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Inflammation Research

Differential immunostimulating effect of Granulocyte-macrophage colony-stimulating factor (GM-CSF), Granulocyte colony-stimulating factor (G-CSF) and Interferon γ (IFNγ) after severe trauma

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Abstract. *Objective:* Severe trauma leads to an increased vulnerability to bacterial sepsis. In the present study, we compared the immunostimulating potential of granulocyte-colony stimulating-factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and interferon-gamma (IFN-γ).
Design: Prospective clinical experimental study.
Setting: University hospital intensive care unit and research facility.
Patients: 6 Patients with an Injury Severity Score (ISS) of more than 25 points.
Interventions: Heparinized blood samples of severely injured patients and 12 healthy volunteers were incubated *in vitro* with 10 ng/ml GM-CSF, 10 ng/ml G-CSF or 10 ng/ml IFN-γ for 6 h.
Measurements: Flow cytometry: HLA-DR expression on monocytes, B- and T-lymphocytes.
ELISA: LPS-induced TNFα and IL-10 production.
Results: In all patients reduced cytokine production and HLA-DR expression on monocytes was established. After administration of GM-CSF and IFN-γ *in vitro*, the level of HLA-DR expression on monocytes and the *ex vivo* TNFα-synthesis increased while only GM-CSF increased significantly IL-10-liberation after LPS-stimulation. However, only IFN-γ had the capacity to enhance HLA-DR on B- and T-Lymphocytes. G-CSF *in vitro* had no significant effect on the measured parameter.
Conclusions: These data suggest that GM-CSF and IFN-γ may serve to support immune functions in severely injured patients.

Key words: GM-CSF – Interferon-γ – G-CSF – Trauma – Immunoparalysis – Sepsis

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Introduction

After severe trauma many changes are observed which can be associated with an immunosuppressive state, characterized by a combination of pro-inflammatory cytokine production and secretion of suppressive inflammatory mediators such as interleukin 10 (IL-10). An exaggerated production of the anti-inflammatory mediators may lead to an impairment of a broad range of immune functions that appear to be necessary for the control and elimination of bacteria. Antigen-presenting cells, namely monocytes, macrophages and dendritic cells are assumed to be primarily affected by the sequels of a severe trauma.

Beside a down regulation of major histocompatibility complex class (MHC) II molecules [e.g. human leukocyte antigen DR (HLA-DR)], a diminished production of pro-inflammatory cytokines such as TNFα may contribute to an impaired function of the immune response after injury. Antigen presentation via MHC class-II molecules as well as an adequate, but not an overshooting cytokine response are both essential for an effective host response. Since infectious complications, especially due to systemic sepsis remain one of the leading cause of death during intensive care treatment after multiple injury, strategies overriding the reduced HLA-DR expression and cytokine-producing capacity might represent a promising approach to prevent such complications in severely injured patients.

Therefore, several compounds, namely GM-CSF, G-CSF and IFN-γ, have been suggested as possible immunostimulating substances in multiply injured patients to restore immune competence and prevent infectious complications [1, 2, 3]. Especially GM-CSF and IFN-γ are known to induce the production of pro-inflammatory cytokines such as TNFα alone or after secondary stimulation [4, 5, 6], while G-CSF results in a mixed pattern with enhanced pro- and anti-inflammatory mediator expression [7]. Both GM-CSF and

IFN-γ upregulate HLA-DR surface expression, although partly different regulatory mechanisms are postulated [8, 9, 10]. G-CSF, GM-CSF and IFN-γ have been analysed separately in terms of immunomodulatory capacity in *in vitro* approaches or preliminary therapeutic trials. However, the effects of these three compounds have never been compared in a well defined patient collective. In the light of growing interest in immunomodulatory strategies in trauma or sepsis patients we therefore compared the effect of these three substances in immunocompromized trauma patients. In an *in vitro* approach we analysed the cytokine production and monocyte HLA-DR-expression after treatment with G-CSF, GM-CSF or IFN-γ.

