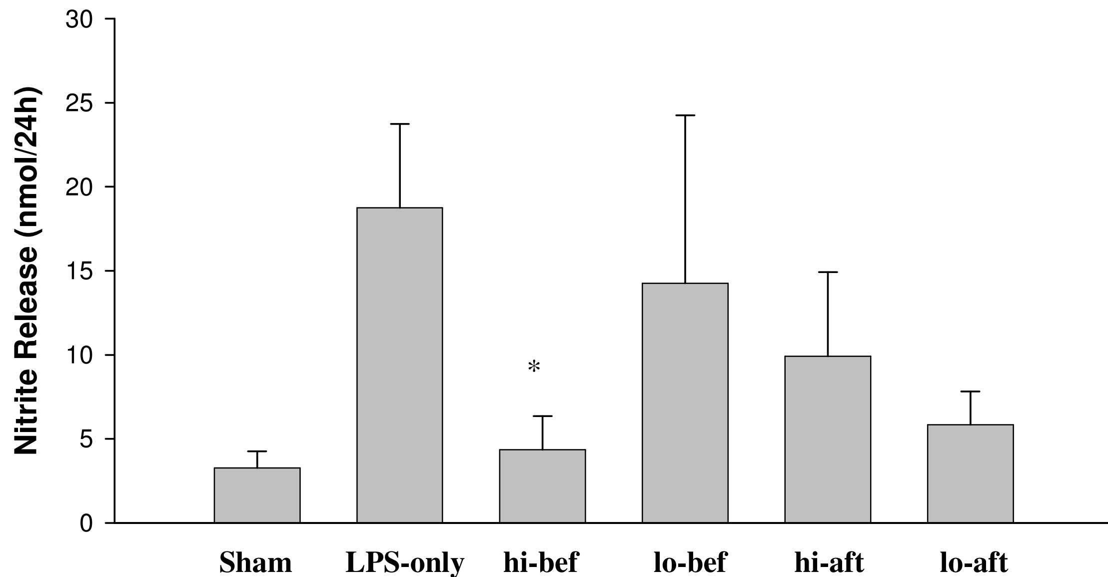

Figure 12. Spontaneous release of nitrite from ex-vivo cultured alveolar macrophages within 24h. Data as median (semi-IQR); * *hi-bef* group vs. *LPS-only* group (p< 0.0125).

3.4. Cytokine concentrations in BALF

Measured proinflammatory and anti-inflammatory cytokine concentrations in BALF are displayed in Figures 13 to 17.

There were no statistically significant differences in TNF-α, IL-1ß, and IL-6 concentrations in BALF when groups receiving IL-10 aerosol were compared to the *LPS-only* group. However, IFN-γ concentrations in BALF from the *hi-bef* (32(26)pg/ml), the *hi-aft* (75(26)pg/ml), the *lo-bef* (17(27)pg/ml), and the *lo-aft*

(55(54)pg/ml) groups were significantly higher than that from the *LPS-only* group (0(0)pg/ml) ($p < 0.0125$).

In the present study, IL-10 was detectable in all BALF samples except for one sample from the *LPS-only* group. As compared to the *LPS-only* group, IL-10 concentration in BALF from the *hi-bef* group was 2.5 fold higher (15(12)pg/ml vs. 38(26)pg/ml) ($p<0.0125$).

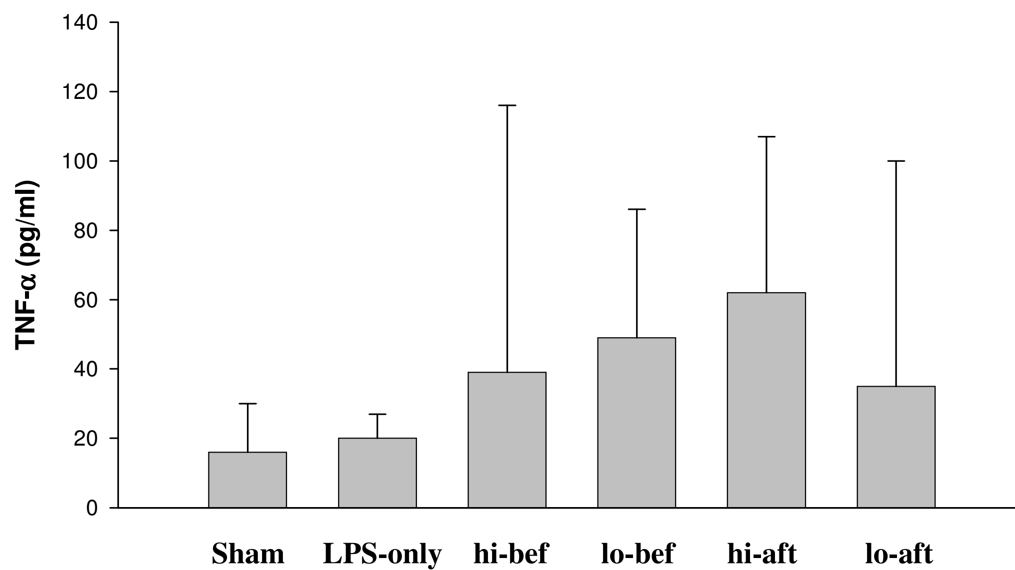

Figure 13. TNF-α concentrations in BALF. Data as median (semi-IQR)

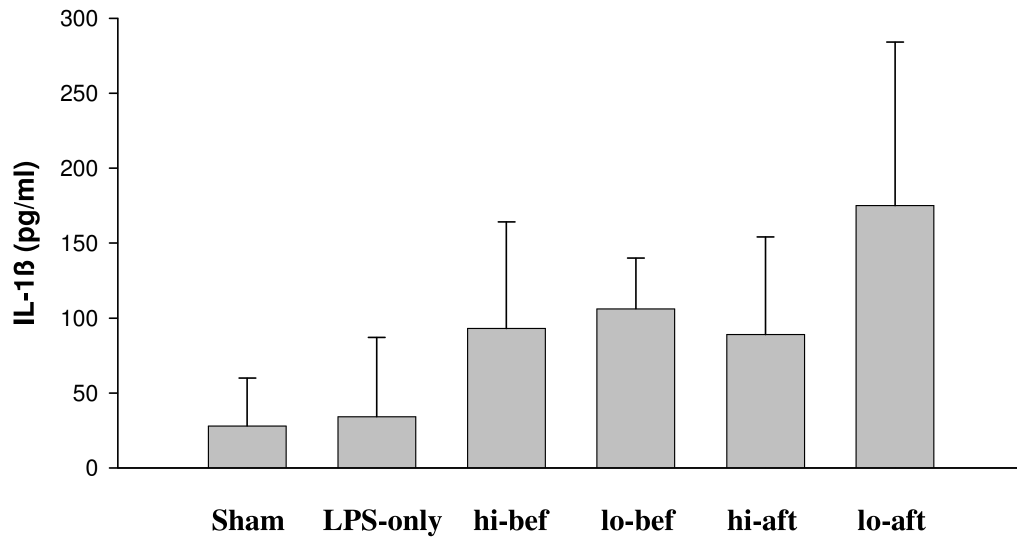

Figure 14. IL-1ß concentrations in BALF. Data as median (semi-IQR).

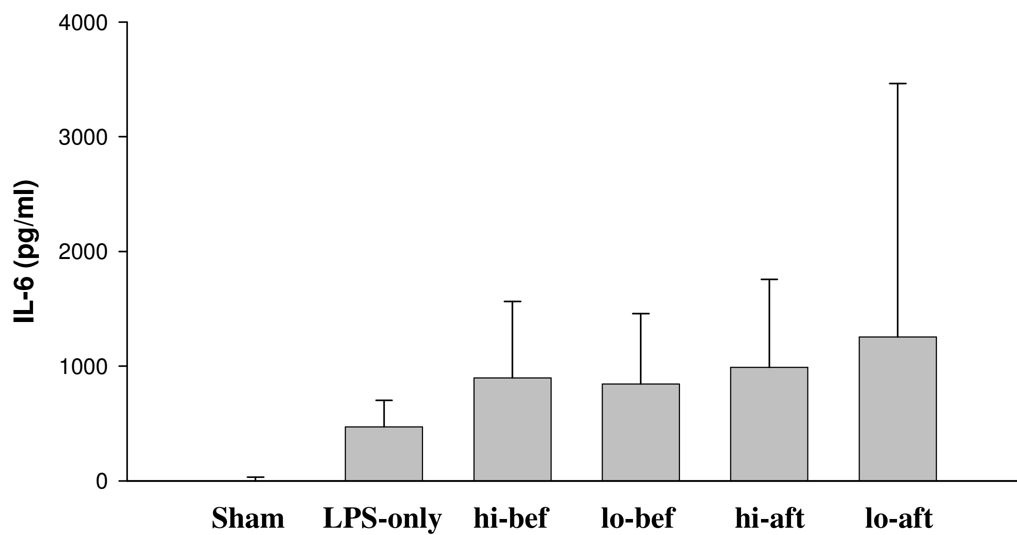

Figure 15. IL-6 concentrations in BALF. Data as median (semi-IQR).

