Platelet Factor 4 Is Highly Upregulated in Dendritic Cells after Severe Trauma

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Dendritic cells (DCs) represent an important linkage between the innate and adaptive immune system and express proinflammatory transcriptomic products early after trauma. The use of a genomic approach recently revealed that platelet factor 4 (PF4) is significantly upregulated in DCs in patients after multiple trauma. However, knowledge about subsequent PF4 alteration and its potential clinical relevance in the context of multiple trauma is still limited. We used quantitative reverse transcription–polymerase chain reaction to analyze PF4 expression in both myeloid DCs (MDCs) and plasmocytoid DCs (PDCs) isolated from 10 patients after multiple trauma. Intracellular PF4 as well as HLA-DR expression were detected by flow cytometry. Furthermore, DCs and peripheral blood mononuclear cells were incubated on a monolayer of human umbilical endothelial cells and their adhesion properties were analyzed. The ratio of the DC subtypes (MDC and PDC) was assessed by flow cytometry. PF4 expression significantly increased on d 1 and d 2 as measured by reverse transcription–polymerase chain reaction. Intracellular PF4 content in MDCs and PDCs was significantly elevated in trauma patients compared with healthy controls. In addition, the surface antigen HLA-DR on MDCs was significantly elevated on d 1 and d 4 after trauma in patients compared with controls. However, cell adhesion of DCs did not show any significant differences between patients and controls. PF4 concentration in MDCs and PDCs significantly correlated with the injury severity score. These results confirm an early and subsequent posttraumatic activation of PF4 in DCs. PF4 also participates in the posttraumatic activation of DCs in relation to injury severity, a role that might be preferably based on the modification of receptor expression, whereas adhesion properties are largely unaffected.
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INTRODUCTION

Platelet factor 4 (PF4) is a CXC chemokine synthesized by megakaryocytes and stored in platelet α-granules (1). PF4 shares up to 60% sequence identity and typical structural properties with other CXC chemokines. Upon platelet activation after trauma, PF4 is immediately released, leading to various immunomodulatory effects, such as monocytic chemotaxis and prevention of monocyte apoptosis. PF4 expression is upregulated in monocytes by thrombin via proteinase-activated receptors (2). PF4 induces natural killer cells to release interleukin-8 (3). Additionally, phagocytosis and the generation of reactive oxygen metabolites in mononuclear phagocytes is promoted by PF4 (4). It has also been demonstrated that PF4 induces $CD4^{+}CD25^{+}$ T regulatory cell proliferation while impairing T regulatory cell function within the adoptive immune system (5). PF4 induces neutrophil cell adhesion and monocyte differentiation into macrophages and dendritic cells (DCs) (6,7). Moreover, PF4 participates as a regulator of hematopoiesis and homeostasis. Both pro- and anticoagulatory properties have been reported for PF4 as well as an impact on thrombomodulation (8). PF4 obviously is involved at different sites of inflammation; however, its functional role following multiple trauma has not been fully elucidated.

Severe trauma induces a systemic inflammatory response that can eventually lead to remote organ dysfunction and secondary organ failure (9,10). The innate immune system is the first line of posttraumatic immune response, initiating further cellular activation. DCs are bone marrow–derived professional antigen-presenting cells and part of the innate immune system. In this context DCs provide an important link between the innate and adoptive immune systems (11) by the initiation and regulation of the immune response (12). Two major subpopulations of DCs can be distinguished, the myeloid DC (MDC) and the

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plasmacytoid DC (PDC). In addition to their phenotypic differences MDCs and PDCs also show some functional distinctions: they express different patterns of pathogen-recognition receptors (Toll-like receptors) and different cytokine receptors, and MDCs and PDCs release different cytokines when reacting to an identical stimulus (13). PDCs are characterized as CD11c-negative and CD303-positive cells, whereas MDCs express CD11c but not CD303 (13,14).

The impact of trauma on DC activation and its subtypes MDCs and PDCs, as well as the subsequent gene expression patterns have been comprehensively studied (15). In the latter study, we demonstrated for the first time a significant upregulation of PF4 expression in DCs during the first day following multiple trauma. Nevertheless, knowledge is still limited regarding the subsequent alteration of PF4 and its potential clinical relevance in the context of multiple trauma. Therefore, we analyzed PF4 expression as well as phenotypic alterations of DCs following multiple trauma during the first days after severe trauma.