Material and methods

Patients

The prospective study include a consecutive series of severely injured patients with more than 25 points on the Injury Severity Score (ISS), aged between 18 and 80 years and primary admission to the surgical intensive care unit within 8 h after accident. Head injuries with an abbreviated injury scale (AIS) >3 points were excluded. ISS and AIS were coded according to the 1998 update of the AIS 90 [11]. In the study period between February and May 2003 42 patients admitted to the shock room of the study hospital were screened during the study period. Six consecutive patients fulfilled the above mentioned criteria. The remaining 36 patients revealed either a lower ISS or presented a predominant severe brain injury or were secondarily transferred to the study centre. Sepsis was defined according to the consensus conference criteria published by the American College of Chest Physicians/Society of Critical Care Medicine [12]. Organ failure was evaluated on a daily basis applying the Sepsis-related Organ Failure assessment (SOFA)-Score. Multiple organ failure (MOF) was defined as SOFA-Score >2 points of two organ systems persisting for more than two days, exclusively of the evaluation of the central nervous system. An ISS >25 points was chosen because incidence of MOF or sepsis in these patients is expected to be over 25 % [13]. Blood samples of healthy donors (n = 12) of the same age and sex distribution were drawn by cubital vein puncture. The study was approved by the ethics committee of the study university hospital.

Blood sampling

Heparinized blood (Sarstedt® Heparin monovettes) was collected on the first day after hospital admission (day 1) and every Monday and Thursday after the accident at 08.00 hours until discharge from the intensive care unit.

Whole Blood stimulation

1 ml of whole blood was diluted 1:1 with 1 ml RPMI 1640 medium containing 100 U/ml penicillin, 100 µg/ml streptomycin, and L-glutamine and incubated in 12-well flat bottom tissue culture plates at 37 °C in an atmosphere of 5 % CO2 in air. Blood cultures were set up as duplicates and incubated with 10 ng/ml recombinant human GM-CSF (Molgramostim; Leucomax®, Boehringer, Mannheim), G-CSF (Filgrastim, Neupogen®, Amgen, München), IFN-γ (Imukin®, Boehringer, Ingelheim) or RPMI medium alone for 6 h. Thereafter, samples were stimulated with 10 ng/ml lipopolysaccharide (from Salmonella friedenau, protein free phenol-extracted, kindly provided by H. Brade, Borstel, Germany) for 14 h before supernatants were collected after centrifugation (800 g for 5 min). Supernatants were stored at –70 °C until cytokine detection by enzyme-linked immunosorbent assay (ELISA).

ELISA

TNFα and IL-10 in the supernatant were quantified using ELISA, (Beckman Coulter Company, Marseille, France). The lower detection limit was 15 pg/ml of recombinant human TNFα and IL-10, respectively.

Flow cytometry

Blood samples were incubated with or without 10 ng/ml GM-CSF, G-CSF and IFN-γ in 5 ml heparin monovettes at 37 °C for 6 h before flow cytometric analysis. Phenotyping of leukocytes was conducted by three or two-colour flow cytometry using whole blood lyses technique and monoclonal antibodies. Antibodies used were: phycoerythrin-conjugated anti-CD14, FITC-coupled anti-CD45, CD19 PE-conjugated mab (Becton Dickinson, Heidelberg, Germany), FITC-coupled anti-HLA-DR and for measurement of GM-CSF receptor levels using (FITC)-conjugated anti-GM-CSF-receptor (all antibodies Becton Dickinson, Heidelberg, Germany). To 20 µl of antibody pairs diluted heparinized blood containing 5,000–10,000 leukocytes/µl was added to a final volume of 300 µl and incubated in the dark for 15 min. The erythrocytes were lysed with lysing solution (Becton Dickinson) and washed with PBS once. Measurement of stained cells was performed with a FACScan (Becton Dickinson). HLA-DR+ CD14+ monocytes were stained by two colours using the mab combination HLA-DR FITC/CD14 PE, HLA-DR+ CD45+ for B-Lymphocytes and HLA-DR+ CD19+ for T-Lymphocytes from Becton Dickinson and with the exception of second antibody handled as previously described [14].These three leukocyte populations were analyzed for HLA-DR expression. Paired isotype controls were run with each samples revealing always less than 2 % unspecific binding. Flow cytometric analysis was conducted using a linear format to measure channel fluorescence intensities as numerals and to calculate mean fluorescence intensity values. The results are expressed as HLA-DR molecules on the cell surface of monocytes, B- and T-lymphocytes given as mean channel fluorescence intensity (MFI).