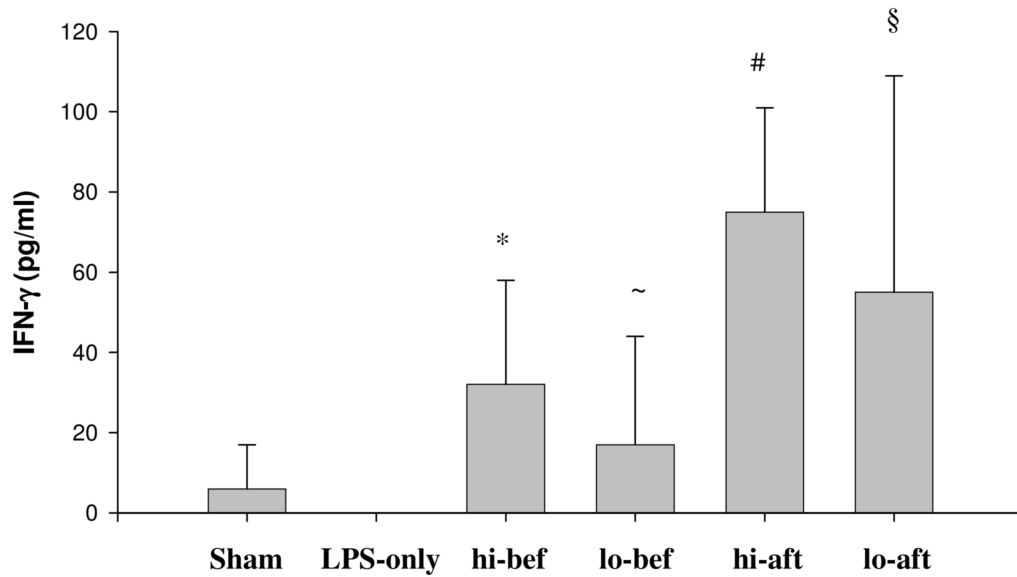

Figure 16. IFN-γ concentrations in BALF. Data as median (semi-IQR). * *hi-bef* group vs. *LPS-only* group; ~ *lo-bef* group vs. *LPS-only* group; # *hi-aft* group vs. *LPS-only* group; § *lo-aft* group vs. *LPS-only* group ($p < 0.0125$).

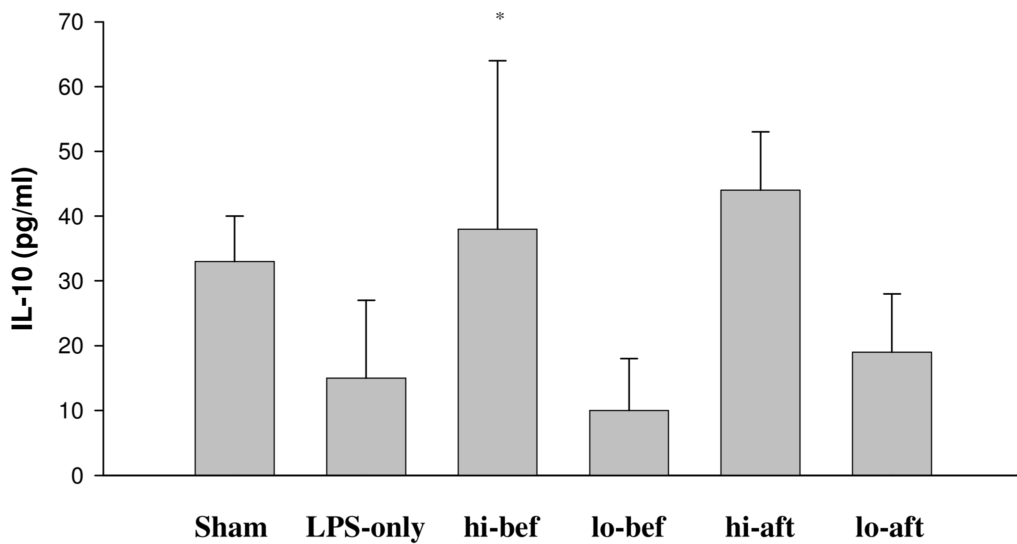

Figure 17. IL-10 concentrations in BALF. Data as median (semi-IQR); * *hi-bef* group vs. *LPS-only* group ($p < 0.0125$).

3.5. Cytokine concentrations in Plasma

Measured proinflammatory and anti-inflammatory cytokine concentrations in plasma are displayed in Figures 18 to 22.

The TNF-α, IL-1ß, and IL-6 concentrations in plasma from the *hi-bef* group were significantly reduced as compared to the *LPS-only* group ($p < 0.0125$). There were no statistically significant differences in the TNF-α, IL-1ß, and IL-6 concentrations in plasma, when the *hi-aft*, the *lo-bef*, and the *lo-aft* groups were compared to the *LPS-only* group.

Specifically, TNF-α concentration in plasma from the *hi-bef* group was significantly reduced by 62.5% as compared to the *LPS-only* group (262(69)pg/ml vs. 700(203)pg/ml) ($p < 0.0125$). IL-1ß concentration in plasma from the *hi-bef* group was significantly reduced by 72.8% as compared to the *LPS-only* group (254(326)pg/ml vs. 936(212)pg/ml) ($p < 0.0125$). IL-6 concentration in plasma from the *hi-bef* group was significantly reduced by 24.8% as compared to the *LPS-only* group (51722(60105)pg/ml vs. 68815(26457)pg/ml) ($p < 0.0125$).

IFN-γ concentrations in plasma from the *hi-bef* (863(529)pg/ml), the *lo-bef* (908(392)pg/ml), and the *lo-aft* (1112(177)pg/ml) groups were significantly reduced by 71.3%, 69.9%, and 63.1%, respectively as compared to the *LPS-only* group (3017(483)pg/ml) ($p < 0.0125$).

IL-10 concentrations in plasma from groups receiving IL-10 aerosol did not reach statistically significant differences as compared to the *LPS-only* group.

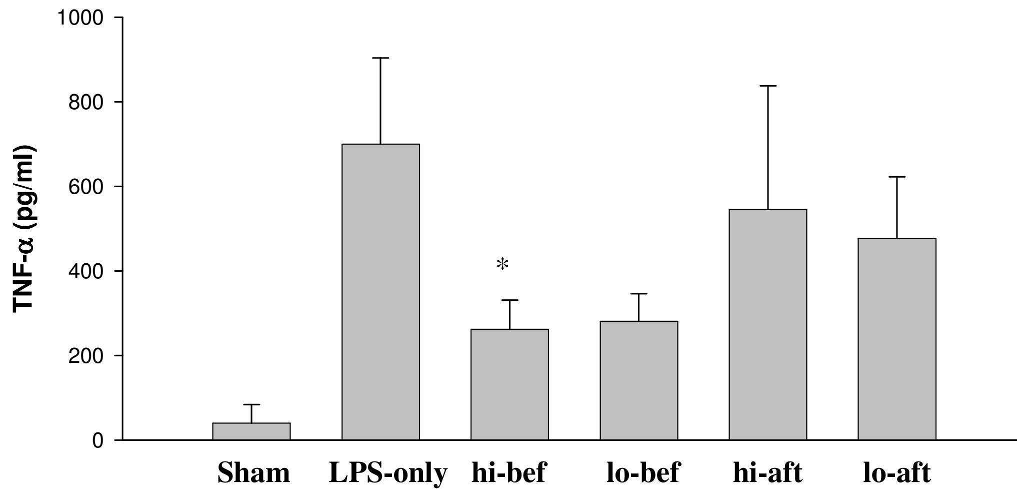

Figure 18. TNF-α concentrations in plasma. Data as median (semi-IQR); * *hi-bef* group vs. *LPS-only* group ($p < 0.0125$).

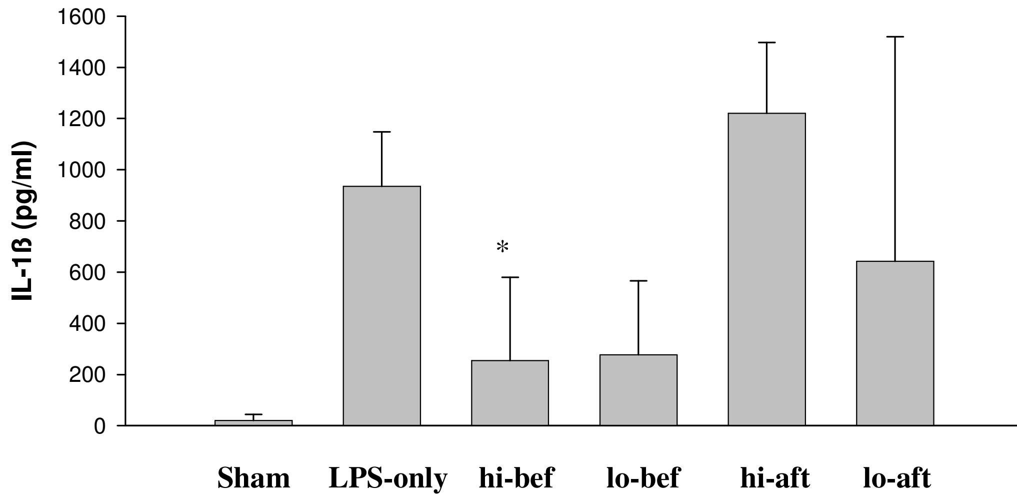

Figure 19. IL-1ß concentrations in plasma. Data as median (semi-IQR); * *hi-bef* group vs. *LPS-only* group ($p < 0.0125$).

