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DISTINCT DYNAMICS OF STEM AND PROGENITOR CELLS IN BLOOD OF POLYTRAUMATIZED PATIENTS

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ABSTRACT—Endogenously mobilized stem and progenitor cells (SPCs) or exogenously provided SPCs are thought to be beneficial for trauma therapy. However, still little is known about the synchronized dynamics of the number of SPCs in blood after severe injury and parameters like cytokine profiles that correlate with these numbers. We determined the number of hematopoietic stem cells, common myeloid progenitors, granulocyte-macrophage progenitors, and mesenchymal stem/stromal cells in peripheral blood (PB) 0 to 3, 8, 24, 48, and 120 h after polytrauma in individual patients (injury severity score $\geq$ 21). We found that the number of blood SPCs follows on average a synchronous, inverse bell-shaped distribution, with an increase at 0 to 3 h, followed by a strong decrease, with a nadir in SPC numbers in blood at 24 or 48 h. The change in numbers of SPCs in PB between 48 h and 120 h revealed two distinct patterns: Pattern 1 is characterized by an increase in the number of SPCs to a level higher than normal, pattern 2 is characterized by an almost absent increase in the number of SPCs compared to the nadir. Changes in the concentrations of the cytokines CK, MDC, IL-8, G-CSF Gro-α, VEGF, and MCP-1 correlated with changes in the number of SPCs in PB or were closely associated with Pattern 1 or Pattern 2. Our data provide novel rationale for investigations on the role of stem cell mobilization in polytraumatized patients and its likely positive impact on trauma outcome.

KEYWORDS—Common myeloid progenitors, granulocyte-macrophage progenitors, hematopoietic stem cells, inflammation, mesenchymal stem cells, mobilization, polytrauma, regeneration

INTRODUCTION

Trauma affects the distribution and activation of cells of the innate branch of the immune system like monocytes, macrophages, neutrophils as well as stem and progenitor cells (SPCs). Adult stem cells reside in their tissue within their niche. A small number of hematopoietic stem and progenitor cells (HSPCs) as well as mesenchymal stem/stromal cells (MSCs) are consistently shed into peripheral blood (PB) in a process called mobilization. Published data support a role for circulating hematopoietic stem cells (HSCs) in immune-surveillance of peripheral tissues, as they enter lymph nodes and other peripheral tissues to quickly differentiate into immune cells to regulate infections and modulate inflammation and infection (1–4). Besides their regenerative function, MSCs present also with an immune-modulatory function. It is thus believed that circulating SPCs have both a regenerative and an immune-modulatory function, and consequently stem cell therapies are discussed as therapeutic approaches in multiple diseases including trauma (1, 4).

MSCs differentiate into several cell types including osteoblasts, chondrocytes, adipocytes, neuronal cells, and cardiomyocytes. In various animal models, the local or systemic application of MSCs revealed positive effects on tissue regeneration. For example, MSCs have been reported to be crucially involved in healing of fractures (5), anterior cruciate ligament rupture (6), acute lung injury with hemorrhagic shock (7), or in burn wound regeneration (8). Hence, MSCs are currently the most widely used adult stem cells in regenerative medicine (9), though clinical trials are still mostly in phase 1 or 2 (9, 10). Immunomodulation is one of the key functions of MSCs. Interacting with adaptive and innate lymphocyte populations (10) and modulating proliferation, differentiation and function of myeloid cells toward a more immunosuppressive phenotype (10) represent strong features of MSCs which therefor influence inflammation and regenerative processes.

HSPCs can give rise to all mature blood cells and are primarily located in the bone marrow. HSPCs are cells that

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are routinely used in the clinic, mostly for their capability to reconstitute the entire hematopoietic system (11). The use of HSPCs for non-hematopoietic tissue repair has been proposed although the underlying mechanisms in trauma regeneration are not well understood so far (4). However, regeneration after myocardial infarction and liver damage was increased after the application of HSPCs in several murine models (12, 13) and first clinical trials for liver damage seem promising, improving at least one liver parameter in human patients (14, 15). In first clinical trials, HSPCs have also been tested for stroke treatment with encouraging results (16). Hematopoietic progenitor cells, preferentially common myeloid progenitors (CMPs) and granulocyte-macrophage progenitors (GMPs), also exhibit proangiogenic activity (17), which might be also beneficial for regenerative processes in the setting of trauma. Chemokines and cytokines like stromal cell-derived factor 1 (SDF-1) are known to initiate migration and thus also mobilization of stem cells (3, 18, 19) and are usually upregulated following tissue damage (20). Elevated numbers of HSPCs in PB were observed upon liver damage (21, 22), myocardial infarction (23), or in ischemia (24). While reports indicate a positive role of SPCs and stem cell mobilization in regeneration post trauma, the mechanisms as well as the biological pathways and the relevance of trauma-associated mobilization have not been determined in detail, especially the relative relationship of mobilization of distinct types of SPCs in a temporal manner in individual patients. Some of the results in addition are controversially discussed, precluding approaches to further improve such a likely positive clinical benefit.

We hypothesized that the number of distinct SPC populations in blood changes in a coordinated/interrelated manner upon trauma. We thus determined the number of MSCs, HSCs, and distinct types of HPCs concurrently in PB of individual patients after severe polytrauma (injury severity score (ISS) $\geq$ 21) as well as the concentration of cytokines/chemokines linked to mobilization and inflammation in blood in the time frame from 0 h to 120 h post-trauma. In summary, we observed a strikingly similar pattern of mobilization for all investigated cell types in the early phase (0–48 h), with an increase in the number of all SPCs early on (0–3 h), followed by a strong reduction of their number in blood at 24 to 48 h. The pattern of combined numbers of SPCs in blood at 48 to 120 h revealed two distinct groups of patients—one with a stable and strong increase and high SPC numbers at 120 h post insult (high late increase in number upon trauma, HiLiNut) and the other with almost no increase and low numbers (low late increase in number upon trauma, LoLiNut). LoLiNut had also the highest creatine kinase (CK) values at later time-points and presented with pneumonia or wound-healing defects and chemokine/cytokine profiles revealed in general increased inflammatory factors, like IL-6, IL-8 and granulocyte-colony stimulating factor (G-CSF) in them early after trauma. Additionally, the immuno-suppressive macrophage derived cytokine (MDC) was significantly lower at the 120 h time-point in the LoLiNut group. Taken together, our data reveal patterns of SPC mobilization early on upon trauma that are similar among patients but also identify distinct patterns at later time-points (HiLiNut an LoLiNut) that might be linked to clinical outcome.