Statistics

Statistical calculations were performed on personal computer with the use of a standard statistical package (SPSS/PC). When more than two dependent subject groups were analyzed, the Friedman-Test was used. Pair wise comparisons between subjects groups were made with the paired Wilcoxon test. For analysis of independent parameters between each individual group the Mann-Whitney-U-test was used. For multiple comparisons the Bonferroni Correction was used. Values are expressed as mean with standard deviation (S.D.) as indicated in the figure legend. P values <0.05 were considered to indicate significant differences.

Results

Effect of GM-CSF, G-CSF and IFNγ on healthy individuals

Pre-incubation of blood samples of healthy donors (n = 12) for 6 h with 10 ng/ml human recombinant IFNγ and GM-CSF resulted in a significant increase of LPS-induced production of TNFα, while G-CSF even lowered the TNFα synthesis after LPS stimulation (Fig. 1A). LPS-induced IL-10 production was enhanced after preincubation with both GM-CSF and IFNγ, while G-CSF preincubation caused no significant changes (Fig. 1B). The enhancing effect of the GM-CSF and IFNγ on LPS-induced TNFα-synthesis was also present after 3 h preincubation and slightly further increased after 20 h (data not shown). Due to logistical reasons we chose a 6 h

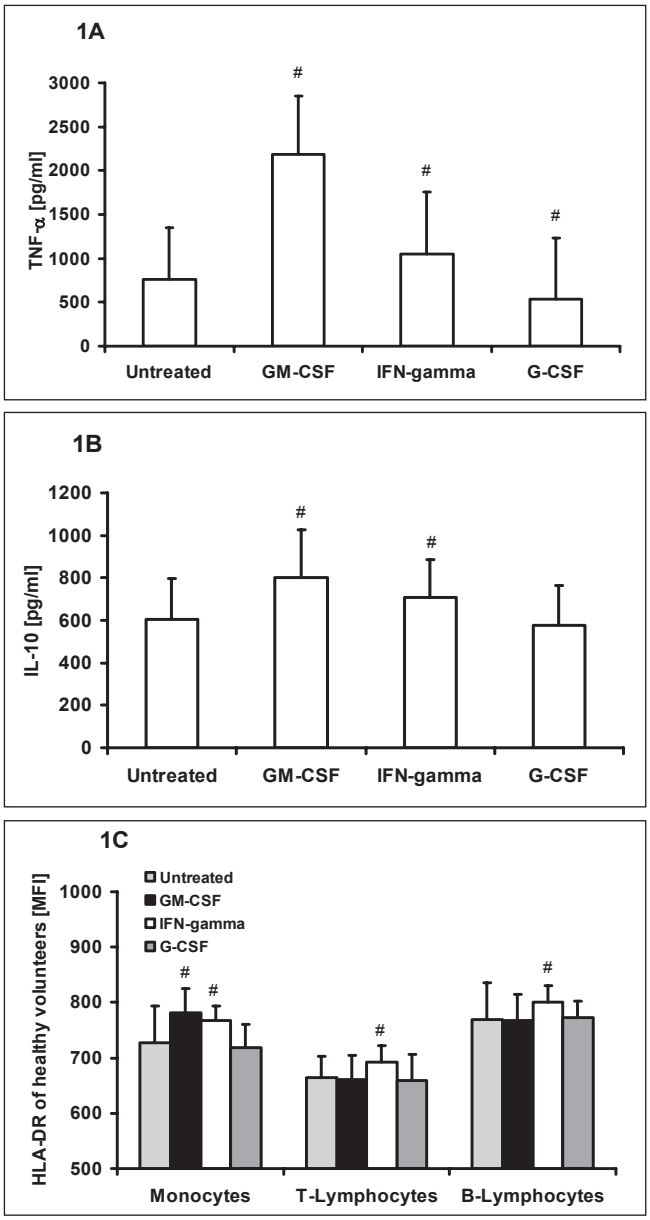

Fig. 1A–C. Effects of 10 ng/ml GM-CSF, 10 ng/ml IFNγ or 10 ng/ml G-CSF in heparinized blood samples of 12 healthy volunteers on LPS induced liberation of TNFα (A) and IL-10 (B) and HLA-DR expression of monocytes, B- and T-Lymphocytes (C). Data are shown as mean ±standard deviation (S.D.). # indicates statistical differences with a $p < 0.05$ compared to untreated control blood samples.

preincubation for all further experiments. HLA-DR expression on the surface of monocytes was also elevated after treatment with both INFγ and GM-CSF. However G-CSF did not influence the expression of this molecule. In contrast, HLA-DR expression on the surface of B-cells and T-cells was only increased by IFNγ and both G-CSF and GM-CSF failed to show any effects (Fig. 1C).