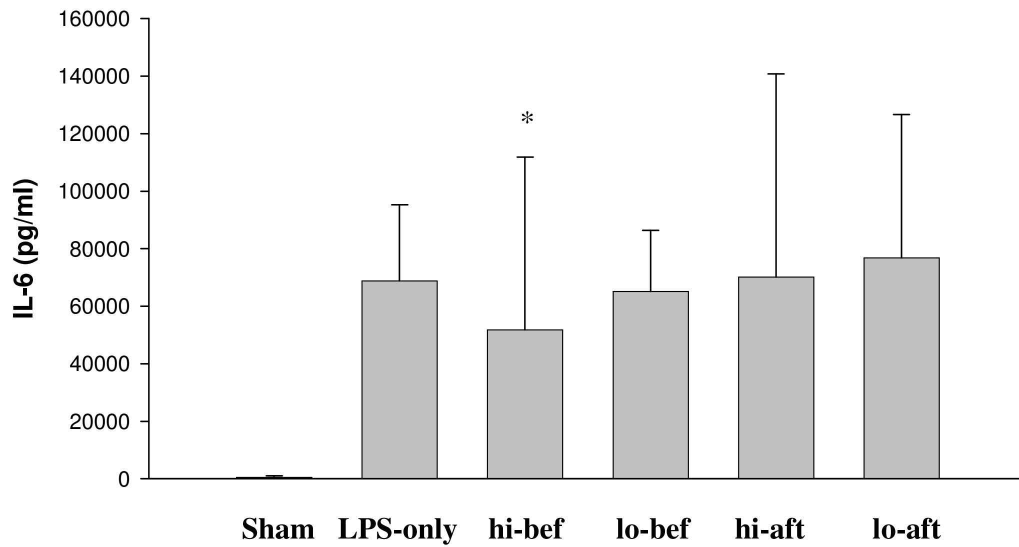

Figure 20. IL-6 concentrations in plasma. Data as median (semi-IQR); * *hi-bef* group vs. *LPS-only* group ($p < 0.0125$).

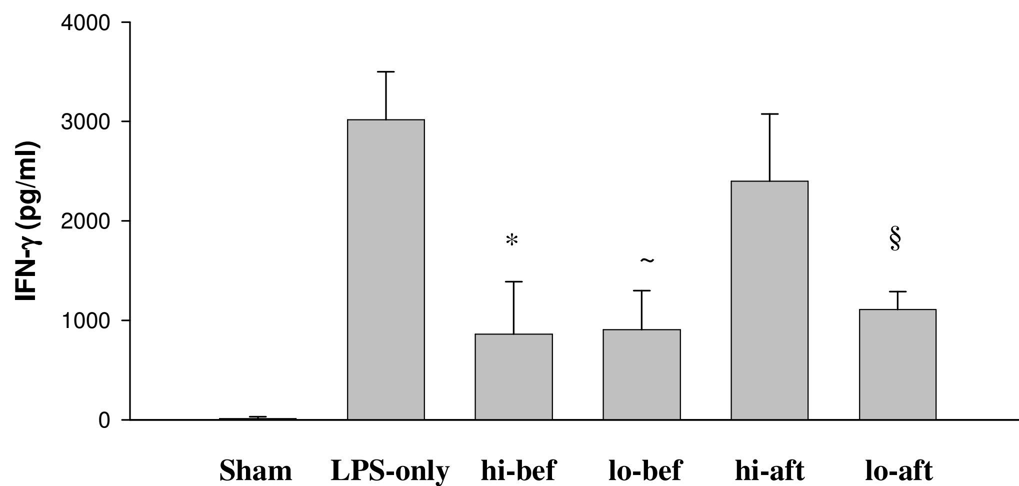

Figure 21. IFN- γ concentrations in plasma. Data as median (semi-IQR); * *hi-bef* group vs. *LPS-only* group; ~ *lo-bef* group vs. *LPS-only* group; § *lo-aft* group vs. *LPS-only* group ($p < 0.0125$).

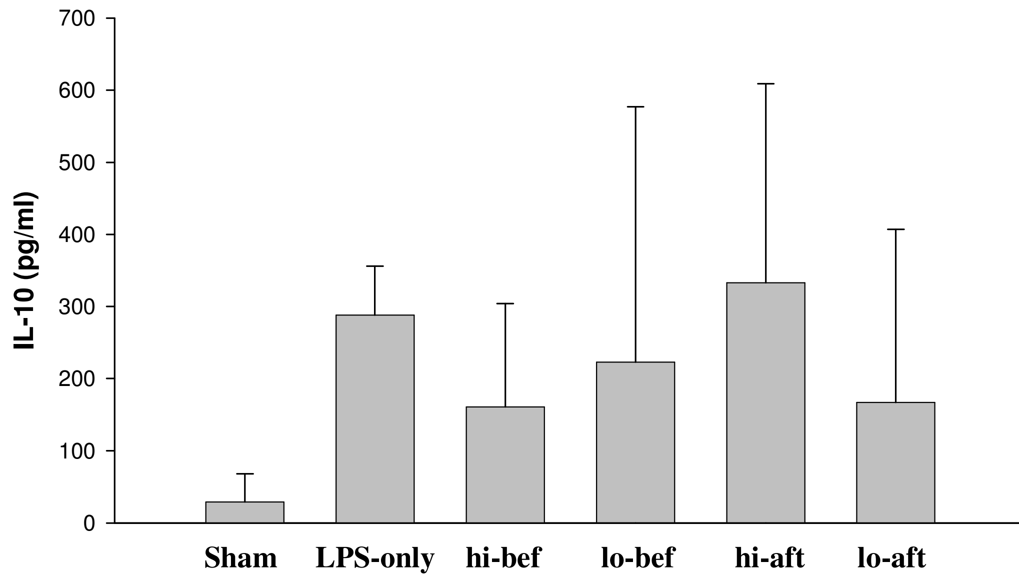

Figure 22. IL-10 concentrations in plasma. Data as median (semi-IQR).

4. DISCUSSION

4.1. Methods

4.1.1. Aerosol Therapy

In pulmonary diseases, direct delivery of drugs is attractive because of reduced dosage and less systemic side-effects. Aerosol production and delivery with specific nebulisers are the adequate and established method.

ARDS patients usually present with acute severe respiratory failure and require endotracheal intubation with mechanical ventilation. Thus, in parallel to that clinical scenario, we performed IL-10 inhalation to mechanically ventilated intubated anesthetized rats.

Actually, two methods are commonly applied to produce aerosols in controlled ventilation: the use of ultrasonic nebulisers and jet nebulisers. However, ultrasonic nebulisers produced a high temperature and highly energetic vibrations during operation. Consequently, the risk of loss of biological activity of proteins such as interferon has been demonstrated [37]. Moreover, the droplet size distribution of ultrasonic nebulisers generally is significantly broader than that of jet nebulisers [61]. Thus, the implementation of a jet nebuliser was more reasonable for aerosol production and delivery from liquid formulation of IL-10 in the present study.

The mass median aerodynamic diameter (MMAD), that denotes the particle size at which half of the total aerosol mass contains larger and half smaller particles, is an important value used for estimating particles' deposition at different anatomic sites within the respiratory tract.

The jet-nebuliser system generated and delivered particles with MMAD of approximately 2.0µm in ventilated intubated rats. Deposition was found in terminal

airways, indicating that aerosol reached the alveolar level [32]. This is consistent with Coleman's report that particles 2-5µm in MMAD were ideal for bronchoalveolar deposition [10].

The effectiveness of aerosol therapy highly depends on the quantity of intrapulmonary drug deposition. Even if aerosolized particles are within a sufficient range to reach terminal airways, only a small percentage of the nebulised dose is effectively deposited. In mechanically ventilated patients, intrapulmonary delivery fractions have been reported to be less than 3% [26] [73]. Our jet-nebuliser system could demonstrate in rats a deposition fraction of 3.8%(1.3%) (median [interquartile range]) of the nebulised dose located in alveoli and small airway thus indicating sufficient drug delivery.

Moreover, the administration of our jet-nebuliser system was safe in ventilated intubated rats by the implementation of the continuously adjustable valve for control and adjustment of peak airway pressure at 1.5kPa during the nebulisation. None of inhaled rats developed detectable acute lung injury (barotrauma and volutrauma), indicated by the absence of alterations in histologically detectable structural tissue damage [32]. This is in line with results from Osier et al. in 1997 and Lizio et al. 2001, who found that peak airway pressure during aerosolisation reaching about 1.5kPa was safe in rats [46] [56].

4.1.2. Experimental Endotoxemia

Intravenous injection of LPS in animals is a commonly applied experimental method to mimic symptoms associated with systemic inflammatory response syndrome, human sepsis, and sepsis-induced ARDS, including pulmonary inflammation [38] [54].

In contrast, intratracheal instillation or inhalation of LPS [19] [44], which are known to also induce acute pulmonary inflammation were not used as a model in the present

study since these approaches do not mimic the most commonly seen pathophysiologic sequences leading to acute lung injury in humans, i.e. the systemic inflammatory response syndrome and sepsis.