PATIENTS AND METHODS

Blood sampling

Patients with polytrauma (ISS from $\geq$21) admitted to Ulm University Hospital between 2016 and 2017 in a prospective, observational cohort study design were included into the study. Blood samples were collected immediately after admission (0–3 h) and subsequently at 8 h $\pm$ 0.25 h, 24 h $\pm$ 1 h, 48 h $\pm$ 2 h, and 120 h $\pm$ 5 h after admission. Citrate-plasma aliquots were stored at -80°C until further analyzed and EDTA-blood was immediately processed for the determination of the frequency of circulating stem and progenitor cells. All patients were recruited into the study under informed consent as approved by the Ethics Committee of Ulm University (number: 94/14). Healthy control cohort: n = 31; 12 males and 19 females; mean age = 32 $\pm$ 10. Cytokine profile healthy control cohort: n = 10; 6 males and 4 females; mean age=37 $\pm$ 13.

Identification and quantification of mesenchymal stromal, hematopoietic stem, and progenitor cells in PB

7.5 mL of PB was drawn into EDTA KE/7.5 mL-Monovette tubes (Sarstedt, Nümbrecht, Germany). Cell counts were taken with a Hemavet multispecies hematology analyzer 950 (Drew, Miami Lakes, Fla). Blood was diluted 1:1 with 0.9% NaCl solution (Fresenius Kabi, Bad Homburg, Germany) and lied on top of lymphoprep (Stemcell Technologies, Vancouver, Canada). Density centrifugation was performed (26 min, RT, no brake/acceleration, 1,600 rpm) and the buffy coat was removed for further progressing. Mononucleated cells were washed twice with PBS (PanBiotech, Aidenbach, Germany) containing 3% FCS (Sigma-Aldrich, St. Louis, Mo). Afterward cell count was taken and 2×10^6 cells were used for hematopoietic staining and mesenchymal staining respectively. For single color controls and unstained control at least 0.1×10^6 cells were used. For the hematopoietic staining, cells were first incubated with 10% human serum (Lonza, Basel, Switzerland) to block unspecific binding, then the following antibodies were added: antihematopoietic lineage eFluor 450 cocktail (eBioscience, San Diego, Calif), CD34 APC (BD Pharmingen, Franklin Lakes, NJ), CD38 PE (BD Pharmingen), CD90 Clone 5E10 Pe-Cy7 (BD Pharmingen), CD123 Clone 7G3 PerCP-Cy5.5 (BD Pharmingen), and CD45RA FITC Ref. A07786 (BeckmanCoulter, Brea, Calif) (all antihuman). Samples and single color controls were incubated at 4°C, washed and analyzed by flow cytometry using a BD LSRII flow cytometer.

For MSC staining cells were resuspended in PBS with 0.5% FCS and 2 mM EDTA (AppliChem, Darmstadt, Germany). Antibodies were added as follows: CD14/CD20/CD34/CD45 Vioblue (all Miltenyi Biotec, Bergisch Gladbach, Germany), CD73 APC (Miltenyi Biotec), CD105 PE (Mitenyi Biotec), and CD90 Clone 5E10 Pe-Cy7 (BD Pharmingen) (all antihuman). Cells were incubated at 4°C for 20 mins and washed twice in medium before analyzed by flow cytometry. For each experiment/sample, voltage adjustments and compensation were newly performed with freshly prepared single color controls and unstained samples. Gates were adjusted to unstained sample. Forward and side scatter was used to exclude cell debris and dead cells. Data analysis was performed with the BD FACS DIVA 8.0.1 software package.

Plasma preparation

Blood was collected and centrifuged at 2,200 g for 15 min at 4°C. Supernatant was frozen at -80°C until further analyzed.

Determination of the cytokines profile

Plasma concentration of the cytokines and chemokines interleukin (IL)-6, IL-8, IL-10, IL-33, IL-1β, fractalkine, macrophage inflammatory protein (MIP)-1β, MIP-1α, monocyte chemoattractant protein (MCP)-1, MDC, stromal cell-derived factor (SDF)-1α, growth-related oncogene (Gro)-α, Gro-β, epidermal growth factor (EGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), G-CSF, and vascular endothelial growth factor (VEGF)-A were determined by human magnetic multiplex bead assay (R&D Systems, Minneapolis, Minn) by the research flow cytometry core CCHMC according to the manufacturer's instructions. Each sample was analyzed in duplicate.

Calculation of the additive number of SPCs (ANSP-score)

The ANSP-score was calculated by adding the changes in the number for HSCs, GMPs, CMPs, and MSCs together to obtain the additive number of SPCs (d0–48 h HSCs + d0–48 h MSCs + d0–48 h CMPs + d0–48 h GMPs = d0–48 h ANSP; d48–120 h HSCs + d48–120 h MSCs + d48–120 h CMPs + d48–120 h GMPs = d48–120 h ANSP).

Statistical analysis

Graph Pad Prism 7.0 was used to perform statistical analysis and to illustrate all graphs. Data, if not otherwise specified, is represented as mean $\pm$ SD and 2

Way ANOVA was used for statistical analysis with $^{*}P <= 0.05$, $^{**}P <= 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Non-parametric Spearman correlation was performed between numbers of SPCs at different time-points, or the amount of the decrease (d0–48 h) or the amount of the increase (d48–120 h) versus clinical parameters or cytokine/chemokine values at different time-points as well as between clinical parameters and cytokine/chemokine values. Relationships were marked as correlating if Spearman coefficient was larger than 0.7 or smaller than −0.7 and *P* values were smaller than 0.05. The *P* value then implies how likely a relationship would occur just by chance.