Patients

Six severely injured patients were analyzed during the complete course of the intensive care unit stay. All patients survived despite the high ISS of 37 points in the mean. However, 5 of them developed a sepsis as a complicating diagnosis with a subsequent multiple organ failure. The patients' data are summarized in Table 1.

Table 1. Patients data.

Number of patients	6
Age	35.8 ± 15.3 years
Sex (male/female)	6/0
Outcome (survived/died)	6/0
ISS	36.7 ± 5.9 pts.
AIS brain/neck	0.8 ± 1.1 pts.
AIS face	0.6 ± 0.9 pts.
AIS thorax	3.2 ± 1.9 pts.
AIS abdomen	2.2 ± 2.1 pts.
AIS extremities	3.6 ± 1.1 pts.
AIS general	0.8 ± 1.3 pts.
ICU days	35.3 ± 22.0
Ventilator days	31.3 ± 18.2
Sepsis days	11.8 ± 13.8
MOF days	8.6 ± 15.1
Clinical relevant microbiological findings	1. Serratia liquefaciens, hafnia alvei 2. Pseudomonas aeruginosa, Staph. aureus 3. Staph. aureus, Corynebacterium 4. Pseudomonas aeruginosa 5. No positive microbial finding 6. Staph. aureus

Patients characteristics, clinical data, injury pattern according to the abbreviated injury scale (AIS) and Injury Severity Score (ISS) are expressed as mean ± standard deviation.

TNFα and IL-10 ex vivo synthesis after trauma with GM-CSF, IFNγ and G-CSF

The synthesis of TNFα after LPS stimulation is significantly reduced in the early phase (day 1–2 and day 3/4) after severe injury and then subsequently normalizes until discharge from the ICU. Obviously, cytokine response recovery parallels the other organ functions. Incubation with both GM-CSF and IFNγ increased TNFα-producing capacity to a similar degree at almost all time points until discharge from the ICU (Fig. 2). G-CSF did not show any modulating effect on *ex vivo* TNFα-production except on day 12/13 after trauma, when G-CSF incubation caused a slight but significant reduction of TNFα-producing capacity similar as in healthy volunteers (Fig. 2). Since immunomodulating strategies in trauma patients may primarily aim for a restoration of the immune response before septic complications are present, we analysed the patients' samples that fulfilled the criteria for severe sepsis separately. In samples of these patients we also observed a reduction of LPS-stimulated TNFα-production in comparison to healthy volunteers. In addition samples collected after the diagnosis of a posttraumatic severe sepsis revealed only responsiveness to GM-CSF in terms of elevated TNFα-production, while IFNγ failed to exert immunostimulating effects in these blood samples (see severe sepsis in Table 2). Ex vivo stimulated IL-10 synthesis was not suppressed after

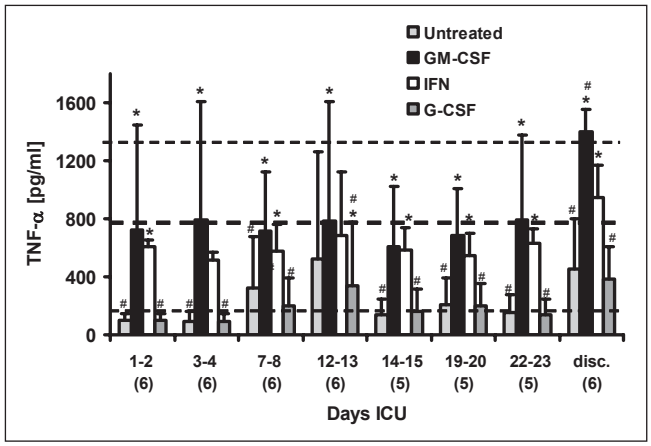

Fig. 2. LPS-induced TNFalpha synthesis in blood samples from six severely injured patients is expressed in pg/ml as mean ±S.D. Day 1 or day 3–5 represent the days after initial injury, recovery includes samples before discharge from ICU. Numbers of patients are shown in parentheses below each group. The horizontal line shows the mean value of control incubations of samples from healthy volunteers with the two dotted lines indicating the standard deviation (n = 12). Number of patients and healthy volunteers are indicated behind each group.* indicates statistical difference between untreated and treated patients samples with GM-CSF, IFN-γ and G-CSF incubation at each specific time point or phase. # indicates statistical difference between patients samples (treated or untreated) and control incubations of healthy volunteers. A p-value <0.05 is considered as significant.

