Abstract. **Objective and design:** To study whether a treatment with the hematopoietic growth factor GM-CSF restores the attenuated ex-vivo cytokine-producing capacity of macrophages after sublethal hemorrhagic shock.

**Subjects:** Male Sprague-Dawley rats.

**Treatment:** 20 μg/animal of recombinant murine GM-CSF after shock via arterial line.

**Methods:** Hemorrhagic shock was established by pressure-controlled bleeding to a mean arterial pressure of 50 mm Hg for 35–40 min and consecutive resuscitation. 24 h after hemorrhage, lipopolysaccharide (LPS)-induced cytokine production of isolated macrophages derived from different compartments was measured.

**Results:** A significant reduction of LPS-induced TNF-α production was found in whole blood cultures (1.0 ± 0.7 ng/ml after sham vs. 0.23 ± 0.08 ng/ml after shock operation), macrophages derived from spleen (0.88 ± 0.23 ng/ml after sham vs. 0.03 ± 0.1 ng/ml after shock operation), peritoneum (2.2 ± 0.7 ng/ml after sham vs. 0.29 ± 0.4 ng/ml after shock operation) and bronchoalveolar fluid (0.65 ± 0.13 ng/ml after sham vs. 0.003 ± 0.027 ng/ml after shock operation, mean ± S.D.). In cells from animals treated with GM-CSF a significantly enhanced LPS-induced TNF-α production in splenic, alveolar and peritoneal macrophages was found after shock compared to the cells derived from untreated animals (peritoneum: 289 ± 366 ng/ml TNF-α after shock vs. 2066 ± 94 ng/ml TNF-α after shock and GM-CSF; lung: 9 ± 12 ng/ml TNF-α after shock vs. 64 ± 17 ng/ml TNF-α after shock and GM-CSF; spleen: 58 ± 96 ng/ml TNF-α after shock vs. 548 ± 47 ng/ml TNF-α after shock and GM-CSF). Blood cultures collected from rats after hemorrhagic shock did not show a significant increase of TNF-α-production after GM-CSF treatment.

**Conclusion:** Hemorrhagic shock caused a depression of the TNF-α response to LPS which was partly counteracted by treatment with GM-CSF. Therefore, GM-CSF represents a promising approach to normalise trauma- and shock-induced immune dysfunction.

**Key words:** Immuno-suppression – hemorrhage – GM-CSF – endotoxin – TNF-α

Introduction

Severe trauma is commonly associated with hemorrhagic shock that causes alterations of the patients' immune system. Experimental hemorrhagic shock in animals causes an immunological status similar to the one observed in patients directly after multiple injury.

Alterations of the immune response following hemorrhagic shock include formation of pro- and anti-inflammatory mediators. After experimental shock as well as in severely injured patients excessive amounts of circulating cytokines such as Interleukin (IL)-6 and IL-8 have been separated. The excessive mediator liberation is counteracted by a suppression of the cytokine-producing capacity. An almost completely suppressed endotoxin-induced Tumour Necrosis Factor (TNF) α production of blood cells has been described in multiply injured patients as well as after experimental hemorrhagic shock in rats and mice [1–3]. In addition to cytokine-production of monocytes, maturation and antibody production of B cells [4] as well as proliferation of T lymphocytes [5] have been reported to be diminished after hemorrhagic shock. The double-edged role of the inflammatory response after hemorrhagic shock becomes most obvious for TNF-α. On one hand, circulating TNF-α has been detected in experimental hemorrhage [6]. Antibodies against TNF-α were shown to antagonise the lethal effects of endotoxin [7]. They also improve survival after lethal hemorrhagic shock [8]. On the other hand, TNF-α is essential for an adequate immune response to infectious invaders. In contrast to the effect of high amounts of circulating TNF-α causing endothelial cell damage resulting in systemic oedema, hypotension and mul-
tiple organ failure [9], local TNFα secretion is absolutely necessary to cope with infectious diseases. Local effects of TNFα include increased migration of macrophages, lymphocytes and polymorphonuclear leukocytes to the site of infection as well as enhanced release of antibodies, complement and acute phase proteins [10]. Finally, TNFα elimination by disruption of the gene of the TNF p55 receptor in ‘knock-out’-mice prevented the establishment of an adequate immune response e.g. against mycobacterium tuberculosis or Listeria monocytogenes [11, 12].

The hematopoietic growth factor Granulocyte-Macrophage Colony-stimulating Factor (GM-CSF) is a 22 kD glycoprotein which stimulates proliferation and differentiation of myeloid bone marrow progenitor cells. Additionally, it also enhances effector functions of mature neutrophilic granulocytes and monocytes [13]. GM-CSF increases the LPS-induced TNFα production of monocytes without stimulating TNFα secretion alone [14]. Furthermore, the formation of reactive oxygen species, leukotrienes and prostaglandins of granulocytes are enhanced by GM-CSF treatment [15–17]. In addition, GM-CSF can reverse a desensitisation of monocytes induced by preculture with IL-10 and TGFβ or low amounts of endotoxin in vitro [18]. GM-CSF has been shown to successfully increase neutrophil counts after myelosuppressive chemotherapy and in neutropenic new-borns [reviewed in 19]. However, GM-CSF so far only reduced mortality in neonates with sepsis and neutropenia [20]. In adult septic patients GM-CSF improved sepsis-related pulmonary dysfunction without reducing mortality [21]. In vitro treatment of blood samples from severely injured patients with GM-CSF restored the diminished TNFα-producing capacity as well as the downregulation of the HLA-DR expression on monocytes [22]. This suggests that GM-CSF may serve as a potential immuno-stimulating agent for these patients.

