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ORIGINAL ARTICLE

P-selectin glycoprotein ligand-1 deficiency augments G-CSF induced myeloid cell mobilization

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Abstract The effect of granulocyte colony-stimulating factor (G-CSF) was investigated in P-selectin glycoprotein ligand-1 (PSGL-1) deficient (PSGL-$1^{-/-}$) and wild-type (PSGL-$1^{+/+}$) mice to establish the role of this mucin in myeloid cell mobilization. G-CSF activates tissue proteases that cleave adhesion molecules, thus enhances the mobilization of myeloid cells and haematopoietic stem cells. Cytopenia was induced with a single dose of cyclophosphamide. In PSGL-$1^{-/-}$ animals, we observed a delayed extravasation of mature myeloid cells from the peripheral vessels into the tissue compartments and their faster mobilization from the bone marrow. Subsequently, animals received G-CSF twice a day for 4 days. Neutrophil and monocyte counts increased upon completion of G-CSF treatment and both values were significantly higher in PSGL-$1^{-/-}$ mice; 47.7 versus 28.3 G/l for neutrophils and 4.1 versus 2.0 G/l for monocytes. The ratio of atypical myeloid cells was also elevated. Analyzing the causes of the above differences, we identified a 4-fold increase in the colony-forming unit (CFU-GM) counts of the peripheral blood in PSGL-$1^{-/-}$ mice, compared to wild-type animals. A significantly elevated number of CFU-GM was detected also in the femurs of PSGL-$1^{-/-}$ mice, 4 and 5 days after cyclophosphamide treatment and these values paralleled with the elevation of CD34+/CD117+ stem cell counts in the peripheral blood. Our data suggest, that in the absence of PSGL-1, G-CSF was more potent in elevating absolute myeloid cell numbers by acting on cell release from the bone marrow, maturation from circulating precursor cells in the peripheral blood and prolonged retainment in the circulation.

Keywords P-selectin glycoprotein ligand-1 · G-CSF · Myeloid cells · Colony-forming units · Stem cells · Mobilization

Abbreviations

ABC	Antibody binding capacity
ACD	Acid citrate dextrose
CFU-GM	Colony-forming unit granulocyte/macrophage
CXCR4	Chemokine C-X-C motif receptor 4
FITC	Fluorescein isothiocyanate
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte–macrophage colony-stimulating factor
HSC	Hematopoietic stem cells
IHC	Immunohistochemical reaction
IL	Interleukin
MFI	Mean fluorescence intensity
PSGL-1	P-selectin glycoprotein ligand-1
R-PE	R-Phycoerythrin
SDF-1	Stromal cell-derived factor 1
VCAM-1	Vascular cell adhesion molecule-1

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Introduction

Mobilization of leukocytes and leukocyte precursors from the bone marrow became a standard method in harvesting haematopoietic stem cells for both autologous and allogeneic transplantation. The circulating mouse stem cell has been described 50 years ago (Goodman and Hodgson 1962) and it was subsequently shown that the number of circulating hematopoietic progenitor cells (HPCs) could be augmented by chemotherapy, endotoxin administration, or stress (Richman et al. 1976; Cline and Golde 1977). Recently, mobilized HPCs have been routinely used in the clinical practice as a source of hematopoietic stem cells (HSCs) (Frenette and Weiss 2000). Multiple agents induce peripheral blood stem cell mobilization, each with different kinetics and efficiencies (Lord et al. 2001; Pelus 2008). The most commonly used mobilizer is the gold standard granulocyte colony-stimulating factor (G-CSF, filgrastim) or its longer acting form pegfilgrastim (Fenk et al. 2006; Gertz 2010). Granulocyte–macrophage colony-stimulating factor (GM-CSF) mobilizes less CD34+ cells than G-CSF (Lane et al. 1995). The bicyclam AMD3100 (plerixafor, Mozobil) induces HSC and HPC mobilization in mice and humans via a different mechanism. It blocks the interaction between the chemokine C-X-C motif receptor 4 (CXCR4) and stromal cell-derived factor 1 (SDF-1); moreover, it synergizes with G-CSF (Broxmeyer et al. 2005; Nervi et al. 2006; Paganessi et al. 2011). In recent trials, the combined use of AMD3100 and G-CSF mobilized more CD34+ cells in fewer days than G-CSF alone and allowed more patients to proceed to autotransplantation (Pelus 2008; Micallef et al. 2009; Dugan et al. 2010).