Table 2. Comparison of five severely injured patients on first day of severe sepsis and healthy Volunteers.

		1st day of severe Sepsis	Healthy donors
HLA-DR	No stimulation	564 ± 122*	728 ± 66
	IFNγ	588 ± 108*	781 ± 38
	GM-CSF	606 ± 120*	767 ± 66
	G-CSF	564 ± 110*	719 ± 43
Ex vivo TNFα	No stimulation	451 ± 478*	759 ± 592
	IFNγ	590 ± 467	2181 ± 671
	GM-CSF	960 ± 541	1048 ± 711
	G-CSF	402 ± 452*	535 ± 695

Effects of 10 ng/ml GM-CSF, 10 ng/ml IFNγ or 10 ng/ml G-CSF in heparinized blood samples of 12 healthy volunteers on LPS-induced liberation of TNFα and HLA-DR expression of monocytes from five severely injured patients at the first day of severe sepsis. Data are expressed as mean standard deviation (S.D.) *indicates statistical differences with a $p < 0.05$ compared to the corresponding sample of healthy volunteers without any additional treatment.

severe injury. In contrast, in the later phase after injury (second and third week) we observed a tendency towards elevated IL-10 producing capacity, which however presented a great variance and therefore did not reach level of significance in the small sample size (Fig. 3). Incubation with both IFNγ and GM-CSF enhanced IL-10 production after *ex vivo* LPS stimulation early after trauma (day 1–2), but only GM-CSF also elevated IL-10-production also at later time points (Fig. 3). In contrast, G-CSF preincubation leads to a decreased IL-10 producing capacity in blood samples of trauma patients collected on day 1 or 2 after trauma. The effect, however, was only present in this very early phase after trauma (Fig. 3).

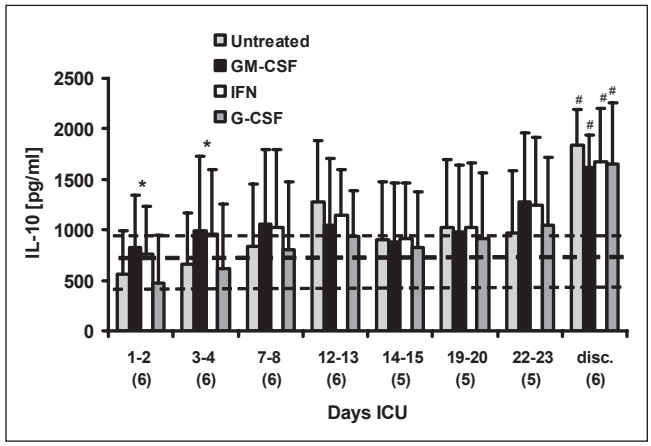

Fig. 3. LPS-induced IL-10 synthesis in blood samples from 6 severely injured patients is expressed in pg/ml as mean ±S.D. with and without preincubation of 10 ng/ml GM-CSF, 10 ng/ml IFN-γ or 10 ng/ml G-CSF. The horizontal line shows the mean values of untreated samples from healthy controls with the two dotted lines indicating the standard deviation. Number of patients and healthy volunteers are indicated below each group.
For statistical symbols and group definitions see Figure 2.

HLA-DR expression on monocytes after trauma with GM-CSF, IFNγ and G-CSF

HLA-DR expression on monocytes was significantly lower after severe injury in comparison to normal individuals during the whole observation period. The decrease of HLA-DR expression was most obvious immediately after trauma and continuously rose until discharge from the ICU, which somehow parallels the course of the *ex vivo* TNFα synthesis. *Ex vivo* treatment with IFNγ and GM-CSF significantly elevated HLA-DR expression at any investigated time (with the exception of Day 22/23 for IFNγ) and also in periods of severe sepsis (Fig. 4, Table 2). In spite of the stimulating effect of GM-CSF and IFNγ the HLA-DR-expression on monocytes stayed below normal values of healthy control after preincubation with these substances. G-CSF applied *in vitro* had no effect on HLA-DR expression at any time point (Fig. 4, Table 2).