4.2. Results

4.2.1. Haemodynamics

Haemodynamic changes were no main parameters of the study because the changes were influenced by fluid and vasopressor therapy to prevent the development of septic shock. Due to consequent therapy of hypotension, no mean arterial pressure lower than 70mmHg was observed. In the present study, we found that the differences in haemodynamic parameters between groups receiving IL-10 aerosol and the *LPS-only* group at the same time point Mea1 or Mea5 have not been statically significant. Our findings were consistent with our previous study, which showed that inhalation of 5µg IL-10 before LPS injection (which corresponds to the *hi-bef* group of the present study) did not attenuate the hypotensive response to endotoxin [31]. There is an evidence that heart rate and mean arterial blood pressure in volunteers who received intravenous injection of IL-10 (25µg/kg) simultaneously with endotoxin (4ng/kg) were not significantly different as compared to volunteers only received intravenous injection of endotoxin [57]. In contrast, Kumar et al. in 2005 reported that heart rate was significantly lower at points between 6.5 and 8h after intravenous injection of LPS (4ng/kg) in the intravenously injected IL-10 group (1, 10, and 25µg/kg) immediately before LPS injection as compared to the placebo group (which corresponds to the *LPS-only* group of the present study) in human endotoxemic model [41]. Difference in heart rate between our result and Kumar's result may be explained by different subjects (rats vs. human volunteers), routes of IL-10 administration (inhalation vs. intravenous

injection), doses of IL-10 administration (5µg or 0.5µg vs. 1, 10, and 25µg/kg), doses of LPS injection (5mg/kg vs. 4ng/kg), and finally, different observation period after LPS injection (4h vs. 24h).

4.2.2. Arterial Blood Gas Analysis

Changes of acid-base balance were no main parameters in the study because if metabolic acidosis occurred, natriumbicarbonat solution in increments was intravenously injected to maintain acid-base homeostasis. Moreover, experimental animals were mechanically ventilated. Hence, results of hourly-performed arterial blood gas analysis were considerably influenced by the ventilatory regime which was adjusted to maintain normocapnia.

In the present study, IL-10 inhalation did not improve hypoxemia and acidosis induced by endotoxemia. Our findings were consistent with our previous study which showed no significant differences in arterial oxygen tension when the *LPS-only* group was compared to the group of inhalation of 5µg IL-10 before LPS injection (which corresponds to the *hi-bef* group of the present study) [31]. Similarly, Knoblach et al. in 1998 demonstrated that the arterial blood gas analysis was not affected by intravenous or subcutaneous injection of IL-10 (100µg) in a model of traumatic brain injury in rats [39].

4.2.3. Alveolar Macrophages and Nitrite Release from cultured Alveolar Macrophages

The main targets of IL-10 are mononuclear cells and tissue macrophages which are a major source of IL-10 production in vivo. Macrophages play a central role during inflammation, as they are also a target for pathogens and of importance for both, the activation of specific and innate immune response. Alveolar macrophages serve a

regulatory role by releasing proinflammatory mediators, which can further increase the intensity of the inflammatory response.

In the present study, LPS injection caused a decrease in the quantity of alveolar macrophage count in the BAL which affirms results from other investigators that showed a decrease in the absolute number of recoverable alveolar macrophages following infusion of endotoxin [9] [40] [65]. In contrast, Li et al. in 1998 found the number of alveolar macrophages only reduced one hour following intratracheal instillation of LPS as compared to baseline in BAL, while thereafter alveolar macrophage count started to rise over time of the ARDS course [44].

In the present study, reduction of the number of alveolar macrophages in BAL after LPS injection was prevented by IL-10 aerosol. The total number and the vital number of alveolar macrophages in BAL from the *hi-bef* group was significantly higher than those from the *LPS-only* group ($p < 0.0125$). This result corresponds with our previous study which showed that the total number of alveolar macrophages in the group of inhalation of 5µg IL-10 before LPS injection (which corresponds to the *hi-bef* group of the present study) was significantly higher than in the *LPS-only* group [31]. In contrast, in a model of immune complex-induced alveolitis, Mulligan et al. in 1993 reported that the number of alveolar macrophages in BAL fell by 65% 4h after IL-10 instilled into the airways [53]. The reason for difference between our present finding and Mulligan's result may be based on different experimental models. While we performed LPS intravenous injection, Mulligan et al. instilled Immunoglobulin (IgA and IgG) into the airway for the induction of lung injury. A further cause may be the different dosage of IL-10. Dosage of IL-10 in Mulligan's experiment (25ng/rat) was lower than that in our present study (0.19µg/rat).

The reason of the significant elevation of the total number and the vital number of alveolar macrophages in BAL from the *hi-bef* group as compared to the *LPS-only* group in our present study has been not explained completely. Bingisser et al. in 1996 found that LPS-induced apoptosis in alveolar macrophages was significantly reduced by the macrophage-deactivating cytokine IL-10 [8]. One may speculate that the LPS-induced apoptosis in alveolar macrophages, which declines after IL-10 administration resulted in significant elevation of the vital number of alveolar macrophages in the BAL in the *hi-bef* group. Fuchs et al. in 1996 reported that there were significant elevations in the number of peripheral blood monocytes (the precursor of alveolar macrophages) in healthy volunteers after intravenous injection of a single bolus of IL-10 (1, 10, and 25µg/kg) [25]. It may be possible that the increased number of peripheral blood monocytes after IL-10 administration leads to a significant elevation of total number of alveolar macrophages in BAL in the *hi-bef* group.

The meaning of the significant elevation of total number and the vital number of alveolar macrophages in BAL from the *hi-bef* group as compared to the *LPS-only* group in the present study has been not completely elucidated. Steinberg et al. in 1994 demonstrated that the number of alveolar macrophages in BAL increased in survivors from ARDS, both in absolute numbers and as a percentage of total cells [67].

Nitric oxide is a ubiquitous messenger molecule that affects various biological functions. The inducible nitric oxide synthase (iNOS) is responsible for the production of large amounts of nitric oxide, an important mediator of inflammation [66]. iNOS is expressed from macrophages, epithelial cells, endothelial cells and regulated at the pretranslational level [74]. LPS or proinflammatory cytokines (like IFN-γ, IL-1ß, and TNF-α) have been shown to induce iNOS expression [16] [69] [75]. Under physical

conditions, nitric oxide is produced in low concentration and participates in cellular metabolism, signal transduction, and cellular protection. In an inflammatory environment, mononuclear cells increasingly release nitric oxide induced by iNOS [40] [44]. Nitric oxide binds with other oxidants (like superoxide anion) generated by inflammatory cells, thus leading to the formation of reactive nitrogen species (like peroxynitrite) that are more reactive and toxic than nitric oxide itself [74]. Reactive nitrogen species exert cytotoxic effects through enzyme inhibition, toxic radical formation, inactivation of metabolic pathways, damage to cell structures as well as proteins, and alteration of gene expression [45]. In septic shock, nitric oxide overproduction is responsible for vasodilatation and cardio depression, which contributes to haemodynamic instability and tissue damage [30]. Therefore, the prevention of excessive formation of nitric oxide in endotoxemia is of therapeutical interest.

Interleukin-10 has been demonstrated to reduce iNOS expression and nitric oxide production by mononuclear cells. Cunha et al. in 1992 reported that maximum inhibition of nitric oxide synthesis by IL-10 was achieved when macrophage cells were preincubated with IL-10 18h before activation with IFN-γ [11]. Thus, IL-10 may contribute to reduce cytotoxic activity of nitric oxide [27].

In the present study, nitrite release was measured in supernatants of ex-vivo cultured cells as an indicator for nitric oxide production resulting from the induction of iNOS of alveolar macrophages. Spontaneous release of nitrite from ex-vivo cultured alveolar macrophages in the *hi-bef* group was significantly lower than that in the *LPS-only* group ($p< 0.0125$). This finding corresponds with our previous study, which showed that inhalation of 5µg IL-10 before LPS injection (which corresponds to the *hi-bef* group of

the present study) suppressed nitrite release from cultured alveolar macrophages by 96% as compared to the *LPS-only* group in a model of endotoxemic rats [31]. In contrast, Perretti et al. in 1995 reported that intravenous injection of IL-10 (1µg) 20min before intraperitoneal injection of LPS (0.3mg/kg) in mice did not cause the reduction in this LPS-stimulated nitric oxide production by peritoneal macrophages ex-vivo [60]. The difference in nitric oxide production between our present study and the study from Perretti et al. may be explained by different routes of LPS exposure (intravenous vs. intraperitoneal injection), doses of LPS (5mg/kg vs. 0.3mg/kg), routes of IL-10 administration (inhalation vs. intravenous injection), time points of IL-10 administration (40min vs. 20min before LPS injection), doses of IL-10 (5µg vs. 1µg), and finally different sources producing nitric oxide (alveolar macrophages vs. peritoneal macrophages).

There is evidence that nitrite levels in BALF may play a certain role for the prognosis in sepsis patients. Sittipunkt et al. in 2001 reported that nitrite levels were significantly elevated in BALF from patients at risk and after the onset of ARDS, but were even more significantly elevated in nonsurvivors than survivors, particularly in ARDS patients after sepsis [66].

Taking into account the reduction of spontaneous nitrite release from ex-vivo cultured alveolar macrophages in the *hi-bef* group as compared to the *LPS-only* group in our present study, it can be reasoned that inhalation of 5µg IL-10 before LPS injection (the *hi-bef* group) appears to be more beneficial than inhalation of IL-10 following LPS injection in the present model.