MATERIALS AND METHODS

Patients

The study protocol was approved by the local ethics committee, and signed informed consent was obtained from all participants or their legal substitutes. Ten consecutive multiple trauma patients (Injury Severity Score [ISS] ≥ 20) (1 female, 9 males; mean age 30.3 ± 3.1 years) were included in this study. Patients suffering from burns and patients under immunosuppressive therapy were excluded. In addition, 10 healthy controls were enrolled (5 females, 5 males; mean age 29 ± 1.6 years). A subanalysis of the control group, which compared female and male controls with respect to PF4 expression, adhesion properties, and HLA-DR expression, did not show any differences associated with sex. The demographic data are summarized in Table 1. For experiments we obtained, from each patient per d, a total volume of 20 mL ethylene diaminetetraacetic acid (EDTA)-anticoagulated whole blood.

Table 1. Demographic data on the study groups.

	Patients	Controls
Female:male, n	1:9	5:5
Age, years	30.3 ± 3.1	29 ± 1.6
Injury severity score, points	28 ± 6	—
DC purity, n	$92 \times 10^5 \pm 6.3 \times 10^5$	$97.5 \times 10^5 \pm 21.3 \times 10^5$
Purity, %	94.7 ± 1.5	93.2 ± 1.2

Determination of Intracellular PF4

Intracellular PF4 was determined by means of flow cytometry. In brief, peripheral blood mononucleated cells (PBMC) were isolated from a total volume of 10 mL blood by Ficoll density-gradient centrifugation. After being washed with phosphate-buffered saline (PBS), cells were resuspended in 2 mL PBS supplemented with 5% fetal calf serum and subdivided into 2 portions of a volume of 1 mL each. One portion was used to determine native and stimulated PF4 synthesis, the other served as control. Subsequently, Brefeldin-A solution (Golgiplug, BD Biosciences, Heidelberg, Germany) was added to both vials according to the manufacturer's instructions. Brefeldin-A prevents the secretion of PF4 and thus leads to intracellular accumulation of PF4. Then phorbol 12-myristate 13-acetate (PMA) (Sigma, Deisenhofen, Germany) was added in a final concentration of 10^{-6} mol/L to one vial. Both preparations were subsequently incubated at 37°C for 4 h. For identification of MDC and PDC the PBMC preparations were preincubated for 20 min with each 10 μL of monoclonal antibodies against CD11c-PerCPCy5 (MDC) and CD303-APC (PDC). To discriminate DC from other PBMC populations, we also added a 10-μL fluoroscein isothiocyanate (FITC)-labeled lineage cocktail that contained antibodies against T cells, B cells, natural killer cells, and monocytes. A parallel preparation contained isotype-identical antibodies.

Intracellular staining requires fixation followed by the permeabilization of the cells. For both procedures the FIX & PERM® kit (Caltag, Burlingame, CA, USA) was used. Briefly, after being washed with PBS containing 5% FCS the cells were fixed and washed once again before permeabilization. Then 10 μL of PE-labeled antibodies against PF4 were added. A parallel preparation served as isotype control. After a last wash the preparations were subjected to flow cytometry. All antibodies used were obtained from BD Biosciences, except the PF4 antibody and the PF4 isotype control (both from R&D Systems GmbH, Wiesbaden, Germany) and the CD303 antibody (Miltenyi Biotech, Bergisch Gladbach, Germany).

A four-color, dual-laser FACScalibur (BD Biosciences) with CellQuest Pro software (V 4.02, BD Biosciences) was used, and at least 30,000 events were collected in a monocyte/lymphocyte region as identified by the appropriate forward/sideward scatter properties.

The gating strategy applied to determine semiquantitatively the mean PF4 content in MDCs and PDCs is illustrated in Figure 1. The final PF4 value was calculated by subtraction of the FL-2 mean fluorescence intensity of the isotype control from the FL-2 mean fluorescence intensity of the test preparation.

Determination of HLA-DR on DCs

The HLA-DR surface expression on MDCs and PDCs was determined in whole blood as follows: 10 μL each of CD11–PerCPCy5, CD303-APC, HLA-DR-PE, and an FITC-labeled lineage-negative cocktail were added to 200 μL of previously cooled EDTA blood (4°C). A parallel preparation contain-