HLA-DR expression on B- and T-Lymphocytes after trauma with GM-CSF, IFNγ and G-CSF

HLA-DR expression on the surface of B-lymphocytes was also significantly decreased after severe injury and stayed low for the first week, before the expression began to normalize. Neither GM-CSF nor G-CSF had an effect on HLA-DR expression on B-lymphocytes at any time point (Fig. 5). In contrast, IFNγ caused a small but significant increase of HLA-DR expression on B-lymphocytes. This effect was present at all investigated time points (Fig. 5). The HLA-DR expression on B-lymphocytes of patients with sepsis was significantly decreased, but could be elevated by IFNγ incubation. HLA-DR expression on T lymphocytes was not influenced by severe injury at any time point. The same holds true for blood samples collected after the diagnosis of a severe sepsis, HLA-DR expression on T-lymphocytes

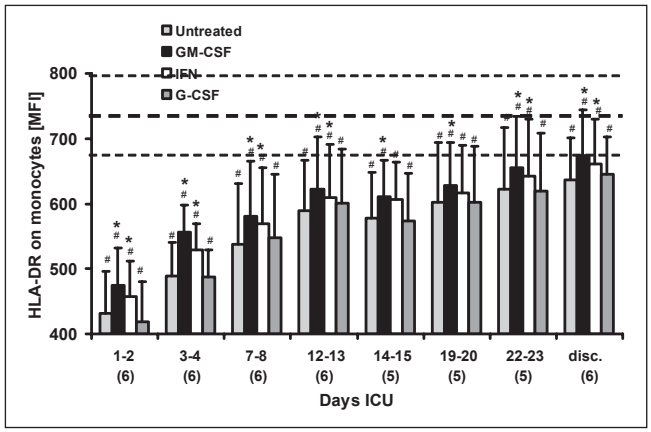

Fig. 4. HLA-DR expression on monocytes of 6 severely injured patients is expressed in MFI as mean ±S.D. with or without preincubation of GM-CSF. The horizontal line shows the mean values of untreated samples of healthy controls with the two dotted lines indicating the standard deviation. Number of patients and healthy volunteers are indicated below each group.
For statistical symbols and group definitions see Figure 2.

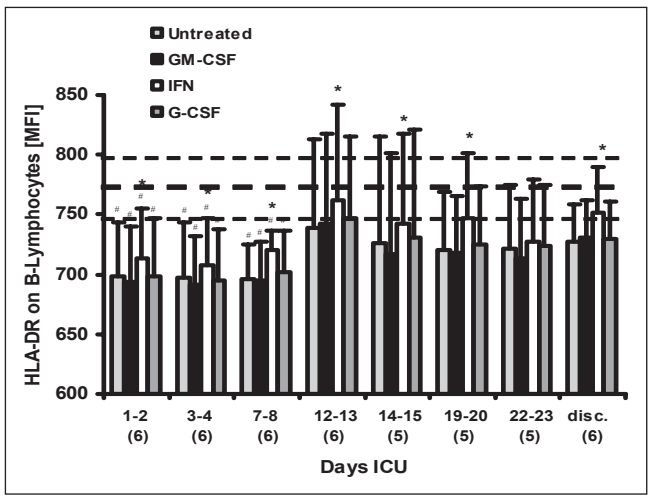

Fig. 5. HLA-DR expression on B-Lymphocytes of 6 severely injured patients is expressed in MFI as mean ±S.D. with or without preincubation of 10 ng/ml GM-CSF, 10 ng/ml IFN-γ or 10 ng/ml G-CSF. The horizontal line shows the mean value of untreated samples of healthy controls with the two dotted lines indicating the standard deviation. Number of patients and healthy volunteers are indicated below each group.
For statistical symbols and group definitions see Figure 2.

was remained unchanged. Corresponding to the observation on B cells, only IFNγ was able to slightly elevate HLA-DR expression on T cells (control: 770 ± 44 MFI vs. 800 ± 46 MFI with IFNγ; p = 0.003, n = 6).

GM-CSF receptor expression on monocytes early after trauma, during sepsis and after recovery

GM-CSF receptor is expressed on circulating monocytes. Severe trauma causes a significant suppression of GM-CSF receptor expression on day 1 and 2 after trauma on monocytes, which began to normalize on day 7–8. However, during sepsis we observed the same levels of GM-CSF receptor as in recovering patients or healthy individuals (Fig. 6).