G-CSF induces the release of a number of proteases into the bone marrow via acting on multiple cell types. Neutrophil elastase, cathepsin G as well as matrix metalloproteases — through mostly unexplored mechanisms — all contribute to this process. These proteases cleave several molecules that are thought to play an important role in HSC tethering and anchorage, including c-kit, VCAM-1 (vascular cell adhesion molecule-1), CXCR4, and SDF-1 (Nervi et al. 2006).

P-selectin glycoprotein ligand 1 (PSGL-1) is a dimeric mucin that is the best characterized ligand for all types of selectins. PSGL-1 binds to P-selectin, E-selectin and L-selectin under flow conditions (Eto et al. 2005; Zarbock et al. 2011; Nagy et al. 2012). This protein is expressed constitutively on the surface of leukocytes and plays an important role in homo- and heterotypic interactions with platelets and endothelial cells contributing considerably to transendothelial migration of leukocytes (McEver 2001; Miner et al. 2008; Figueiredo et al. 2013). In a previous study we have established the usefulness of a PSGL-1 knockout (PSGL-$1^{-/-}$) mouse strain in studying multicellular interactions during thrombus formation (Miszti-Blasius et al. 2011). In our study, we hypothesized, that in the same animal model, the lack of PSGL-1 would modulate leukocyte release from the bone marrow via perturbed cellular interactions of leukocytes and endothelial or stromal cells. The effect of G-CSF on peripheral blood myeloid cell counts, peripheral blood and bone-marrow colony-forming units and stem cells were investigated in PSGL-$1^{+/+}$ and PSGL-$1^{-/-}$ mice after cyclophosphamide-induced cytopenia.

Here, we provide for the first time morphological and flow cytometric evidence that in the absence of PSGL-1, mature myeloid cells, their precursors and mouse stem cells display faster mobilization from the bone marrow and delayed extravasation in the peripheral vessels.

Materials and methods

Laboratory animals

C57B6/126 PSGL-1 PSGL-$1^{+/+}$ and PSGL-$1^{-/-}$ mice were used as it was previously described (Xia et al. 2002). PSGL-1 KO mice are healthy, have a normal lifespan, have moderately elevated total leukocyte count. Mice were weaned at 4 weeks, maintained on a 12-h light/12-h dark cycle at 21 °C, and fed water and standard rodent chow (VRF1 Charles River, Germany) ad libitum. All procedures were approved by the Animal Care and Use Committee of University of Debrecen and tissues were obtained in accordance with the guidelines (1/2006 DE MÁB, 8/2011 DE MÁB).

Blood collection and cell counting

Mice were anesthetized with inhaled halothane (Sigma-Aldrich, St. Louis, MO, USA) and 200 μl blood was collected into a cup containing 40 μl ACD (acid citrate dextrose) (from Vacutainer tube Becton Dickinson Diagnostics-Preanalytical Systems, Plymouth, UK) by puncture of the retrobulbar venous plexus with a 30 mm long glass capillary. ACD anticoagulated whole blood was analysed by Siemens Advia-120 hematology analyzer (Deerfield, IL, USA) with Multi Species software (Palicz et al. 2013). Blood smears were prepared manually and stained with May–Grünwald Giemsa stain by Wescor Aerospray 7120 (Wescor, Utah, USA) and evaluated with Zeiss AxioStar (Carl Zeiss, Jena, Germany) microscope.