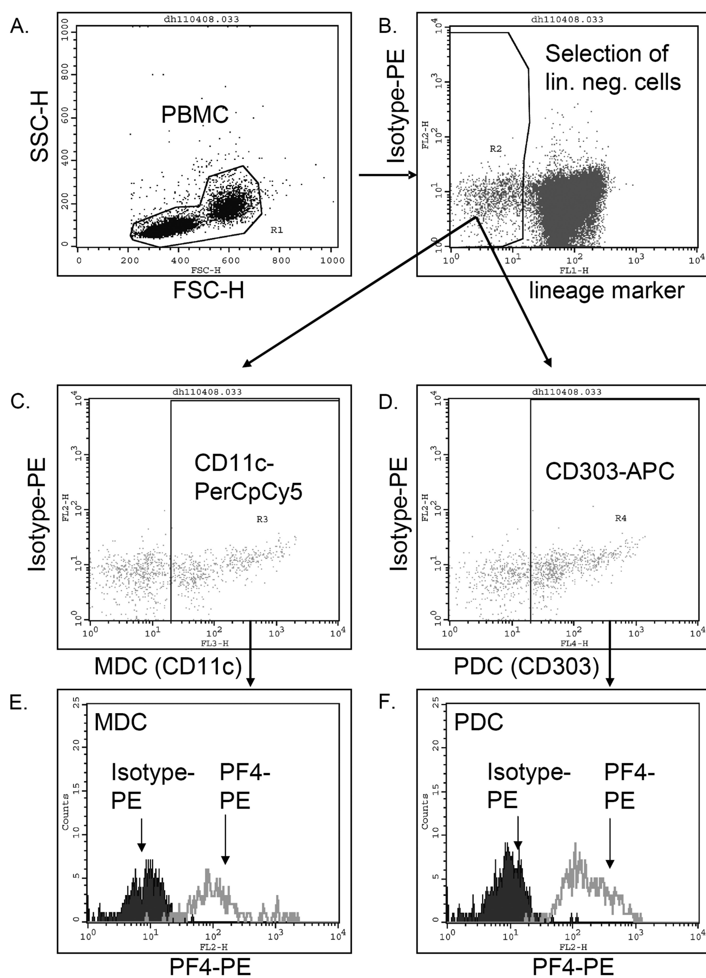

Figure 1. Gating strategy for the determination of intracellular PF4 in MDC and PDC. Representative FACS diagrams are presented. Mononucleated cells were identified by their forward/sideward properties (A) and subsequently analyzed with regard to the expression of lineage markers such as CD3, CD14, and CD19 (B). The lineage-negative cells were further tested for expression of CD11c (C) and CD303 (D). Then the PF4 content of the CD11c-positive (E) and CD303-positive cells (F) was evaluated using histogram analysis. The surface expression of HLA-DR was measured accordingly.

ing fluorochrome-conjugated isotype-identical antibodies served as control. After 15 min of incubation red cells were lysed and the preparations were washed twice and subjected immediately to flow cytometry. The general gating strategy applied is described in Figure 1.

Determination of DC Adhesion on Endothelial Cells

The adhesion assay was performed as described in (16). Briefly, PBMC derived from trauma patients or controls were incubated in a density of 2×10^5 per cm^2 on a confluent monolayer consisting of human umbilical vein endothelial cells (HUVEC) for 1 h. The HUVEC were a kind gift from Dr. Roman Blaheta, Department of Urology, Johann Wolfgang Goethe-University, Frankfurt, Germany.

The nonadherent cells were removed by aspiration of the culture medium. To remove weak adherent cells the monolayer with the remaining adhering PBMCs was gently rinsed three times with warm PBS (containing Mg^{2+} and Ca^{2+}; $PBS^{+/+}$) and then subjected to phase-contrast microscopy.

Adhering cells were counted in 10 randomized fields of view (FOV) at a power of 100× and the mean cell number/FOV was calculated. Based on the mean PBMC number per FOV the absolute number of PBMC per well was extrapolated. Next, HUVEC and adherent cells were detached by thorough and repeated aspiration, and the percentages of MDCs and PDCs per PBMC within this preparation were determined by flow cytometry as indicated above. PBMC and HUVEC were distinguished by their forward and sideward scatter properties. We also calculated the absolute number of MDCs and PDCs by multiplying the corresponding percentage with the microscopically determined absolute number of PBMC.

Isolation of Peripheral Blood DCs for Gene Expression Analysis and Purity Check

The analysis of PF4 gene expression was performed using highly purified DCs obtained from 10 mL anticoagulated peripheral blood. Blood samples were drawn into EDTA tubes on the d of admission (d 0) as well as on d 1 and d 4. The blood samples were placed on ice and were immediately subjected to the DC isolation procedure, which was carried out at continuously low temperatures (4°C) to prevent changes of PF4 gene expression.