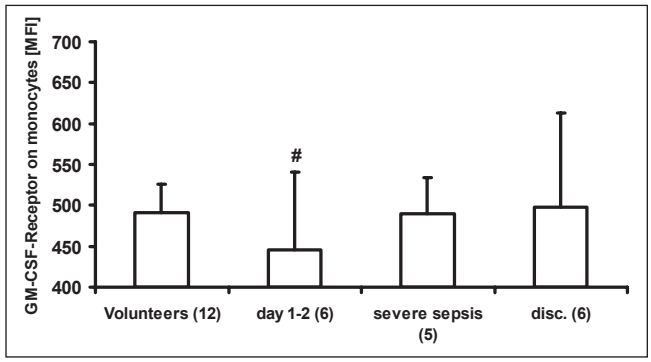

Fig. 6. GM-CSF receptor level on monocytes is expressed as mean ±S.D. of the MFI of healthy donors, blood samples from patients on day 1 after trauma and in patients samples with severe sepsis. Statistically significant changes were only found between healthy volunteers and day 1 after trauma (P = 0.048). Number of patients and healthy volunteers are indicated below each group.

Discussion

In this study the immunomodulating effects of GM-CSF, G-CSF and IFNγ were compared in an *in vitro* approach in severely injured patients. Numerous clinical studies with multiply injured patients as well as animal models have described a broad range of immunological deteriorations after trauma or haemorrhagic shock. Excessive mediator production of both pro- and anti-inflammatory cytokines has been reported in combination with a deregulation of almost all parts of the innate and adoptive immune response including a reduced expression of antigen-presenting MHC-class-II molecules and cytokine-production of monocytes [15, 16, 2], impaired mitogen-induced T-cell proliferation [17] as well as B-cell-dependent functions such as immunoglobulin secretion [18].

In the present investigation analysis of the immune response was focused on the HLA-DR expression on monocytes and lymphocytes and the LPS-induced TNFα-production. Reduced HLA-DR expression on monocytes and low TNFα-synthesis after *in vitro* stimulation with LPS are considered to reflect the status of immunoincompetence and have been demonstrated to be predictive for complications such as sepsis or multiple organ failure in trauma patients and after major surgery [16, 19, 20]. In the present study we observed changes in the immune response with low HLA-DR on monocytes and low TNFα-production of endotoxin-spiked blood cultures that resembles in degree and time frame earlier reports of various groups [21, 22, 23]. Interestingly, we observed only a rather modest reduction of TNFα-producing capacity in the samples of septic patients, which is in contrast to earlier studies with severe septic patients [24]. In all these studies the origin of sepsis was not restricted to a situation after trauma. HLA-DR expression on monocytes was more depressed early after trauma in comparison to samples collected during severe sepsis. These facts possibly illustrate a

special situation in patients with posttraumatic sepsis where a mixture of deteriorating effects caused by the trauma itself and the secondary bacterial infection is present. In addition we only analysed the blood samples after the first diagnosis of a severe sepsis, which means that we examined a rather early phase of sepsis. Early sepsis is generally considered to be accompanied with the effects of a severe systemic inflammatory response, while the later phase of a sepsis is thought to be dominated by immunoincompetence. In attempts to reactivate these functions of the immune system both IFNγ and GM-CSF turned out to be similary effective *in vitro*, while no relevant effects could be demonstrated with G-CSF. Both, IFNγ and GM-CSF elevated HLA-DR expression on monocytes, however, neither of these was able to completely normalize the levels of HLA-DR expression *in vitro*. In therapeutic trials IFNγ significantly elevated HLA-DR expression on monocytes on day three after onset of the treatment. This is in line with *in vitro* findings from murine bone marrow-derived macrophages that showed increased expression of MHC class-2 molecules up to 72 h after IFNγ incubation [25]. Surprisingly, this effect of IFNγ was less pronounced in macrophages from 20-month-old mice in comparison to cells derived from 6-week-old animals suggesting an age-dependent mechanisms [25].

Clinical recovery is accompanied by normalizing HLA-DR expression and cytokine-producing capacity, which reflects a functional reconstitution of the innate immune response. Its remains unclear whether this recovery is caused by a restoration of the functions of circulating monocytes or whether new cells are attracted into the circulation either from the bone marrow in the framework of haematopoesis or from other peripheral lymphoid organs.