Induced cytopenia and G-CSF treatment

Wild-type and knockout male (Nemeth et al. 2010) mice matched for age (12–16 weeks) were anesthetized with Halothane (Sigma-Aldrich, St. Louis, MO, USA) and a single dose of cyclophosphamide (Endoxan, Baxter, IL, USA) was administered intraperitoneally (250 mg/kg) to reach cytopenia. G-CSF (Neupogen; Amgen, Thousand Oaks, CA,

USA) treatment was applied twice a day at 7.8 μg/kg body weight subcutaneously for 4 days.

Flow cytometric analysis

For flow cytometric analyis of mouse peripheral blood samples three-color stainings were applied by using rat anti-mouse CD34/CD162/CD45 and CD34/CD117/CD45 combinations. R-Phycoerythrin (R-PE)-conjugated rat anti-mouse CD162 (PSGL-1), R-Phycoerythrin (R-PE)-conjugated rat anti-mouse CD117, fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD34 and Peridinin-chlorophyll proteins (PerCP)-conjugated CD45 monoclonal antibody were from BD Pharmingen (Becton Dickinson, Franklin Lakes, NJ, USA). Flow cytometric analyses were performed on a FACSCalibur flow cytometer (50 000 events were collected) and data were analysed by the CELLQuest 3.2 software (Becton Dickinson, Franklin Lakes, NJ, USA).

Quantitative evaluation of PSGL-1 expression

We have determined surface PSGL-1 expression on mouse and human myeloid cells and analysed the effect of G-CSF added in vitro to blood samples on the expression of this mucin. Measurements were carried out by using PE conjugated mouse anti-human CD162 antibody (BD Pharmingen, Becton Dickinson, Franklin Lakes, NJ, USA) and in PSGL-$1^{+/+}$ mice a rat anti-mouse CD162-PE (BD Pharmingen, Becton Dickinson, Franklin Lakes, NJ, USA) was applied. Mean fluorescence intensity (MFI) values were recorded and values were compared to MFI values obtained by measuring the QantiBRITE-PE beads (Becton Dickinson Biosciences, San Jose, CA, USA). This enabled us to evaluate the PSGL-1 expression in terms of antibody binding capacity (ABC) values similarly as previously described by using different beads for calibration (Kappelmayer et al. 2001).

Tissue processing for quantitative immunohistological analysis and immunohistochemistry

The applied method has been described earlier (Miszti-Blasius et al. 2011). Shortly, after harvesting the mice, femurs were removed for histological processing. After fixation, tissues were placed into 10 % EDTA; pH 7.2 (Solon, Ohio) for approximately 2 weeks. Decalcified and dehydrated tissue samples were embedded into wax at 54 °C and 5–6 μm thick longitudinal sections were cut (Microm HM335E; Microm International GmbH, Walldorf, Germany) perpendicular to the long axis of femur. Paraffin sections were placed on gelatin-coated glass slides and left dried overnight at 37 °C. To identify CD34, standard immunohistochemical (IHC) reaction and ABC techniques were performed as follows: Before IHC staining, sections were deparaffinised in xylene and hydrated through graded alcohols to water. After rehydration, sections (5–7 μm) were subjected to 0.5 % H_2O_2 in absolute methanol to block the endogenous peroxidase activity. After PBS washing, slides were rinsed in 2 % BSA in PBS for 30 min at 37 °C in a humid chamber to minimize non-specific staining. Then, the sections were incubated for 1 h at 37 °C with the rabbit anti-CD34 antibody (Chemicon, Temecula, CA, USA) in PBS (1:75, containing 0.1 % BSA). After a brief wash in PBS, the samples were treated for 1 h at room temperature with biotin-conjugated anti-rabbit secondary antibody (Vectastain Elite ABC Kit; Vector, Burlingame, CA, USA) diluted in 1:200 PBS containing 0.1 % BSA. Then, the slides were treated for 30 min at room temperature with streptavidin–biotin peroxidase complex (Vectastain Elite ABC Kit, Vector). After rinsing in PBS, the reaction was visualised with Vector peroxidase substrate Kit from Vectastain Kit. After the last PBS wash, slides were rinsed with dH_2O and mounted in Aquamount (Thermo Scientific, Rockford, IL, USA). Appropriate negative control reactions were performed as follows: control sections were stained similarly, but the primary antibody was omitted and either replaced by nonimmune IgG (IgG2a; Sigma, St. Louis, MO, USA) or with the non-immune serum of that rabbit in which the CD34 antibody was produced. The different negative controls revealed no labelling. Images were recorded by a digital camera (Olympus DP50, Tokyo, Japan) connected to Nikon Eclipse 800 (Nikon, Tokyo, Japan). Acquired and presented images are representative of all the samples examined.