In the present study it was investigated whether GM-CSF applied in vivo counteracts the hemorrhagic shock-induced suppression of the cytokine-producing capacity of different macrophage populations.

Material and methods

Hemorrhagic shock

Male Sprague-Dawley rats (Harlan Winkelmann, Borchen, Germany) were housed under standard condition with 12 h day/Night circle and food as well as drinking water ad libitum until the day of experiment. The responsible federal government institutions approved the animal experiments. To induce a hemorrhagic shock rats were anaesthetised by i.m. injection of 120 mg/kg ketamin hydrochloride and 10 mg/kg xylazin after inhalation of ether. After anaesthesia a groin incision was performed, the femoral artery was isolated and a 24 Gauge flexible catheter (Introcan, Braun-Melsungen, Germany) was inserted into the vessel. A hypovolemia was induced by bleeding via the femoral arterial line to a mean arterial pressure (MAP) of 50 ± 5 mm Hg. MAP was measured invasively using the same femoral arterial line. The hypovolemia lasted for 35–40 min. Resuscitation was performed by transfusion of shed blood 1:2 diluted with warm (37°C) Ringer-solution. After reperfusion the catheter was removed, the femoral artery was ligated and the groin incision was closed. Survival was observed for 24 h. Control animals (sham operation) were treated in the same manner without bleeding.

GM-CSF treatment

Endotoxin-free recombinant murine GM-CSF (specific activity 12 × 106 U/mg, PC-Gen S.A. Buenos Aires, Argentina) was given as a bolus injection of in a volume of 200 μl sterile phosphate-buffered saline (PBS) in a concentration of 20 μg per rat (270–300 gram bodyweight) via the arterial line at the end of the shock immediately before the reperfusion period. Control animals received the equivalent amount of PBS. The sham-operated animals treated with GM-CSF received the same amount of GM-CSF 30–40 min after catheter treatment.

Cell preparations and stimulation

24 h after hemorrhagic shock animals were sacrificed by cervical dislocation. Blood was collected via cardiac puncture with a 10 ml heparin-sprinkled syringe. 5–10 ml blood could be extracted. Afterwards, the trachea was cannulated with a flexible catheter after preparation and incision. A bronchoalveolar lavage (BAL) was performed with 2 ml sterile PBS. The recovery was approximately 50%. For preparation of peritoneal macrophages 30 ml ice cold RPMI-Puffer were instilled in the peritoneal cavity after midline skin incision and mini-laparotomy under view and sterile conditions to rule out accidental injury of the intestine. The injected buffer was aspirated from the peritoneum after 2 min with an electrical pipette under sterile conditions. Afterwards, the peritoneal cavity was completely opened and the spleen was removed. Spleen were stored in ice cold RPMI-1640 medium, BAL fluid as well as the recovered peritoneal cells were stored on ice as well, blood was diluted 1:1 with RPMI-1640 medium and stored at room temperature until further incubation or cell preparations.

Cultures of heparinised blood diluted (1:2; vol:vol) with RPMI-1640 medium were set up in 96-well flat-bottom microtiter plates and incubated with or without 500 ng/ml lipopolysaccharide (LPS) from Salmonella friedenau (S. fried., kindly provided by Dr. H. Brade, Borstel, Germany) at 37°C for 20 h before supernatants were collected after centrifugation and stored at −20°C until cytokine determination. Macrophages from spleen, BAL and peritoneum were enriched by means of adherence. First, single spleen cell preparation were generated by passing spleens through a stainless steel mesh. Afterwards, peritoneal-, spleen- and alveolar cell suspensions were dissolved in 5 ml cold PBS-buffer and centrifuged with 2000 rpm for 5 min. After centrifugation, the supernatant was discharged and the cell pellet was re-suspended in 5 ml RPMI-1640 medium. This step was repeated once before cells were re-suspended in RPMI-1640 medium with 10% fetal calf serum. Cell number was adjusted to 1 × 106 cells/ml. The viability of the collected cells was confirmed by trypan-blue staining and revealed always more than 95% vital cells. Cultures of 200,000 cells were set up in flat-bottom microtiter plates and incubated for 3 h to allow macrophages to adhere. After 3 h medium with non adherent cells was removed by vigorous pipetting and cells were fed with RPMI containing 2,5% FCS. Enriched splenic, peritoneal and alveolar macrophages were stimulated as described above for whole blood cultures for 3 h at 37°C before supernatants were removed and stored at −20°C until cytokine detection. Each incubation was carried out in duplicate.

To check viability of the enriched macrophages and to rule out changes in the amount of adherent macrophages caused by the experimental design, a viability test was performed after stimulation and removal of the supernatants. Therefore, cells were incubated with 100 μl RPMI with 1 mg/ml of the tetrazolium salt MTT (Sigma, Deisenhofen, Germany) which is reduced to formazan by the activity of mitochondrial dehydrogenases. After 3 h of incubation and cell lysis with isopropanol formazan formation was measured at 550 nm with a reference wave length of 690 nm.

Rat IL-6 and TNFα ELISA

Rat IL-6 and TNFα were determined in cell cultures supernatants by a commercially available ELISA with specific monoclonal antibodies.