RESULTS

Patient cohort information and establishment of the characterization of the distinct stem-progenitor cell (SPC) populations in blood

The patient cohort contained eight patients (seven males and one female) with a mean age of 36.75 ± 18 and a mean ISS of 32.5 ± 9.5. Table 1 summarizes clinical parameters. Blood was collected at 0 to 3, 8, 24, 48, and 120 h after admission to the hospital. We next developed a marker panel and a gating strategy that allows for the simultaneous determination of the in general low-frequency of HSCs (Lin^-, $CD38^-$, $CD45ra^-$, $CD34^+$, $CD90^+$), GMPs (Lin^-, $CD38^+$, $CD45ra^+$, $CD34^+$, $CD123^+$), CMPs (Lin^-, $CD38^+$, $CD45ra^-$, $CD34^+$, $CD123^+$), and MSCs ($CD45^-, CD14^-, CD34^-, CD20^-, CD105^+, CD90^+, CD73^+$) in PB (Fig. 1, A and B) of polytraumatized patients.

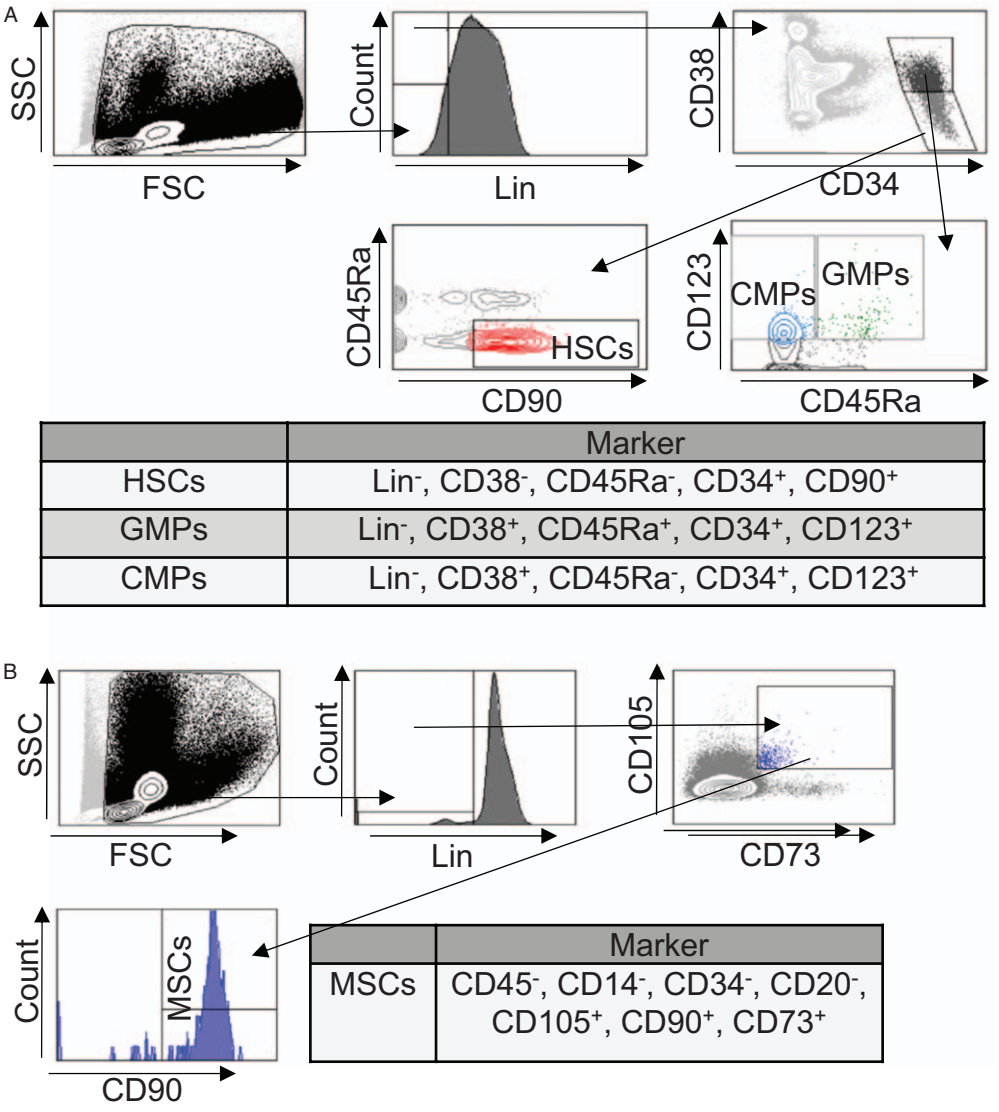
Frequencies of SPCs in PB after severe injury follow distinct patterns

The frequency of HSCs, GMPs, CMPs, and MSCs (SPCs) in PB from individual patients for our five time-points up to 120 h post trauma was determined by multicolor flow cytometry. A synchronous, inverse bell-shaped pattern of the number of distinct SPCs in PB at different time-points was identified in polytraumatized patients (Fig. 2, A–D, F), whereas the number of SPCs in healthy controls over these five time-points remained almost flat and steady (Fig S1, http://links.lww.com/SHK/A763). Shortly after trauma (0–3 h), the number of HSCs and MSCs per milliliter of blood was increased in all patients. For GMPs five out of seven and for CMPs three out of seven traumatized patients showed an increase compared to the average numbers of a healthy volunteer cohort. This was followed by a decrease in the numbers at 8, 24, and 48 h. The lowest numbers of SPCs in PB for each patient and SPC populations were found at 24 or 48 h, with the exception of MSCs in PT7 and GMPs in PTs 3, 5, 6, 8. Patients presented then at 120 h with a general increase in the number of SPCs relative to the nadir of 24 to 48 h, with the exception of GMPs reduced in PT3,8 and HSCs reduced in PT5. This number of SPCs in blood at 120 h was on average higher than normal. HSCs (Fig. 2A), MSCs (Fig. 2B), and GMPs (Fig. 2C) were significantly increased at 0 to 3 h and also at 120 h. Also, a significant decrease in the number of CMP (Fig. 2D) as well as in HSCs (Fig. 2A) was observed at 24 h compared to healthy controls. The average number of the distinct SPCs in PB upon trauma followed an inverse bell-shaped pattern (Fig. 2F). Hematocrit (Ht) values for each patient and time-point did not correlate with the inverse bell-shaped pattern of the number of SPC in PB (Fig. 2, E). There was also no correlation in the number of SPCs in PB with the number of packed red blood cells (PRBC) units, of Fresh Frozen Plasma (FFP) units (with the exception MSC at 24 h) or of platelet concentrate (TK) units (with the exception of HSC at d0–48 h) given to the patients. There was also only one positive correlation between the fluid balance and the different SPC numbers (CMPs 0 h). This implies that changes in the number of SPCs in PB are in general independent of these parameters and also of the blood volume. A correlation matrix (Table S1, http://links.lww.com/SHK/A763) of the number of SPCs of individual patients with the clinical parameters (Table 1) revealed that both the absolute number of distinct types of SPCs as well as the difference in numbers (decrease) between 0 and 48 h and the difference in number (increase) between 48 and 120 h did not correlate consistently with other clinical parameters. Interestingly though, the number of HSCs at 0 and 24 h in PB showed a strong association with the age of the patients (Table S1, http://links.lww.com/SHK/A763).