DCs were isolated by using a magnetic separation technique (Blood Dendritic Cell Isolation Kit II; Miltenyi Biotech) according to the manufacturer's instructions. In brief, the isolation was performed in a two-step procedure. First, B cells and monocytes were magnetically labeled and depleted using a cocktail of CD19 and CD14 microBeads. B cells and monocytes were depleted in advance because a subpopulation of B cells expresses CD1c (BDCA-1), and monocytes express CD141 (BDCA-3) at low levels. Subse-

quently, the pre-enriched DCs in the nonmagnetic flow-through fraction were magnetically labeled and enriched using a cocktail of antibodies against the DC markers CD304 (BDCA-4/Neuropilin-1, PDC), CD141 (BDCA-3, PDC), and CD1c (BDCA-1, MDC). The highly pure enriched cell fraction comprises plasmacytoid DCs, CD1c (BDCA-1)+ type-1 myeloid DCs (MDC1s), and CD1c (BDCA-1)– CD141 (BDCA-3)+ bright type-2 myeloid DCs (MDC2s). The whole procedure required approximately 2 h. After magnetic separation, the cells were counted and the purity was checked by antibody staining (Blood Dendritic Cell Enumeration Kit II; Miltenyi Biotech), according to the instructions of the manufacturer. In brief, PDCs, MDC1, and MDC2 were identified by staining 50 μL of the yielded DC fraction with anti-BDCA-1-(CD1c)-PE, anti-BDCA-2-FITC (CD303), and anti-BDCA-3-APC monoclonal antibodies, respectively. B lymphocytes and monocytes as well as defective cells were excluded by simultaneously staining with anti-CD19-PE-Cy5 and anti-CD14-PE-Cy5 together with a fluorescent dead cell discriminator reagent. A parallel preparation, which served as control vehicle, contained a mixture of all FITC-, phycoerythrin (PE)-, and APC-conjugated isotype-identical antibodies. The cells were then washed, fixed with a formaldehyde solution, and finally, analyzed using a four-color dual laser flow cytometer (FACScalibur, Becton Dickinson, Heidelberg, Germany). A total of 100,000 events were acquired. Samples with a DC fraction below 90% of all vital cells were discarded.

Isolation of DC Total RNA and Whole Blood Total RNA

Total RNA of purified DCs obtained from patients and controls during the follow-up period was isolated using the RNeasy-system (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA was stored immediately at –80°C. Quality and amount of the RNA were determined using the NanoDrop ND-1000 device (NanoDrop Technologies, Wilmington, DE, USA).

Table 2. Recovery of purified DCs.

Day	MDC	PDC	RNA, ng/μL
0	$1.4 \times 10^4 \pm 0.3 \times 10^4$	$8.1 \times 10^4 \pm 1.4 \times 10^4$	47.4 ± 4.1
1	$1.9 \times 10^4 \pm 0.7 \times 10^4$	$5.3 \times 10^4 \pm 2.2 \times 10^4$	42.1 ± 3.1
4	$0.8 \times 10^4 \pm 0.5 \times 10^4$	$4.6 \times 10^4 \pm 1.0 \times 10^4$	42.9 ± 3.7

Real-Time Quantitative Polymerase Chain Reaction

In brief, each 100 ng of patient's RNA was reversely transcribed using the Affinity script QPCR-cDNA synthesis kit (Stratagene, La Jolla, CA, USA) thereby following the instructions of the manufacturer. Subsequently, the real-time quantitative reverse-transcription–polymerase chain reaction (qRT-PCR) was performed on a Stratagene MX3005p QPCR system (Stratagene) with each 100 ng reverse-transcribed total RNA per reaction. The PCR was performed using a commercial primer assay for human PF4 (NM_002619, unigene: Hs. 81564) purchased from SABiosciences (Cat# PPH00562B; SuperArray, Frederick, MD, USA). The sequence of this primer is not available. As reference gene, we measured the expression of *GAPDH* (upstream: AAG CTC ATT TCC TGG TAT GAC AAC G; downstream: TCT TCC TCT TGT GCT CTT GCT GG; MWG-Biotech, Martiensried Germany).

A melting-curve analysis was applied to ensure the specificity of the PCR reaction. Relative quantification of the mRNA levels of the target genes was determined using the comparative CT (threshold cycle values) method ($2^{-\Delta\Delta CT}$ method) In brief, the amount of target was first normalized to the reference gene *GAPDH* and then to a calibrator consisting of isolated DCs obtained from patients directly after hospital admission.

Statistical Analysis

Data are presented as median and interquartile range. Differences between the groups were compared using the nonparametric Kruskal–Wallis test followed by Dunn *post hoc* analysis. Demographic data as well as information about purity and RNA content of DCs were expressed as mean and standard error of the mean.