MHC class-2 molecules are regulated co-ordinately, in part through dependence on expression of class II transactivator (CIITA). CIITA expression is regulated transcriptionally and both IFNγ and GM-CSF were shown to elevate MHC class-2 via increased expression of CIITA molecules in isolated human monocytes [26]. In this *in vitro* study the effects of GM-CSF on HLA-DR expression peaked 24–48 h after incubation, which again suggests that the *in vitro* incubation period of 6 h was not sufficient to reach maximal effects in our study. Interestingly, Hornell et al. also report that GM-CSF and IFNγ raise HLA-DR expression via different mechanisms partly independently by elevating different subtypes of the CIITA [27]. In addition, it has been shown, that GM-CSF up regulates the IFNγ-receptor on human monocytes [27]. These findings suggest that a combination of IFNγ and GM-CSF as immunostimulating compounds may be helpful, which to our knowledge has not been performed in clinical studies or animal models in the field of trauma and sepsis. However, a recent study in patients with acute pancreatitis analysed the immunomodulatory effects of GM-CSF and IFNγ alone and in combination in an *in vitro* approach comparable to the presented study. This group indeed reported, that IFNγ and GM-CSF had additive effects on both LPS-induced TNFα-production and monocytes' HLA-DR expression and only the combination of both substances was sufficient to fully restore this immune functions [28].

Although the effects of GM-CSF and IFNγ on whole blood cultures are very similar, exclusively IFNγ could influence lymphocyte HLA-DR expression. This can be explained by the fact that neither GM-CSF-receptors nor G-CSF receptors have been demonstrated on mature circulating lymphocytes in contrast to monocytes, which express high levels of both receptors [29]. In contrast, IFNγ receptors are expressed on the surface of T- and B-lymphocytes and monocytes [30, 31]. While no GM-CSF receptor could be found on circulating B-cells, GM-CSF has been shown to be produced by normal B cells and also act as an autocrine antiapoptotic factor [32].

Regarding G-CSF treatment *in vitro*, we could not demonstrate any effect on HLA-DR expression, neither in monocytes nor on lymphocytes. In the literature, the effects of G-CSF therapy *in vivo* on HLA-DR expression are inconsistent. While a prophylactic approach with G-CSF in patients undergoing major surgery pre- and postoperatively prevented surgery-induced decrease of HLA-DR expression on monocytes and LPS-induced TNFα-production [3], a therapeutic trial in septic neonates with G-CSF failed to show any effect on HLA-DR expression on monocytes [33].

There exists a report of IFN-γ therapy in septic patients with diminished monocytic HLA-DR expression and low LPS-induced TNFα-production [34]. In this clinical trial IFN-γ treatment reversed both impaired immune responses. In contrast to this report we only observed an increase in HLA-DR expression and no significant effect on TNFα-producing capacity in the present *in vitro* approach in blood samples of severe sepsis patients. A possible explanation for this discrepancy may be the involvement of secondary mediator as e. g. IL-12 in the priming of the TNFα-response by IFNγ.

G-CSF somehow differs from the former substances in terms of its immunostimulating effect. On one hand G-CSF represents a potent bone marrow stimulating substance – an effect that is completely neglected in the present study, since only *in vitro* interventions were performed, on the other hand the effects on circulating leukocytes are double-edged. So G-CSF enhances effector functions of neutrophilic granulocytes such as oxidative burst. In terms of cytokine production the effects of G-CSF reflect a predominantly anti-inflammatory profile with a reduced production of TNFα, IL-1 and IL-8 release but elevated levels of IL-1 receptor antagonist after G-CSF therapy [35]. A reduction of TNFα-producing capacity was also observed in the present study at some time points, which somehow suggests an anti-inflammatory potential of this substance in trauma patients. On the other hand this anti-inflammatory potential seems to make G-CSF a saver substance with potentially fewer adverse effects compared to GM-CSF.

In summary, we demonstrated that both IFNγ and GM-CSF revealed immunostimulating activities in severely injured patients in the presented *in vitro* approach. GM-CSF and IFNγ counteracted suppressed HLA-DR expression and TNFα-producing capacity in severely injured patients. Although the presented data show that immunostimulation in severely injured patients principally works, a clear evidence for a beneficial clinical effect of such an immunotherapeutic approach in trauma patients is still missing.

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