Table 1. **Patient cohort—clinical parameters**

	Age (years)	Sex	ISS (AIS)	Catechol-amines	PRBC	FFP	TC	Fluid balance (ICU first 48 h)	Infectious complication	Length ICU (days)
PT1	47	F	41 (Thorax 4; neck 5)	SR: no Course: n.a.	SR: 2 Course: —	SR: 3 Course: —	SR: — Course: —	+ 2,604 mL	Yes (urinary tract infection)	12
PT2	62	M	21 (Thorax 4; extremities 2; extern 1)	SR: yes Course: yes	SR: 8 Course: —	SR: 8 Course: —	SR: 2 Course: —	+ 1,555 mL	Yes (wound infection)	10
PT3	23	M	48 (thorax 4; head 4; extremities 4)	SR: yes Course: n.a.	SR: 24 Course: 1	SR: 25 Course: —	SR: 2 Course: 2	+ 4,005 mL	Yes (pneumonia; wound healing disorder)	18
PT4	19	M	22 (thorax 3; head 3; extremities 2)	SR: n.a Course: no	SR: n.a. Course: —	SR: n.a. Course: —	SR: n.a. Course: —	+ 1,890 mL	Yes (urinary tract infection)	12
PT5	48	M	34 (thorax 4; abdomen 3; extremities 3)	SR: yes Course: yes	SR: 28 Course: 13	SR: 22 Course: 10	SR: 4 Course: 2	+ 5,090 mL	Yes (wound infection)	30
PT6	18	M	27 (thorax 3; extremities 3; cervical spine 3)	SR: no Course: n.a.	SR: 10 Course: 2	SR: 10 Course: —	SR: — Course: —	+ 1,160 mL	No	10
PT7	22	M	38(thorax 2; abdomen 5; extremities 3)	SR: no Course: no	SR: 3 Course: 2	SR: 3 Course: 3	SR: - Course: —	+ 620 mL (only 24h)	No	1
PT8	55	M	29 (Thorax 4; head 2; extremities 3)	SR: n.a. Course: yes	SR: 1 Course: —	SR: — Course: —	SR: — Course: —	+ 1,540 mL	Yes (n.a.)	5

F indicates female; FFP, fresh frozen plasma; ICU, intensive care unit; ISS, Injury Severity Score; M, male; n.a., not available; PRBC, packed red blood cells erythrocyte concentrate; PT, polytrauma; SR, shock-room; TC, thrombocyte concentrate.



	Marker
HSCs	Lin$^-$, CD38$^-$, CD45Ra$^-$, CD34$^+$, CD90$^+$
GMPs	Lin$^-$, CD38$^+$, CD45Ra$^+$, CD34$^+$, CD123$^+$
CMPs	Lin$^-$, CD38$^+$, CD45Ra$^-$, CD34$^+$, CD123$^+$

	Marker
MSCs	CD45$^-$, CD14$^-$, CD34$^-$, CD20$^-$, CD105$^+$, CD90$^+$, CD73$^+$

Fig. 1. **Gating strategy for (A) HSCs, GMPs, CMPs and (B) MSCs and the marker panels linked to the respective populations.** CMPs indicates common myeloid progenitors; GMPs, granulocyte-macrophage progenitors; HSCs, hematopoietic stem cells; MSCs, mesenchymal stem/stromal cells.

Inflammatory and SPC mobilizing factors in PB after polytrauma

We next analyzed, by multiplex ELISA, cytokine and chemokine levels in plasma at 0 to 3, 24, 48, and 120 h as well as in controls. The following cytokines were analyzed: EGF, Fractalkine, G-CSF, GM-CSF, Gro-α, Gro-β, IL-1β, IL-6, IL-8, IL-10, IL-33, MCP-1, MDC, MIP-1α, MIP-1β, SDF-1α and VEGF-A. Fig S2 and Table S2 (see http://links.lww.com/SHK/A763) report data per cytokine and per patient. *IL-33, IL-1*β, GM-CSF, and EGF were below the level of detection and thus not further pursued. On average, the concentration of inflammatory cytokines like IL-6, IL-8, MCP-1, and IL-10 was significantly increased in polytraumatized patients at the 0 to 3 h time-point but also cytokines that can be in addition linked to mobilization of SPCs like Gro-α, Gro-ß, and G-CSF were increased (Fig. 3). Both IL-8 and IL-6 were also significantly increased at 24 h, MCP-1 even up to 48 h. VEGF and MDC presented with an increased concentration in blood only at the 120 h time-point. The overall pattern of changes in cytokine concentration upon trauma revealed that actually none of the cytokine concentrations analyzed followed the inverse bell-shaped pattern observed with respect to SPCs, suggesting that a combination of the action of multiple cytokines might be linked to the changes in the number of SPCs in PB.