4.2.4. Cytokine concentrations in BALF

In the present study, we found that there were no statistically significant differences in proinflammatory cytokine TNF-α, IL-1ß, and IL-6 concentrations in BALF between groups receiving IL-10 aerosol and the *LPS-only* group. IFN-γ concentrations in BALF from groups receiving IL-10 aerosol were significantly increased as compared to the *LPS-only* group ($p < 0.0125$). The findings implicate that IL-10 inhalation with any dosage (0.5µg or 5µg) and at any time point (before or after LPS injection) did not act anti-inflammatory within the lungs in the used model. There is evidence that endbronchial administration of IL-10 has been found effective in animal models of acute pulmonary inflammation. Escofier et al. in 1999 reported that intranasal administered IL-10 (0.1µg) inhibited TNF-α level in BALF of rats exposed to LPS-aerosol [19]. In a previous study, our group could demonstrate that inhalation of 5µg IL-10 before LPS injection (which corresponds to the *hi-bef* group of the present study) reduced levels of TNF-α, IL-1ß, IL-6, and IFN-γ in BALF in a comparable model of endotoxemic rats [31]. A reason for the different results between our studies may be explained by the fact that our observation period after LPS injection in the present study was shorter than that in the previous study (4h vs. 6h). Therefore, it may be speculated that we did not detect a significant suppression of proinflammatory cytokine concentrations in BALF from the *hi-bef* group in the present study due to kinetic reasons.

In addition, IL-10 concentration in BALF from the *hi-bef* group was significantly higher than that from the *LPS-only* group ($p < 0.0125$) in the present study. In contrast, in a previous study, we could demonstrate that there was no statistically significant difference in IL-10 level in BALF between the group of inhalation of 5µg IL-10 before LPS injection (which corresponds to the *hi-bef* group of the present study) and the *LPS-*

only group [31]. However, each dosage of IL-10 was completely administered within 40 minutes in both studies, thus different kinetics of deposition of IL-10 within the lung do not seem the presumable cause. In lungs, IL-10 can be mainly formed from alveolar macrophages [72] and as mentioned above, the vital number of alveolar macrophages in BAL from the *hi-bef* group was significantly higher than that from the *LPS-only* group in the present study. We speculate, that this is due to the deposition from nebulised IL-10. Moreover, the meaning of the significant elevation of IL-10 concentration in BALF from the *hi-bef* group as compared to the *LPS-only* group in the present study has been not comprehensively understood. It may be speculated that animals of the *hi-bef* group have better outcome than those of the *LPS-only* group. Donnelly et al. in 1996 reported that low IL-10 concentration in BALF from ARDS patients increased significantly the mortality rate [17].

4.2.5. Cytokine concentrations in Plasma

Experimental endotoxemia resulted in significant elevation of TNF-α, IL-1ß, IL-6, and IFN-γ cytokine concentrations in plasma [31] [41] [57]. However, IL-10 administered before LPS exposure significantly reduced the LPS-induced release of proinflammatory cytokines in plasma [19] [41] [57]. IL-10 is known to inhibit the production and release of proinflammatory cytokines by affecting both mRNA synthesis and degradation [77].

In the present study, we found that only the *hi-bef* group decreased significantly TNF-α, IL-1ß, IL-6, and IFN-γ cytokine concentrations in plasma as compared to the *LPS-only* group ($p < 0.0125$). In contrast, in a previous study, we reported that there was no statistically significant difference in TNF-α concentration in plasma between the group of inhalation of 5µg IL-10 before LPS injection (which corresponds to the *hi-bef* group of the present study) and the *LPS-only* group [31]. Kinetics of TNF-α might be a reason

for different results between our studies. In sepsis, TNF-α, one of the most important early inflammatory response cytokines, was released into plasma during the first 30-90min after LPS exposure [44] and TNF-α level in plasma peaked 90min after LPS challenge [28].

The decline of proinflammatory cytokine concentrations in plasma from the *hi-bef* group implicates that inhaled IL-10 before LPS injection did not act exclusively within the lungs but also in the systemic circulation. A significant part of deposited IL-10 aerosol particles may have passed the alveolar capillary barrier into the circulatory system. It can be hypothesized that the systemic concentration of IL-10 reached a sufficient level following inhalation of 5µg IL-10 before LPS injection to inhibit the release of endogenously synthesized proinflammatory cytokines following LPS injection.

In addition, in the present study, IFN-γ concentrations in plasma from the *lo-bef* and *lo-aft* groups were significantly lower than that from the *LPS-only* group ($p < 0.0125$) which is in line with other reports. Marchant et al. in 1994 found that administration of IL-10 (1000U) 30min before or 3h after LPS administration (100µg) decreased peak IFN-γ serum levels in rats [49]. The influence of IL-10 administration on inflammatory reactions is different and depends on the time point of its administration; in some scenarios the expected immunosuppressive activities are observed, while in others IL-10 enhances immune or inflammatory responses [42] [57]. Lauw et al. in 2000 found that IL-10 intravenously injected 1h after LPS injection not only had no suppression of LPS-induced proinflammatory cytokine release, but also increased significantly IFN-γ level in plasma as compared to the placebo group (which corresponds to the *LPS-only* group of the present study) [42]. The difference in IFN-γ levels in plasma between the *lo-aft*

group in the present study and the group receiving IL-10 after LPS injection in the study from Lauw et al. may be explained by different subjects (rats vs. human volunteers), doses of LPS injection (5mg/kg vs. 4ng/kg), routes of IL-10 administration (inhalation vs. intravenous injection), doses of IL-10 (0.5µg vs. 25µg/kg), and time points of IL-10 administration (just after LPS injection vs. 1h after LPS injection). However, Lauw et al. in 2000 also reported that a stimulation of IFN-γ production was found in whole blood cultured with LPS for 24h and the addition of IL-10 resulted in a strong inhibition of LPS-induced IFN-γ release. The intensity of inhibition was dependent on dose and time points of IL-10 administration [42].

In the present study, LPS injection resulted in an elevation not only of proinflammatory cytokine concentrations but also of IL-10 concentration in plasma (288(68)pg/ml in the *LPS-only* group vs. 29(39)pg/ml in the *Sham* group). Normally, endogenous concentrations of IL-10 in plasma are found below the detectable limit [58]. ARDS and sepsis increase the endogenous IL-10 synthesis [43] [59]. Levels of endogenous IL-10 increased rapidly in endotoxemic rats peaking within 1h after LPS challenge and persisting in plasma for at least 6h [5] [49]. Interleukin-10 may be important for directly suppressing late as well as early events leading to endotoxic shock.

In the present study we found that IL-10 concentration in plasma four hours after LPS injection from groups receiving IL-10 aerosol was not significantly different from the *LPS-only* group. Our finding was conformed to other reports which showed that IL-10 levels in serum did not differ between rats receiving injection of LPS alone as compared to rats receiving simultaneous injection of LPS (12mg/kg) *and* IL-10 (20µg/kg or 40µg/kg) [36] or rats receiving IL-10 inhalation (5µg) prior to LPS injection (5mg/kg) in experimental models of LPS-induced acute lung injury [31]. A reason for that may be

that local binding and absorption of aerosolized cytokines by resident cells may further limit spilling over of aerosolized cytokines into the systemic circulation [12] or accumulation of exogenously administered proteins following aerosolisation to the airway. Another possibility may be that administration of exogenous IL-10 may lead to negative feed back regulation of endogenous IL-10 synthesis.

There is evidence that IL-10 concentration in plasma is directly related to the severity of inflammation and mortality rate in patients with trauma, sepsis, and ARDS [43] [59] [64]. Patients with septic shock presenting high IL-10 levels in plasma had higher organ failure scores than those with low IL-10 levels [22]. Moreover, both the IL-10 level in serum and the ratio of IL-10 to TNF-α were higher in nonsurvivors during severe sepsis [29]. In the present study, IL-10 concentration in plasma from the *hi-bef* group tended to decline as compared to the *LPS-only* group (161(143)pg/ml vs. 288(68)pg/ml). Thus, it may be speculated that the inhalation of 5µg IL-10 before LPS injection improved the severity of inflammation in the endotoxemic model.

Taking into account the decline of proinflammatory cytokine concentrations in plasma in the endotoxemic model in the present study, inhalation of 5µg IL-10 *before* LPS injection may be more beneficial than inhalation of IL-10 during experimental endotoxemia. This would also underline the hypothesis of Berg et al. in 1995 who suggested that IL-10 must act early in order to block the pathogenic effects of LPS [5]. Interleukin-10-mediated protection of mice from lethal endotoxemia was achieved when IL-10 was administered either prior to, or concurrently with LPS or staphylococcal enterotoxin B [4] [33].

The present study did not include a group in which IL-10 was nebulised without a subsequent injection of LPS, which would allow the evaluation of local and systemic effects of nebulised IL-10 itself. This may limit the present study.

4.3. Conclusions

In summary, only the nebulisation of IL-10 at 5µg (1ml, 5µg/ml, corresponding to 0.19µg/rat) before induction of experimental endotoxemia resulted in reduced plasma levels of TNF-α, IL-1ß, IL-6, and IFN-γ as well as reduced nitrite release of cultured alveolar macrophages following 4h of experimental endotoxemia. In contrast, the inhalation of IL-10 at the same dose administered subsequently to the induction of experimental endotoxemia had no effect on proinflammatory plasma cytokines or nitrite release from alveolar macrophages.

The inhalation of IL-10 at 0.5µg (1ml, 0.5µg/ml, corresponding to 0.019µg/rat) before or following induction of experimental endotoxemia did not result in a significant reduction of proinflammatory plasma cytokines or nitrite release from alveolar macrophages.

IL-10 aerosol at either dose or time point had no significant effects on cytokine levels in the bronchoalveolar fluid.

We conclude, that IL-10 has anti-inflammatory effects on alveolar macrophages and plasma cytokines only when it is applied before induction of endotoxemia at 5µg (1ml, 5µg/ml, corresponding to 0.19µg/rat). Effects of IL-10 are not bounded to the pulmonary compartment, they rather appear systemically.