To analyze changes in *PF4* gene expression and intracellular protein content, as well as the surface expression of HLA-DR and adhesion abilities during the follow-up period (d 0 to 4), we applied Wilcoxon matched-pair analysis followed by Bonferroni correction. A *P*-value below 0.05 was considered significant. Correlation analyses were carried out using the Spearman rank test. All statistical analyses were performed employing SigmaStat 2.03 (Systat Software, Point Richmond, CA, USA).

RESULTS

In this study 10 patients were investigated after severe multiple trauma (Table 1). The mean ISS was 28 ± 6 points. The age ranged from 26 to 46 years (mean 30.3 ± 3.1 years). This patient group was compared with 10 healthy controls aged from 26 to 35 years (mean 29 ± 1.6 years). The harvested RNA content in multiple traumatized patients (n = 10) was 47.4 ± 4.1 ng/μL (d 0), 42.1 ± 3.1 ng/μL (d 1), and 42.9 ± 3.7 ng/μL (d 4) extracted from $8.2 \times 10^4 \pm 1.2 \times 10^4$ DCs (d 0), $8.9 \times 10^4 \pm 1.4 \times 10^4$ (d 1), and $9.7 \times 10^4 \pm 2.1 \times 10^4$ (d 4) isolated DCs (Table 2).

The ratio of MDCs to PDCs tended to decline in multiple trauma patients on d 1 after admission in comparison with healthy volunteers (control 0.92 ± 0.07, trauma d 1 0.78 ± 0.04, $P = 0.1$). On d 4 after admission the MDC/PDC ratio (0.55 ± 0.07) was significantly reduced in comparison with d 1 and with healthy volunteers ($P < 0.05$).

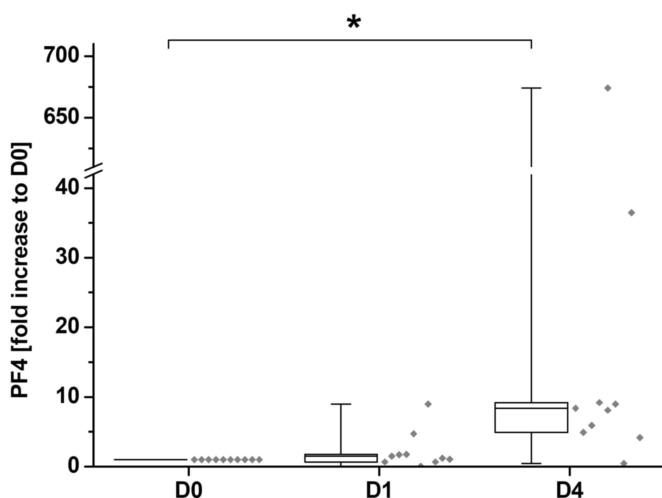

Figure 2. Realtime RT-PCR for PF4; median and interquartile ranges are shown. Results are presented as fold change of *PF4* gene expression on the d of admission (d 0). Messenger RNA was isolated from highly purified DCs obtained from whole blood of multiple trauma patients on d 0, 1, and 4 after admission. Gene expression was determined by the comparative 2-ΔΔCt method as described in the Materials and Methods section. A significant increase of PF4 expression on d 4 compared with d 0 was found (*$P < 0.05$).

Gene Expression of *PF4*

The real-time PCR showed an increase of PF4 expression in DCs obtained from trauma patients which was significant on d 4 after admission in comparison with d 0 ($P < 0.05$) (Figure 2).

PF4 Synthesis Is Enhanced in MDCs and PDCs

Intracellular PF4 content in MDCs of multiple trauma patients was significantly altered compared with healthy controls ($P < 0.05$). PF4 content in patients was significantly elevated both at d 1 and d 4 after trauma ($P < 0.05$, Figure 3A). Between d 1 and d 4, however, PF4 content of MDCs did not show a relevant difference. Similar results were obtained for intracellular PF4 content of PDCs (Figure 3B).

PMA Stimulation of MDCs and PDCs

Following PMA stimulation, the absolute PF4 content in MDCs of trauma patients and controls was significantly elevated as compared with unstimulated cells. Also, the stimulated cells of trauma patients showed significantly higher PF4 content compared with stimulated cells of healthy controls ($P < 0.05$, Figure 4A).

With PMA stimulation in PDCs, a significant elevation of PF4 content compared with the unstimulated PDCs occurred in healthy controls but not in trauma patients ($P < 0.05$, Figure 4B).

We also observed a significant correlation between the injury severity and PF4 concentration in MDCs as well as in PDCs (Table 3, $P < 0.05$).