We next tested for correlations between cytokine concentrations and clinical parameters (from Table 1) as well as for correlations between cytokine concentrations and the number of SPCs in PB for all individual time-points and cell types as well as for the change in numbers (decrease/increase). Basic clinical parameters such as age and ISS did not consistently correlate with the cytokine pattern (Table S3, http://links.lww.com/SHK/A763). Interestingly, we found that G-CSF levels correlated with the length of stay in intensive care unit (ICU), not only at 0 h but also at 48 h and 120 h postadmittance (Table S3, http://links.lww.com/SHK/A763). The amount of FFP provided did not consistently correlate with cytokine

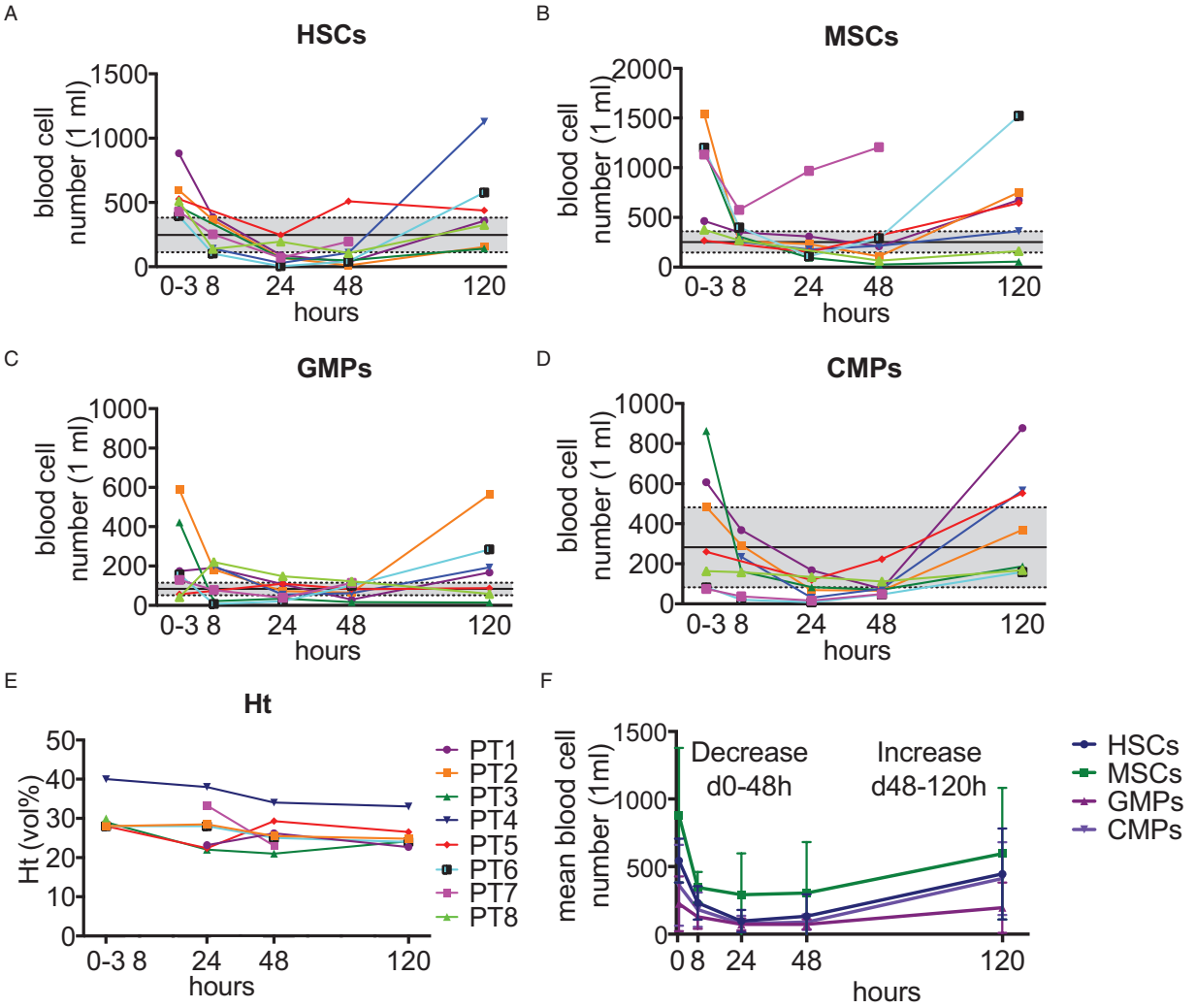


FIG. 2. **Absolute cell number of (A) HSCs, (B) MSCs, (C) GMPs, and (D) CMPs between 0 and 120 h postadmittance of individual patients per milliliter of blood.** The black dotted lines represent the average SPC number in healthy controls, the gray area the respective standard deviation (HSCs: 247 ± 134, n = 31; MSCs: 252 ± 107, n = 30; GMPs: 83 ± 32, n = 31; CMPs: 282 ± 200, n = 31). **E**, Hematocrit (Ht) values between 0 and 120 h of individual patients. **F**, Average number of HSCs, MSCs, GMPs, and CMPs between 0 and 120 h in patients with polytrauma. Light grey box: time area defined as decrease—d0-48 h and dark grey box: time area defined as increase—d48-120 h. CMPs indicates common myeloid progenitors; GMPs, granulocyte-macrophage progenitors; HSCs, hematopoietic stem cells; Ht, Hematocrit; MSCs, mesenchymal stem/stromal cells; SPC, stem and progenitor cell.

concentrations, implying that plasma transfusion did not contribute to changes in cytokine levels in blood. Levels of creatine kinase (CK, primarily a marker for muscle injury) showed consistently correlations with multiple cytokines, including G-SCF, Gro-α, IL-6, IL-8, IL-10, and MCP-1 (Table S3, http://links.lww.com/SHK/A763).