5. SUMMARY

To determine the effects of inhaled IL-10 at different doses and different time points on the pulmonary and systemic inflammatory response during endotoxemia, 48 ventilated, anaesthetized rats (mean body weight ± standard deviation, 500 ± 33g) were randomly assigned to six groups (n = 8, each). Interleukin-10 was nebulised either prior to or following the intravenous injection of LPS (5mg/kg) at two doses (5.0µg or 0.5µg) in four groups. Eight rats received the same insult with no further treatment (*LPS-only* group). Another eight rats served as controls without endotoxemia but with aerosolized phosphate-buffered saline, the solvent of IL-10 (*Sham* group).

Concentrations of TNF-α, IL-1ß, IL-6, and IFN-γ were analyzed in plasma and bronchoalveolar lavage fluid (BALF). In addition, the nitrite release from ex-vivo cultured alveolar macrophages was determined.

As compared to the *LPS-only* group, the concentrations of the proinflammatory cytokines TNF-α, IL-1ß, IL-6, and IFN-γ in plasma were significantly reduced in the group, which inhaled 5µg IL-10 before LPS injection ($p < 0.0125$). Spontaneous nitrite release from ex-vivo cultured alveolar macrophages was suppressed in this group ($p < 0.0125$).

Inhalation of 0.5µg IL-10 before LPS injection and both dosages of IL-10 inhalation (5µg or 0.5µg) after LPS injection did not significantly influence either inflammatory cytokine concentrations in BALF, in plasma or the nitrite release from ex-vivo cultured alveolar macrophages.

In this study, inhaled IL-10 only demonstrated anti-inflammatory effects when it was administered at 5µg prior to the induction of experimental endotoxemia. Interleukin-10 aerosol had no effect when it was given either following induction of endotoxemia or

given at a lower dosage (which here was 0.5µg) either before or following injection of lipopolysaccharide.

ZUSAMMENFASSUNG

Das „Acute Respiratory Distress Syndrome“ (ARDS) ist eine akut auftretende, überwiegend Sepsis-induzierte, inflammatorische Erkrankung der Lunge mit hoher Letalität. Ein komplexes Netzwerk aus Zytokinen und anderen proinflammatorischen Mediatoren unterhält die pulmonale Entzündungreaktion. Dem Zytokin Interleukin-10 (IL-10) könnte in diesem Zusammenhang aufgrund seines spezifisch antiinflammatorischen und immunmodulierenden Wirkspektrums eine therapeutische Bedeutung zukommen. In tierexperimentellen Untersuchungen konnten die protektiven Wirkungen von systemisch appliziertem Interleukin-10 bezüglich verringerter Wirkspiegel proinflammatorischer Mediatoren sowie des Überlebens der Versuchstiere bei Sepsis belegt werden. In einer Untersuchung an ARDS-Erkrankten wiesen Patienten, deren bronchoalveoläre Lavage (BAL) hohe Konzentrationen an Interleukin-10 enthielt, eine signifikant niedrigere Letalität auf als Patienten mit niedriger IL-10 Konzentration.

Die Inhalation von IL-10 über den Luftweg könnte lokal in der Lunge die Freisetzung von Entzündungsmediatoren verringern und so den Verlauf eines ARDS positiv beeinflussen.

Im Rahmen einer bereits durchgeführten Studie der eigenen Arbeitsgruppe konnte gezeigt werden, dass die Inhalation von IL-10 (0.19µg/Tier) vor Induktion einer experimentellen Endotoxinämie (Beobachtungszeitraum 6h) zur signifikanten Reduktion proinflammatorischer Zytokine im Plasma sowie der BAL führte. Daneben bewirkte IL-10 Aerosol eine signifikante Verringerung der Nitritproduktion aus ex vivo kultivierten Alveolarmakrophagen (AM).

Mit der vorliegenden Studie sollte untersucht werden, ob vernebeltes IL-10 auch in einer geringeren Dosierung als in der ersten Studie angewandt antiinflammatorisch wirksam ist. Daneben sollte geklärt werden, ob der Zeitpunkt der Applikation des IL-10 Aerosol relevant ist. Konkret sollte untersucht werden, ob die Inhalation von IL-10 erst *nach* Induktion der experimentellen Endotoxinämie ebenfalls antinflammatorisches Potential besitzt.

Die Generierung und Verneblung des IL-10 Aerosols erfolgte in der vorliegenden Untersuchung mittels eines speziell für diesen Einsatz von der eigenen Arbeitsgruppe entwickelten und charakterisierten Verneblersystems. Der Vernebler produziert stabil Aerosopartikel, deren Größenverteilung (rund 2µm) mit hoher Wahrscheinlichkeit in alvelären Bereichen deponiert. Die alveoläre Depositionsfraktion des Verneblers beträgt rund 4% und liegt damit in einem Bereich, der aus der Humanmedizin für die Verneblung bei intubierten Patienten bekannt ist.

An 48 narkotisierten, kontrolliert beatmeten Ratten wurde die antiinflammatorische Wirkung eines IL-10-Aerosols untersucht. Die Induktion des experimentellen Lungenschadens erfolgte durch intravenöse Injektion von Endotoxin (Lipopolysaccharid; LPS, in einer Dosierung von 5mg/kg). Als löslicher Bestandteil der Membran gram-negativer Bakterien führt LPS über die Freisetzung proinflammatorischer Substanzen zu entzündlichen Reaktionen, die lokal beschränkt oder auch systemisch auftreten können. Bei Versuchstieren unterschiedlicher Spezies bewirkt die systemische Applikation von LPS pulmonale Veränderungen, die denen des septisch bedingten ARDS des Menschen qualitativ entsprechen. Die Tiere wurden zufällig in eine der sechs Versuchsgruppen eingeteilt (je n=8): Die LPS-Gruppe erhielt eine LPS-Injektion (5 mg/kg/KG) ohne anschließende therapeutische Intervention.

Die mit IL-10 behandelten Tiere erhielten das IL-10-Aerosol entweder vor oder nach Induktion der experimentellen Endotoxinämie nach unten genanntem Protokoll. In einer Kontroll (Sham)-Gruppe wurde die Auswirkung von Narkose, chirurgischer Präparation, Beatmung und Aerosolapplikation (IL-10-Trägerlösung; phosphatgepufferte Kochsalzlösung) evaluiert. Neben der in vivo Beobachtung von Hämodynamik (Herzfrequenz, mittlerer arterieller Blutdruck), Lungenmechanik, arteriellen Blutgasen und Blutbild, untersuchten wir anhand von Blutproben und einer Bronchoalveolären Lavage (BAL) die systemische und pulmonale Inflammation (inflammatorische Zytokine TNFα, IL-1ß, IL-6 und IFNγ in Plasma und BAL).

Die Verneblung von IL-10 erfolgte in zwei Dosierungen und zu zwei Zeitpunkten: in einer Gruppe wurde IL-10 in einer Dosierung von 5.0µg (0.19µg/Tier) *vor*, und in einer weiteren Gruppe *nach* Induktion der experimentellen Endotoxinämie vernebelt. In zwei weiteren Versuchgruppen wurde IL-10 in einer Dosierung von 0.5µg (0.019µg/Tier) ebenfalls *vor* sowie *nach* Injektion von LPS vernebelt.

Die Endotoxinämie führte nur zu geringen Verschlechterungen der klinischen Parameter, aber sowohl pulmonal (BAL) als auch systemisch (Plasma) zeigte sich ein Anstieg proinflammatorischer Mediatoren. Gegenüber den Tieren, deren Endotoxinämie unbehandelt blieb, bewirkte nur die Inhalation von IL-10 in der höheren Dosierung (5µg) das signifikante Abfallen der proinflammatorischen Zytokine TNFα, IL-1ß, IL-6 und IFNγ im Plasma sowie der Freisetzung von Nitrit aus kultivierten AM. IL-10 Aerosol hatte in keiner Dosierung respektive zu keinem Applikationszeitpunkt antiinflammatorische Effekte auf die Zytokinkonzentrationen in der BAL.

Die präemptive Vernebelung von IL-10 in einer Dosierung von 5 µg (0.19µg/Tier) *vor* Induktion einer experimentellen Endotoxinämie zeigte sowohl auf die AM Kultur als

auch systemisch (Plasma) antinflammatorisches Wirkprofil. Demgegenüber zeigte IL-10 keine antinflammatorische Effekte, wenn es erst nach Injektion von LPS oder aber in geringerer Dosierung vernebelt wurde.

Zur antiinflammatorischen Therapie der experimentellen Endotoxinämie durch LPS Injektion in der Spezies Ratte erscheint IL-10 Aerosol nur wirksam, wenn es in ausreichender Dosierung (5 µg) sowie vor Injektion von LPS appliziert wird.