CFU-GM colony formation assay

Mononuclear cells were separated both from peripheral blood and bone marrow and were grown in special soft gel cultures as described earlier (Benko et al. 1999, 2001; Géresi et al. 2012). Briefly, peripheral blood was collected from the retro-orbital venous plexus into tubes containing 50 units of heparin and was diluted with equal volume of phosphate buffered saline solution. After exterminating the mice, bone marrow was obtained by expulsion from their femurs under sterile conditions and single cell suspensions were prepared in McCoy's 5A medium. The mononuclear cell fraction, from both peripheral blood and bone marrow, was separated by density gradient centrifugation using Ficoll-Paque™ Plus density gradient media (specific gravity, 1.077 g/ml) (GE Healthcare Biosciences AB, Uppsala, Sweden). The separated mononuclear cells from peripheral blood and bone marrow were plated in Petri dishes (Greiner, Nürtingen, Germany) at a density of 6×10^5 and 1×10^5 cells per dish, respectively. Cells were grown in McCoy's 5A modified medium (Sigma-Aldrich, Budapest, Hungary) supplemented with amino acids, Na pyruvate, $NaHCO_3$, antibiotics (streptomycin, penicillin) and 20 % fetal bovine serum. In the presence of WEHI-3B conditioned medium, a crude source of interleukin-3 and

granulocyte-colony stimulating factor, granulocyte–macrophage progenitor cells (colony forming unit-granulocyte–macrophage [CFU-GM]) form colonies in special soft gel cultures. Methylcellulose (Methocel, 3,000–5,000 cP; Fluka, Buchs, Switzerland) at 1.2 % was used as the support matrix for these semi-solid cultures. Cultures were grown in duplicates for 7 days at 37 °C at 100 % relative humidity in an atmosphere containing 5 % CO_2. Colonies were defined as groups of at least 50 cells and were counted under a stereomicroscope (Olympus, Hamburg, Germany) at the end of the incubation period.

To further characterize granulopoiesis, in addition to measuring the frequency of granulocyte–macrophage progenitors by counting the colonies in the CFU-GM colony assay, the total CFU-GM content of femoral bone marrow was also estimated. The number of nucleated cells per femur (cellularity) was calculated from nucleated cell density and the volume of bone marrow suspension, and the total CFU-GM content is the product of cellularity and CFU-GM frequency.

Statistical analysis

For comparison of data showing Gaussian approximation Student's *t*-test was used. In case of nonparametric distribution results were evaluated by Mann–Whitney test with GraphPad Prism 4.0 software (La Jolla, CA, USA).

Results

Leukocyte elimination after induced cytopenia

Leukocyte count decreased by 80–90 % in both mouse strains after cyclophosphamide administration however, the elimination kinetics differed in the two strains. In PSGL-$1^{+/+}$ animals, the absolute counts of all cell types decreased while in PSGL-$1^{-/-}$ mice the neutrophil and monocyte absolute numbers were elevated on day 1. It started to decline on day 2 and only returned to baseline value. Both strains showed the lowest values on day 3. Knockout mice displayed a significantly larger rebound effect on day 6 peaking at three times the baseline values (Fig. 1).