Multiple single correlations were also detected among cytokines and numbers of distinct SPCs in blood. Thus, we focused in the following on correlations that allowed for causative interpretations. IL-8 and G-CSF are both known besides their role in inflammation also for their role in inducing migration and mobilization of stem and progenitor cells (25, 26). The level of both IL-8 as well as G-CSF in PB at the 0 h time-point correlated positively with the number of CMPs at 48 h (Fig S3a, http://links.lww.com/SHK/A763). We also observed a positive correlation of Gro-α levels and MCP-1 at 0 h with the number of CMPs in PB at 48 h (Fig S3b, http://links.lww.com/SHK/A763). VEGF-A concentrations at 0 h also correlated with the number of HSCs at 0 and 24 h (Spearman $r = 0.786$; $P = 0.048$ and $r = 0.857$; $P = 0.024$, data not shown). Although a large number of cytokines linked to inflammation but also migration like IL-6, IL-8, IL-10, G-CSF, Gro-α, and Gro-β presented on average with elevated levels in PB upon trauma (Fig 3, Fig S2, http://links.lww.com/SHK/A763) they did not show strong correlations to the number or changes in the number of SPCs at the 0 to 48 h time frame. It is thus likely that a yet unknown combination of the factors analyzed or even not yet determined factors might be ultimately involved in determining the number of distinct SPCs in the first 48 h after trauma.

We then focused on the relative increase in the number of distinct types of SPCs in the 48 to 120 h time frame. Concentrations of CK, IL-6, G-CSF, IL-8, as well as VEGF-A (linked to inflammation but also endothelial progenitor mobilization) at the 48 h time-point correlated inversely with the change in the number of HSCs (Fig S3c–e, http://links.lww.com/SHK/A763), but not with any other cell type. Higher levels of these cytokines at 48 h

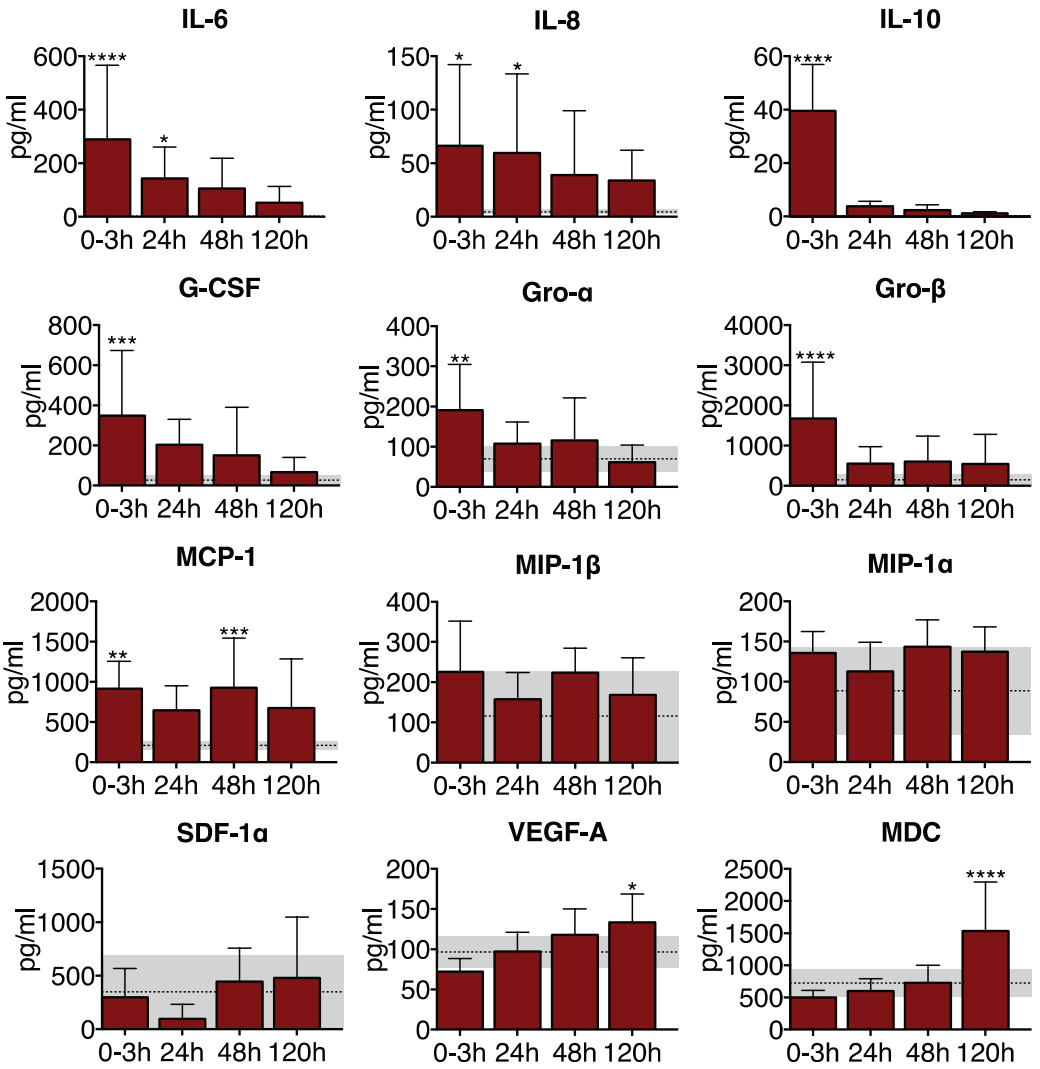


Fig. 3. **Average concentration of selected inflammatory and mobilizing factors in PB of the patients with polytrauma 0 and 120 h postadmittance.** The based line of the factor in healthy controls (n = 10) is represented as black dotted line, the gray areas indicate the standard deviation of the concentration in controls.

were linked to a low increase in the number of HSCs between 48 and 120 h. Correlations of cytokine concentrations with the number of MSCs in PB were rare and not really consistent and mostly negative (Gro-β, MDC, and MIP-1β, data not shown). In summary, an intricate interplay of multiple cytokines and chemokines might be linked to changes in the number of distinct types of SPCs upon the first 120 h post polytrauma.