REFERENCES

1. Abraham E, Glaser MP, Butler T, Garbing J, Gellman D, Latherer PF, et al. p55 tumour necrosis factor receptor fusion protein in the treatment of patients with severe sepsis and septic shock. A randomized controlled multimember trial [abstract]. *JAMA.* 1997;277:1531-1538

2. Armstrong L, Millar A. Relative production of tumor necrosis factor alpha and interleukin-10 in adult respiratory distress syndrome. *Thorax*. 1997;52:442-446

3. Ashbaugh DG, Bigelow DB, Petty TL, Levine BE. Acute respiratory distress in adults. *Lancet* 1967;2:319-23

4. Bean AGD, Freiberg RA, Andrade S, Menon S, Zlotnik A. Interleukin-10 protects mice against Staphylococcal Enterotoxin B-Induced Lethal Shock. *Infection and Immunity* 1993;61:4937-4939

5. Berg DJ, Kuehn R, Rajewsky K, et al. Interleukin-10 is a central regulator of the response to LPS in murine models of endotoxic shock and the Shwartzman reaction but not endotoxin tolerance. *The American Society for Clinical Investigation* 1995;96:2339-2347

6. Bernard GR, Wheeler AP, Naum CC, et al. A placebo controlled, randomized trial of IL-10 in acute lung injury [abstract]. *Chest* 1999;116:260S

7. Bernard GR, Artigas A, Brigham KL, et al. The American-European Consensus Conference on ARDS: definitions, mechanisms, relevant outcomes, and clinical trial coordination [abstract]. *Am J Respir Crit Care Med.* 1994;149:818-824

8. Bingisser R, Stey C, Weller M, Groscurth P, Russi E, Frei K. Apoptosis in human alveolar macrophages is induced by endotoxin and is modulated by cytokines [abstract]. *Am J Respir Cell Mol Biol* 1996;15:64-70

9. Chang JC, Lesser M. Quantification of leukocytes in bronchoalveolar lavage samples from rats after intravascular injection of endotoxin [abstract]. *Am Rev Respir Dis.* 1984;129:72-75

10. Coleman DM, Kelly HW, McWilliams. Therapeutic aerosol delivery during mechanical ventilation. *The Annals of Pharmacotherapy* 1996;30:644-655

11. Cunha FA, Moncada S, Liew FY. Interleukin-10 inhibits the induction of nitric oxide synthase by interferon-γ in murine macrophages. *Biochemical and Biophysical Research Communications* 1992;182:1155-1159

12. Debs RJ, Fuchs HJ, Philip R, et al. Lung-specific delivery of cytokines induces sustained pulmonary and systemic immunomodulation in rats. *The Journal of Immunology* 1988;140:3482-3488

13. de Waal MR, Abrahams J, Bennett B, Figdor CG, de Vries JE. Interleukin 10 inhibits cytokine synthesis by human monocytes-an autoregulatory role of interleukin 10 produced by monocytes. *J Exp Med.* 1991;174:1209

14. de Waal MR, Haanen J, Spits H, et al. IL-10 and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. *J Exp Med.* 1991;174:915-924

15. Dickensheets HL, Freeman SL, Smith MF, Donnelly RP. Interleukin-10 upregulates tumor necrosis factor receptor type-II (p75) gene expression in endotoxin-stimulated human monocytes. *Blood* 1997;90:4162-4171

16. Ding AH, Nathan CF, Stuehr D. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. *J Immunol.* 1988;141:2407-2412

17. Donnely SC, Strieter RM, Reid PT, et al. The association between mortality rates and decreased concentration of Interleukin-10 and Interleukin-1 receptor antagonist in the lung fluids of patients with the adult respiratory distress syndrome. *Ann Intern Med.* 1996;125:191-196

18. Donnelly SC, Strieter RM, Kunkel SL, et al. Interleukin-8 and development of adult respiratory distress syndrome in at-risk patient groups [abstract]. *Lancet* 1993;341:643-647

19. Escofier N, Boichot E, Germain N, et al. Effects of interleukin-10 and modulators of cyclic AMP formation on endotoxin-induced inflammation in rat lung. *Fundam Clin Pharmacol.* 1999;13:96-101

20. Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A. IL-10 inhibits cytokine production by activated macrophages. *J Immunol.* 1991;147:3815-3822

21. Fiorentino DF, Bond MW, Mosmann TR. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med.* 1989;170:2081-2095

22. Friedman G, Jankowski S, Marchant A, Goldman M, Kahn RJ, Vincent JL. Blood interleukin-10 levels parallel the severity of septic shock [abstract]. *J Crit Care*. 1997;12:183-187

23. Fisher CJ, Jr., Agosti JM, Opal SM, et al. Treatment of septic shock with the tumor necrosis factor receptor:Fc fusion protein. The soluble TNF receptor sepsis study group. *N Engl J Med.* 1996;334:1697-1702

24. Fisher CJ, Jr., Dhainaut JF, Opal SM, et al. Recombinant human interleukin-1 receptor antagonist in the treatment of patients with sepsis syndrome. Results from a randomized, double-blind, placebo-controlled trial. Phase III rhIL-1ra sepsis syndrome study group [abstract]. *JAMA.* 1994;271:1836-1843

25. Fuchs AC, Granowitz EV, Shapiro L, et al. Clinical, hematologic, and immunologic effects of interleukin-10 in humans. *J Clin Immunol.* 1996;16:291-303

26. Fuller HD, Dolovich MB, Posmituck G, Pack WW, Newhouse MT. Pressurized aerosol versus jet aerosol delivery to mechanically ventilated patients. Comparison of dose to the lung [abstract]. *Am Rev Respir Dis.* 1990;141:440-444

27. Gazzinelli RT, Oswald IP, James SL, Sher A. IL-10 inhibits parasite killing and nitrogen oxide production by IFN-gamma-activated macrophages. *J Immunol.*1992;148:1792-1796

28. Ge'rard C, Bruyns C, Marchant A, et al. IL-10 reduces the release of tumor necrosis factor and prevents lethality in experimental endotoxemia. *J Exp Med.* 1993;177:547

29. Gogos CA. Drosou E, Bassaris HP, Skoutelis A. Pro-versus Anti-inflammatory Cytokine Profile in Patients with Severe Sepsis: A Marker for Prognosis and Future Therapeutic Options. *The Journal of Infetious Diseases* 2000;181:176-80

30. Gryglewski RJ, Wolkow PP, Uracz W, Janowska E, Bartus JB, Balbatun O, et al. Protective role of pulmonary nitric oxide in the acute phase of endotoxemia in rats. *Circ Res.* 1998;82:819-827

31. Hofstetter C, Flondor M, Hoegl S, Muhl H, Zwissler B. Interleukin-10 aerosol reduces proinflammatory mediators in bronchoalveolar fluid of endotoxemic rat. *Critical Care Medicine* 2005;33(10):2317-2322

32. Hofstetter C, Flondor M, Thein E, et al. Aerosol delivery during mechanical ventilation to the rat. *Exp Lung Res.* 2004;30:735-651

33. Howard M, Muchamuel T, Andrade S. IL-10 protects mice from lethal endotoxemia . *J Exp Med.* 1993;177:1205-1208

34. Hsu DH, Moore KW, Spits H. Differential effects of IL-4 and IL-10 on IL-2 induced IFN-gamma synthesis and lymphokine-activated killer activity. *Int Immunol.* 1992;4:563-569

35. Hudson LD, Milberg JA, Anardi D, Maunder RJ. Clinical risks for development of the acute respiratory distress syndrome [abstract]. *Am J Respir Crit Care Med.* 1995;151:293-301

36. Inoue G. Effect of IL-10 on experimental LPS-induced acute lung injury. *J Infect Chemother.* 2000;6:51-60

37. Ip A, Arakawa YT, Silvers H, Ransome CM, Niven RW. Stability of recombinant consensus interferon to air-jet and ultrasonic nebulisation [abstract]. *J Pharm Sci.* 1995;84:1210-1214

38. Kabir K, Gelinas JP, Chen M, et al. Characterization of a murine model of endotoxin-induced acute lung injury. *Shock* 2002;17:300-303

39. Knoblach SM and Faden AI. IL-10 improves outcome and alters proinflammatory cytokine expression after experimental traumatic brain injury. *Experimental Neurology* 1998;153:143-151

40. Kobayashi A, Hashimoto S, Kooguci K, et al. Expression of inducible nitric oxide and inflammatory cytokines in alveolar macrophages of ARDS following sepsis. *Chest* 1998;113:1632-1639

41. Kumar A, Zanotti S, Bunnell G, et al. IL-10 blunts the human inflammatory response to lipopolysaccharide without affecting the cardiovascular response. *Crit Care Med.* 2005;33:331-340

42. Lauw FN, Pajkrt D, Hack CE, Kurimoto M, van Deventer SJH, van der Poll T. Proinflammatory effects of IL-10 during human endotoxemia. *The Journal of Immunology* 2000;165:2783-2789

43. Lehmann AK, Halstensen A, Sornes S, Rokke O, Waage A. High levels of interleukin-10 in serum are associated with fatality in meningococcal disease. *Infect Immun.* 1995;63:2109-2112

44. Li XY, Donaldson K, MacNee W. Lipopolysaccharide-induced alveolar epithelial permeability. *Am J Respir Crit Care Med.* 1998;157:1027-1033

45. Liaudet L, Soriano FG, Szabo C. Biology of nitric oxide signalling. *Crit Care Med.* 2000;28:N37-N52

46. Lizio R, Marx D, Nolte T, Lehr CM, Sarlikiotis AW, Borchard G, Jahn W, Klenner T. Development of a new aerosol delivery system for systemic pulmonary delivery in anaesthetized and orotracheal intubated rats. *Lab Anim.* 2001;35:261-270.