PF4 synthesis of MDCs but not PDCs showed a significant correlation with patient outcome measured by the Glasgow Outcome Score (GOS) (MDC 0.7; $P < 0.04$ / PDC 0.4; $P < .24$). PF4 of the MDC subtype after LPS stimulation was also significantly correlated with the GOS (MDC 0.7, $P < 0.05$; PDC 0.4, $P < .35$).

Cell Adhesion Properties

Cell adhesion as tested by the cell adhesion assay (DC on HUVEC monolayer) was quantified as number of adherent DCs (MDCs or PDCs) per cm^2. Despite a slight but not significant decrease of adhesion of MDCs (adhering MDC d 1, 2808 ± 763; d 4, 2307 ± 596) and PDCs (adhering PDC d 1, 2973 ± 885; d 4, 2578 ± 778) on d 1 and d 4 after severe trauma no significant difference was found in patient cells compared to controls (adhering MDC, 4179 ± 1043; adhering PDC, 4926 ± 1484).

HLA-DR

Expression of HLA-DR on circulating MDCs was significantly increased in multiple trauma patients on d 1 and d 4 after admission compared with healthy controls ($P < 0.05$, Figure 5A). The surface density was inversely correlated with the patient ISS ($P < 0.05$, Table 3). HLA-DR surface expression on PDCs did not show differences between controls and trauma patients on d 1 and 4 (Figure 5B).

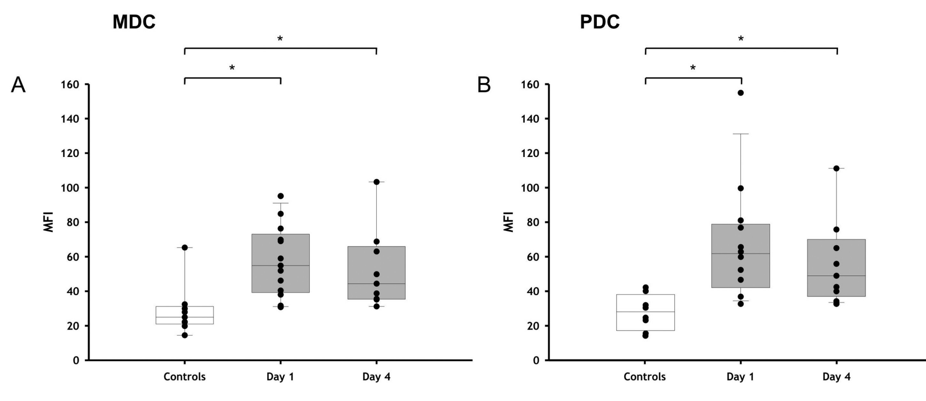

Figure 3. Intracellular PF4 content in MDCs (A) and PDCs (B) of patients and healthy controls. These experiments were performed with freshly isolated PBMC by means of flow cytometry. (*$P < 0.05$).