Comparison between patients revealed low and high increaser between 48 and 120 h

Displaying the number of distinct cell populations per patient in PB along our time-axis revealed a novel interesting pattern per individual patient. Patients like PT3 (Fig. 4A) and PT5 and PT8 (Fig S4, http://links.lww.com/SHK/A763) displayed a low increase in the number of all SPCs analyzed between 48 and 120 h, while patients like PT1 (Fig. 4B) and PT2, 4 and 6 (Fig S4, http://links.lww.com/SHK/A763) showed a noticeable increase in the number of all cell types analyzed between the 48 and 120 h time frames. This observation promoted us to introduce a novel additive score of the change in the number of SPCs in PB between 0 and 48 h and between 48 and 120 h, in which changes in the number for HSCs, GMPs, CMPs, and MSCs were added together to obtain the additive number of SPCs (d48–120 h ANSP score).

While the ANSP score of individual patients reflecting the difference in numbers of SPCs between 0 and 48 h presented with a heterogeneous pattern (Fig. 4C), the score for the difference in numbers between 48 and 120 h marked two very distinct groups of patients—one with a high ANSP score and thus a stable and strong increase and high SPC numbers at 120 h post insult (high late increase in numbers upon trauma termed HiLiNut patients, PT1, 2, 4, and 6) and another group with a low ANSP score and thus overall lower numbers at 120 h (low late increase termed LoLiNut patients, PT3, 5, and 8) (Fig. 4C). Two of the three LoLiNut patients had the longest ICU stay and developed infectious complications such as pneumonia or wound-healing problems and might thus be related to clinical outcome. There was no difference in the ISS score between these two groups (Fig. 4D). Finally, levels of cytokines and chemokines were compared between LoLiNuts and HiLiNuts (Fig. 4D). In agreement with our initial correlative analyses, we found in LoLiNuts pro-inflammatory cytokines like IL-6, IL-8,

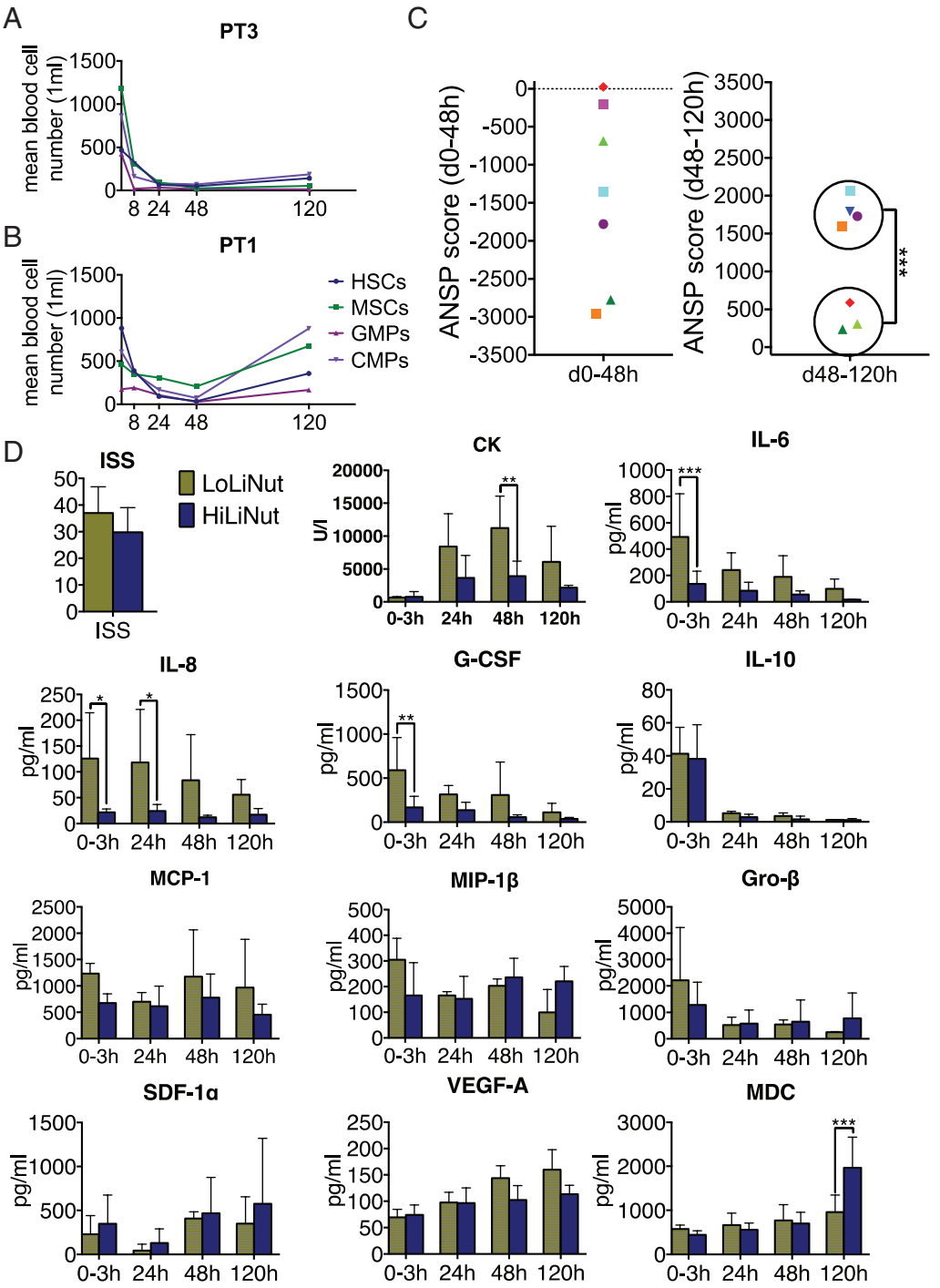


Fig. 4. **Number of HSCs, GMPs, CMPs, and MSCs in (A) PT3 and (B) PT1 between 0 and 120 h. C, Additive number of SPCs (ANSP)-score for changes in the number of SPCs between 0 and 3 h and 48 h (d0–48 h) and 48 h and 120 h (d48–120 h).** The ANSP score (d48–120 h) reveals two groups of patients with either a low late increase of the SPC number upon trauma (LoLiNut) or with a high late increase in the SPC number upon trauma (HiLiNut). D, Average concentration of selected inflammatory and mobilizing factors in PB between 0 and 120 h in LoLiNut and HiLiNut patients.

and G-SCF and the damage parameter CK to be increased at the early stages of trauma (at 0–3 h IL-6, IL-8, and G-CSF, at 24 h IL-8 and at 48 h CK), implying that high levels of these cytokines at early time points after trauma are linked to a low ANSP score in the 48 h to 120 h interval. Macrophage-derived chemokine (MDC), which is a ligand for CCR4 and has been already implemented as a mediator of lung inflammation upon trauma (27) while being on average elevated at the 120 h time-point (Fig. 3), was the only factor of all the mediators analyzed that presented with a higher concentration at the 120 h time-point in HiLiNuts. These data link an elevated level of MDC to a high ANSP score.