47. Lo C-J, Fu M, Cryer HG. Interleukin-10 inhibits alveolar macrophage production of inflammatory mediators involved in adult respiratory distress syndrome. *J Surg Res* 1998;79:179-184

48. Manzano F, Yuste E, Colmenero M, Aranda A, Garcia-Horcajadas A, Rivera R, Fernandez-Mondejar E, Granada Respiratory Failure Study Group. Incidence of acute respiratory distress syndrome and its relation to age [abstract]. *J Crit Care* 2005;20:274-80

49. Marchant A, Bruyns C, Vandenabeele P, et al. Interleukin-10 controls interferon-gamma and tumor necrosis factor production during experimental endotoxemia [abstract]. *Eur J Immunol.* 1994;24:1167-1171

50. Martin TR. Lung cytokines and ARDS. *Chest* 1999;116:2S-8S

51. Meduri GU, Kohler G, Headley S, et al. Inflammatory cytokines in the BAL of patients with ARDS: persistent elevation over time predicts poor outcome. *Chest* 1995;108:1303-1314

52. Meduri GU, Headley S, Kohler G, et al. Persistent elevation of inflammatory cytokines predicts a poor outcome in ARDS. Plasma IL-1 beta and IL-6 levels are consistent and efficient predictors of outcome over time. *Chest* 1995;107:1062-1073

53. Mulligan MS, Jones ML, Vaporciyan AA, et al. Protective effects of IL-4 and IL-10 against immune complex-induced lung injury. *The Journal of Immunology* 1993;151:5666-5674

54. Murakami K, Okajima K, Uchiba M. The prevention of lipopolysaccharide-induced pulmonary vascular injury by pre-treatment with cepharanthine in rats. *Am J Respir Crit Care Med.* 2000;161:57-63

55. O'Garra A, Stapleton G, Dhar V, et al. Production of cytokines by mouse B cells: B lymphomas and normal B cells produce interleukin 10. *Int Immunol.*1990;2:821-832

56. Osier M, Oberdoerster G. Intratracheal inhalation vs. intratracheal instillation: differences in particle effects. *Fundam Appl Toxicol.* 1997;40:220-227

57. Pajkrt D, Camoglio L, Monique CM, et al. Attenuation of proinflammatory response by recombinant human IL-10 in human endotoxemia: effect of timing of recombinant human IL-10 administration. *J Immunol.* 1997;158:3971-3977

58. Park WY, Goodman RB, Steinberg KP, et al. Cytokine balance in the lungs of patients with acute respiratory distress syndrome. *Am J Respir Crit Care Med.* 2001;164:1896-1903

59. Parsons PE, Moss M, Vannice JL, et al. Circulating IL-1ra and IL-10 levels are increased but do not predict the development of acute respiratory distress syndrome in at risk patients [abstract]. *Am J Respir Crit Care Med.* 1997;155:1469-1473

60. Perretti M, Szabo C, Thiemermann C. Effect of interleukin-4 and interleukin-10 on leukocyte migration and nitrite oxide production in the mouse [abstract]. *Br J Pharmacol.* 1995;116:2251-2257

61. Riedler J, Robertson CF. Effect of tidal volume on the output and particle size distribution of hypertonic saline from an ultrasonic nebuliser [abstract]. *Eur Respir J.* 1994;7:998-1002

62. Rubenfeld GD, Caldwell E, Peabody E, et al. Incidence and outcomes of acute lung injury. *N Engl J Med.* 2005;353:1685-1693

63. Sheng WS, Hu S, Kravitz FH, Peterson PK, Chao CC. Tumor necrosis factor alpha upregulates human microglial cell production of interleukin-10 in vitro. *Clin Diagn Lab Immunol.* 1995;2:604-608

64. Sherry RM, Cue JI, Goddard JK, Parramore JB, DiPiro JT. Interleukin-10 is associated with the development of sepsis in trauma patients. *The Journal of Trauma* 1996;40:613-617

65. Simons RK, Maier RV, Chi EY. Pulmonary effects of continuous endotoxin infusion in the rat. *Circulatory Shock* 1991;33:233-243

66. Sittipunkt C, Steinberg KP, Ruzinski JT, et al. Nitric oxide and nitrotyrosine in the lungs of patients with acute respiratory distress syndrome. *Am J Respir Crit Care Med.* 2001;163:503-510

67. Steinberg KP, Milberg JA, Martin TR, et al. Evolution of bronchoalveolar cell population in the adult respiratory distress syndrome [abstract]. *Am J Respir Crit Care Med.* 1994;150:113-22

68. Stoeber W, Flachsbarth H. The aerodynamic diameter of aggregates of uniform spheres. *J Colloid Interface Sci.* 1969;29:710-717

69. Stuehr DJ, Marletta MA. Synthesis of nitrite and nitrate in murine macrophage cell lines [abstract]. *Cancer Res.* 1987;47:5590-5594

70. Suter PM, Suter S, Girardin E, et al. High bronchoalveolar levels of tumor necrosis factor and its inhibitors, interleukin-1, interferon, and elastase, in patients with adult respiratory distress syndrome after trauma, shock, or sepsis [abstract]. *Am Rev Respir Dis*.1992;145:1016-1022

71. Thein E, Raab S, Harris AG, Messmer K. Automation of the use of fluorescent microspheres for the determination of blood flow. *Comput Methods Programms Biomed* 2000;61:11-21

72. Thomassen MJ, Divis LT, Fisher CJ. Regulation of human alveolar macrophage inflammatory cytokine production by interleukin 10. *Clinical Immunology and Immunopathology* 1996;80:321-324

73. Thomas SH, O'Doherty MJ, Fidler H, Page CJ, Treacher DF, O'Nunan T. Pulmonary deposition of a nebulised aerosol during mechanical ventilation [abstract]. *Thorax*. 1993;48:154-159

74. van der Vliet A, Cross CE. Oxidants, nitrosants, and the lung. *Am J Med.* 2000;109:398-421

75. Xie Q, Nathan C. The high-output nitric oxide pathway: role and regulation [abstract]. *J Leukoc Biol.* 1994;56:576-582

76. Yssel H, de Waal MR, Roncarolo MG, et al. IL-10 is produced by subsets of human CD4+ T cell clones and peripheral blood T cells [abstract]. *J Immunol.* 1992;149:2378-2384

77. Wang P, Wu P, Siegel MI, Egan RW, Billah MM. IL-10 inhibits transcription of cytokine genes in human peripheral blood mononuclear cells. *The Journal of Immunology* 1994;153:811-816

78. Ware LB and Matthay MA. The acute respiratory distress syndrome. *The New England Journal of Medicin* 2000;342:1334-1349

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LEBENSLAUF

Name und Vorname : NGUYEN NGOC THACH

Anschrift : Nr.406, Ev. Studentenwohnheime, Henriette-Fürth-Str.2

60529 Frankfurt

Staatsangehörigkeit : vietnamesisch

Geschlecht : männlich

Geburtsdatum : 06. August 1970

Geburtsort : Hanoi, Vietnam

Familienzustand : verheiratet

Arbeitsstelle : Abteilung für Anästhesiologie, Intensivmedizin
Hochschule für Medizin Hanoi
Adresse : Khuong Thuong – Str.79, Stadtbezirk Dong Da, Hanoi, Vietnam

E-mail : thachanesthetist@yahoo.com

Urkunden : Arzt f.Allgemeine Medizin - im September 1993
Magister für Medizin – im October 2001

STUDIENVERLAUF

* Hochschule : Hochschule für Medizin Hanoi

Stelle : Hanoi, Vietnam

Fachrichtung : Medizin (Direkstudium - 6 Jahre)

Urkunden : Arzt f.Allgemeine Medizin

Studienzeit : von September 1987 bis September 1993

* Hochschule : Hochschule für Medizin Hanoi

Stelle : Hanoi, Vietnam

Fachrichtung : Anästhesiologie, Intensivmedizin (Direkstudium - 2 Jahre)

Urkunden : Magister für Medizin

Studienzeit : von Oktober 1999 bis September 2001

ARBEITSVERLAUF

Oktober 2004 bis heute: studiert an der Klinik für Anästhesiologie, Intensivmedizin und Schmerztherapie, Klinikum der Johann Wolfgang Goethe – Universität Frankfurt am Main

Oktober 2001 bis September 2004: Hochschullehrer der Abteilung f. Anästhesiologie, Intensivmedizin and der Hochschule für Medizin Hanoi, Vietnam

Oktober 1999 bis September 2001: Student an der Hochschule für Medizin Hanoi, Vietnam, Magister für Medizin mit dem Prädikat „gut“

Oktober 1993 bis September 1999: Hochschullehrer der Abteilung f. Anästhesiologie, Intensivmedizin and der Hochschule für Medizin Hanoi, Vietnam

September 1987 bis September 1993: Student an der Hochschule für Medizin Hanoi, Vietnam, Abschlussurkunde Arzt für Allgemeine Medizin mit dem Prädikat „gut“

Frankfurt am Main, den 24.08.2007

Nguyen Ngoc Thach

EHERENWÖRTLICH ERKLÄRUNG

Ich erkläre hiermit ehrenwörtlich, daß ich die dem Fachbereich Medizin der Johann Wolfgang Goethe-Universität Frankfurt am Main zur Promotionsprüfung eingereichte Dissertation mit dem Titel

"Inhaled Interleukin-10 before and after induction of experimental endotoxemia in the rat. Effects on the inflammatory response."

in der Klinik für Anästhesiologie, Intensivmedizin und Schmerztherapie unter Betreuung und Anleitung von Prof. Dr. med. Bernhard Zwissler mit Unterstützung durch Dr. med. Christian Hofstetter ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe.

Ich habe bisher an keiner in-oder ausländischen Universität ein Gesuch um Zulassung zur Promotion eingereicht noch die vorliegende Arbeit als Dissertation vorgelegt.

Frankfurt am Main, den 24.08.2007

Nguyen Ngoc Thach