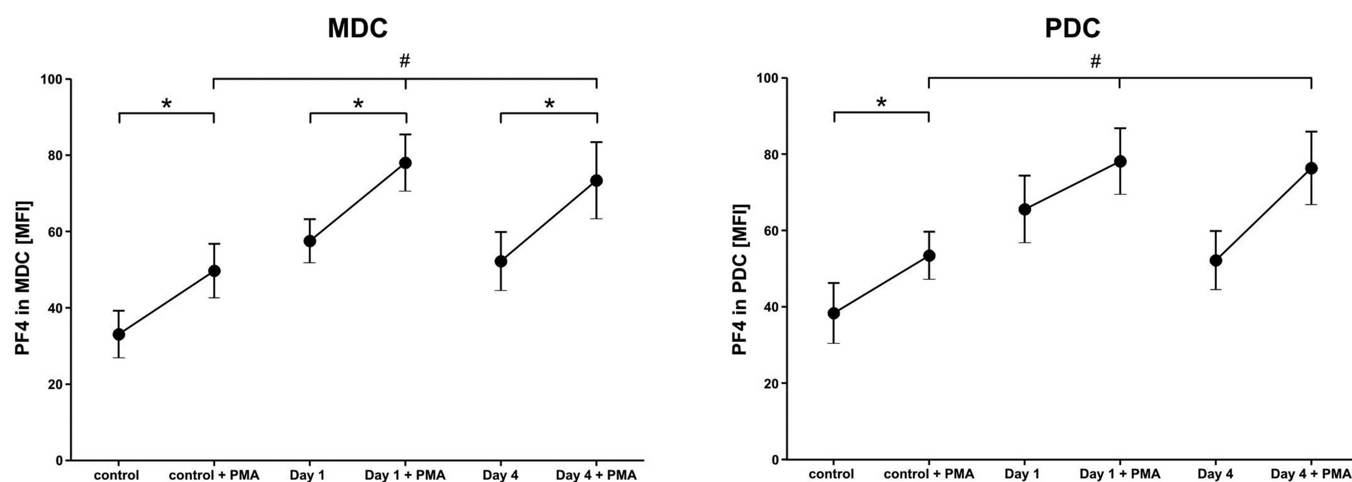

Figure 4. Intracellular PF4 content of patients and healthy controls before and after stimulation with PMA of MDCs (A) and PDCs (B) as measured by flow cytometry (*$P < 0.05$).

DISCUSSION

The present study shows for the first time that PF4 expression in the main DC subtypes—PDCs and MDCs—is significantly increased starting on day 1 and is maintained until day 4 after multiple trauma. A similar time scheme has been observed with other markers (for example, C-reactive protein, procalcitonin, and lipopolysaccharide-stimulated interleukin-1β synthesis) of inflammation during the posttraumatic inflammatory response (17,18). Moreover, a trauma-associated differential activation of DC subtypes with regard to the surface expression of HLA-DR can be found with enhanced expression on MDCs in contrast to PDCs. These results confirm and significantly extend the findings of our recent study, in which we demonstrated that PF4 is upregulated in DCs following multiple trauma.

The DC subtypes show tremendous differences concerning their cell surface receptors as well as the production of chemokines (13). This leads to different migratory properties and individual capacity to recruit different cell types at the site of inflammation (14). In a recent study we showed that the clinical impact of trauma leads to a decreasing number of MDCs whereas PDCs are not affected, which seems to be related to increased apoptosis of this cell type (17,19). Therefore the results of this study are presented with the analysis of the two subtypes—MDCs and PDCs.

Preliminary microarray analysis during the 5-day period following trauma revealed a significant increase of *PF4* gene expression until day 4 and a slight decrease afterward (day 5). We therefore chose to analyze samples obtained on day 1 and day 4. Therefore, however, we still do not know how PF4 is regulated during the subsequent period, for example, when secondary complications like sepsis or organ failure occur.

Originally PF4 was thought to be solely produced in platelets and this production was thought to be related to platelet-associated diseases like heparin-induced thrombocytopenia and autoimmune thrombocytopenic disorders (20). However, a number of recent studies revealed the complex role of PF4 within the microcirculation and immune system (7,21–23). Different immune-competent cells like monocytes, macrophages, neutrophils, and T cells have been shown to produce and interact with PF4 (2,5,24). It has been speculated that the role of PF4 after trauma might be the increase of cell adhesion, because this has been reported for neutrophils in other studies (7,25,26). However, cell-specific data regarding PF4 in a human population are rare and to our knowledge have not been reported in trauma.

Severe trauma leads to an early activation of DC transcription with a significant increase over the first day as qRT-PCR showed in this study. Intracellular PF4 was significantly increased in both DC subtypes (MDCs and PDCs) on day 1 compared with controls. However, in both DC subtypes the stimulatory response of DCs concerning the production of PF4 observed on day 1 does not further increase until day 4 after trauma. It seems that the activation that occurs with the trauma impact does not allow further stimulation. Although this finding has not yet been observed in DCs, experimental and clinical data both indicate that the overactivation of the

Table 3. Correlation of HLA-DR and PF-4 with ISS evaluated separately for MDCs and PDCs.*

	MDC	PDC
HLA-DR	Rho = –0.6; $P < 0.05$	Not significant
PF4	Rho = 0.54, $P < 0.05$	Rho = 0.65, $P < 0.05$

*Results presented with correlation coefficient (rho) and level of significance (*P*).