DISCUSSION

Adult stem cells play a central role in regenerative medicine, and MSCs as well as HSPCs and their distribution via the blood have already been investigated in the trauma setting in both

animals models and humans, but in most cases individually and primarily when provided exogenously (see, for example, (28)). Here, we provide novel insights into the dynamics of hematopoietic and mesenchymal SPCs in individual patients over the first 120 h post trauma. We determined the number of distinct types of SPCs at the same time-point, as we anticipated that beneficial aspects of changes in the number of individual types of circulating SPCs might be additive or even synergistic. Several studies demonstrated that either local, distant, or multiple trauma results in elevated (higher than steady-state levels) numbers of hematopoietic as well as endothelial stem cells in PB with a primarily positive correlation to a clinical benefit (29–31), primarily determined though in the context of fractures. Simultaneous changes in the number of multiple types of SPCs in PB upon polytrauma have not been determined in greater detail. In our patient cohort, SPC numbers in PB follow a specific inverse bell-shaped pattern over the first 120 h. It is likely that in the first hours after injury the mobilization or maybe also the mechanical release of SPCs is increased, before they migrate toward damaged tissue. The increase of SPCs in blood at later time-points might be due to either increased mobilization or reduced migration into tissues. As both migratory as well as inflammatory cytokine levels are linked to these changes in SPCs numbers, also combinations of distinct mechanisms are likely. Thus, mechanism of SPC recruitment in the early phase post trauma might be based on the extent of the inflammation and the subsequent release of cytokines/chemokines. Specific SPC subpopulations are then recruited or suppressed by the interaction and the activation of many different pathways. We used a Ficoll density centrifugation step to enrich for SPCs from blood. As it has been reported that in such a Ficoll step SPCs can be lost (32), the total number of SPCs in blood might be even higher than reported by us.

All patients were treated according to the S3 guidelines for polytrauma patients (AWMF registry no 012/19) and thus obtained fluids and/or blood products to resuscitate the hemodynamically active blood loss. PRBCs and other blood products like FFP or thrombocytes might influence PB SPC numbers; however, we observed no correlations between changes in the number of any SPC population and the number of PRBCs or FFPs or the Ht, strongly implying that the number of SPCs in PB was independent of these parameters.

In addition to the well-known chemotactic cytokines G-CSF, IL-8, and VEGF-A (linked to angiogenesis and endothelial cell recruitment), concentrations of the pro-inflammatory cytokines correlated with the increase of the number of CMPs and later with a change in the number of HSCs between 48 h and 120 h postinjury, which implies a direct or even indirect chemotactic role of inflammatory cytokines on CMPs and HSCs. In this context it is noteworthy that CMPs might also be able to support angiogenesis and might be even able to trans-differentiate into endothelial cells (17). It has been reported that MCP-1 was associated with MSC recruitment in gliomas (33), supporting that chemotaxis of SPCs could be increased with higher levels of pro-inflammatory cytokines. The inverse correlation of the level of VEGF-A with the number of HSCs was surprising, as in general the concentration of VEGF-A increased between 48 and 120 h (Fig. 3). CK levels, in contrast, could be either causative or also a consequence with respect to levels of HSCs. Surprisingly, the concentration of SDF-1α, a well-known chemokine usually linked do SPC mobilization, did not correlate with SPC numbers in PB of polytraumatized patients.

Introducing a novel additive d48–120 h SPC parameter (ANSP score), two distinct patient groups could be identified, one with a strong increase in SPC numbers between 48 and 120 h (HiLiNuts) and the other with only a marginal/low increase (LoLiNuts). Out of the three patients of the LoLiNut group, two patients had the longest stay in ICU, the highest remaining CK values at 48 h/120 h and presented with pneumonia or wound-healing defects. The cytokine/chemokine profiles for these groups were quite distinct, while trauma-impact parameters like the ISS were very similar (Fig. 4D). Early systemic inflammation seems to be more pronounced in the low ANSP group, as they present with higher levels of pro-inflammatory cytokines/chemokines (IL-6, IL-8, and G-SCF). This might indicate that an initial strong inflammatory response negatively impacts the later recovery of SPC numbers in PB. Surprisingly, MDC was the only chemokine of all the analyzed factors which showed an increase in the HiLiNut group at 120 h. Our data thus imply a positive role for MDC in enhancing SPC recovery. MDC plays a central role for Th_2 lymphocytes, monocytes, monocyte-derived dendritic cells, and natural killer cell recruitment and binds to the CCR4 (34). MDC was previously also shown to influence MSC migration (35). We speculate that the number of SPCs in PB in the time-frame of 48 to 120 h postinsult might be clinically relevant parameter for trauma outcome. The ANSP score and its implementation of HiLiNuts and LoLiNuts are interesting novel concepts and parameters based on our data. A limitation of our study is the low number of patients analyzed. Therefore, further investigations in additional cohorts are necessary to determine the role and predicate value of the ANSP score and the role of MDC in influencing the HiLiNut and LoLiNut groups.

In conclusion, the contribution of HSPCs and MSCs in PB and their hypothetical therapeutic potential for trauma regeneration will need to be further investigated, as well as the interactions of distinct types of SPCs in trauma. However, the recurrent, inverse bell-shaped pattern of SPCs in PB in the first 120 h post trauma suggests that SPCs are mobilized at distinct time-points by overlapping but also distinct mechanisms. It also implies that the migration of SPCs into damaged tissue might happen in waves rather than being a continuous process.

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