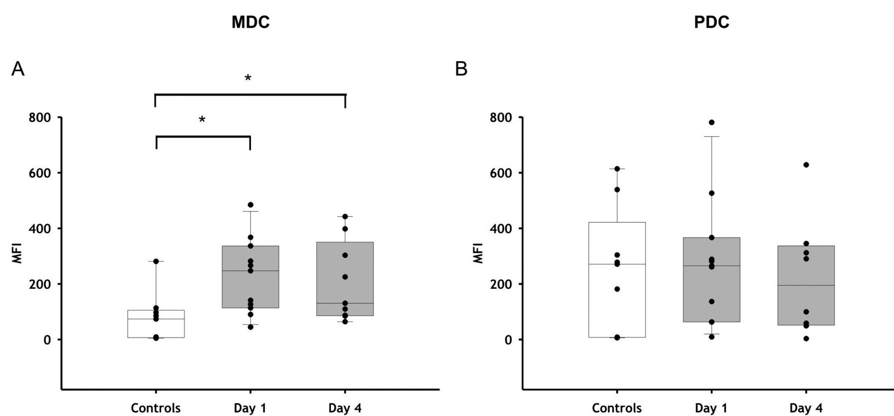

Figure 5. Surface expression of HLA-DR in MDCs and PDCs, median and interquartile ranges are shown. Measurement of HLA-DR-expression was performed using flow cytometry in whole blood obtained from multiple trauma patients on d 1 and d 4 after admission and healthy controls as described in the materials and methods section (*$P < 0.05$).

immune system after severe trauma is often followed by a period of immunosupression (27,28). Our data might support this observation in DCs; however, more specific investigations are necessary to confirm this hypothesis. Intracellular PF4 synthesis did not show any difference between the subtypes of MDC and PDC. This finding is in agreement with the results of an earlier report that some chemokines are predominantly produced by MDC (CCL17, CCL22) and PDC (CCR1, CCR5) whereas others, like CCL4 (PF4) and CXCL8, are produced in both subtypes (13). However, *in vitro* stimulation of MDCs and PDCs with PMA showed a further significant increase of intracellular PF4 only for MDCs, and this increase was observed on day 1 and day 4 after trauma (Figure 4). PMA is a reliable stimulator of DCs, and although it is primarily used in cancer research, it provides valuable insight into the pathophysiology of various inflammatory conditions (29,30). Using PMA, we observed that the DCs—and especially the MDCs—can be further activated to produce PF4 even several days after trauma, when the initial trauma impact does not further support this activation. MDC subsets are considered "naive" immature cells that are migrating from bone marrow to the site of inflammation, whereas PDCs are predominantly localized in secondary lymphoid organs. Hypoxia seems to promote the migratory functions of DCs (31). Focusing on the systemic DC content, it seems obvious that the MDC subtype is more adjustable or more sensitive toward changes of the environment, at least at this early stage after trauma. Nevertheless activation of DCs *in vivo* certainly depends on various factors that need to be elucidated.

Increased cell adhesion is observed in a number of cells as part of the posttraumatic inflammatory response, and increased DC adhesion on PF4 was observed in former *in vitro* studies (7,26,32). In addition, PF4 also interacts with the vessel wall and upregulates E-selectin (32,33). Therefore, we analyzed the posttraumatic DC adhesiveness on HUVEC. However, the cell adhesion of MDCs and PDCs *in vitro* did not show any changes over the observed time period compared with the control group in our study. Although previous studies suggest a relation between PF4 and cell adhesion, apparently this finding does not relate to posttraumatic DC activation and PF4 upregulation.

We found a significant correlation between ISS and HLA-DR only for MDCs but not for PDCs, a difference that highlights the functional variabilities of DC subtypes (Table 3). Previously, HLA-DR surface expression *in vitro* was significantly increased on circulating MDCs but not on PDCs, and only MDC HLA-DR showed a significant inverse correlation with the ISS. Taken together, such findings provide increasing evidence for a possible connection between HLA-DR and PF4 content in DCs (4,6); however, a pathophysiological link between HLA-DR and PF4 after trauma remains to be elucidated.

As we showed earlier, the systemic MDC fraction decreases after trauma compared with the constant PDC fraction (34). The activated HLA-DR expression on MDCs compared with PDCs might therefore further highlight the high sensitivity of this DC subtype after trauma, a characteristic that has also been observed on MDCs after other activation triggers (35). Because HLA-DR is essential for DCs to present antigenic peptides to $CD4^+$ T cells, the observed upregulation of HLA-DR on MDCs indicates the activated process of antigen presentation to the immune system to elicit or suppress T-(helper)–cell responses and DC-enforced participation in the immune response after trauma (36).

PF4 expression of both DC subtypes was significantly correlated with the ISS. Interestingly, *PF4* expression of MDCs but not PDCs was significantly correlated with the outcome as measured with the GOS. Because of the limited number of patients in this study, the interpretation of this result is critical; however, the injury severity has an influence on the immune response and this might also include PF4 production in DCs (37).

CONCLUSION

DCs situated at the interface between the innate and the acquired immune systems show an immediate and subsequent increase of *PF4* expression and intracellular translation early after severe trauma. The receptor modulation emphasizes an individual and specific DC reaction of the DC subtypes after trauma that might also be related to trauma severity and outcome. Further studies are necessary

to evaluate the mechanisms leading to the processes we observed in this study.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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