Spontaneous leukocyte mobilization

When this rebound effect was analysed in detail in seven wild-type and seven knockout mice it was observed that in PSGL-$1^{-/-}$ animals at day 7 both neutrophils and monocytes displayed significantly higher counts. Seven days after the injection of cyclophosphamide the neutrophil counts were 3.0 G/l in wild-type and 12.5 G/l in knockout animals (Fig. 2a) and decreased considerably by day 11. The monocyte count decrease could be observed only in the PSGL-$1^{+/+}$ strain after the seventh day (Fig. 2b) but it also returned to

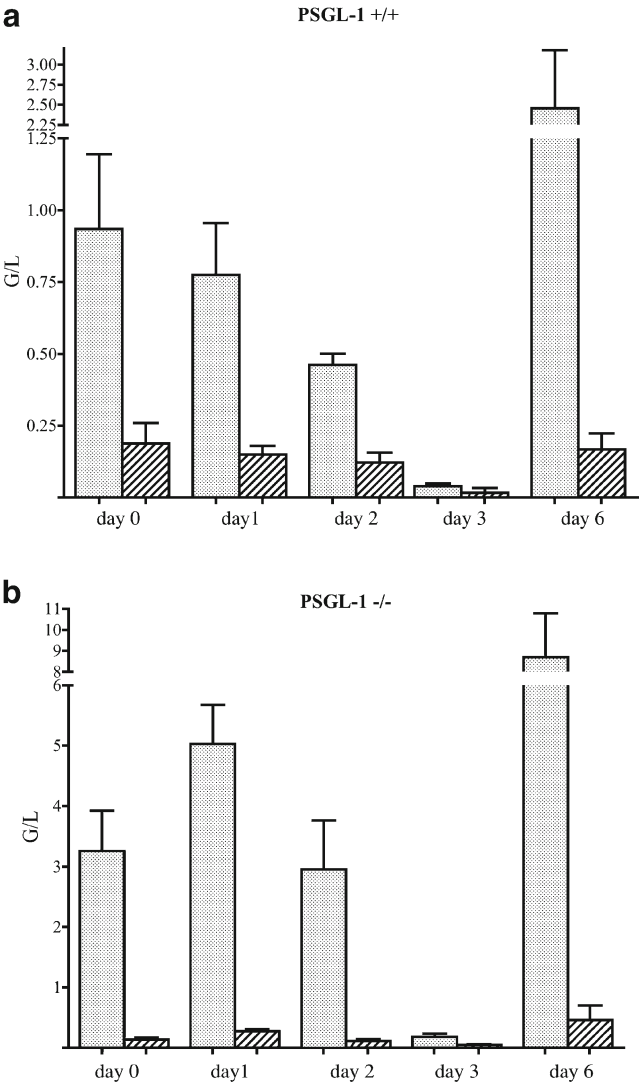

Fig. 1 Elimination of leukocytes from peripheral circulation upon induced cytopenia is different in wild-type and knockout animals. The effect of a single dose of cyclophosphamide was used in this experiment ($n=4$). The *dotted bar* shows neutrophil; *striple bar* represents monocyte absolute cell count changes in PSGL-$1^{+/+}$ (A) and knockout (B) mice

normal in the PSGL-$1^{-/-}$ mice when recovery was followed up to day 16 (data not shown). Contrary to these cells, eosinophilic granulocytes displayed a delayed response and were nearly undetectable at day 7 and only became elaveted by day 11 being much higher in knockout animals too (Fig. 2c). Furthermore at day 7, morphologically atypical neutrophils (precursor cells) also appeared that were barely detectable before the induced cytopenia (Fig. 2d).

G-CSF induced leukocyte mobilization

In another series of experiments, mice received 7.8 μg/kg body weight exogenous G-CSF during 4 days (twice a day). Four hours after the last injection of G-CSF, retro-orbital blood samples were drawn and the WBC count was determined. In 15 wild-type mice and in 15 knockout mice the

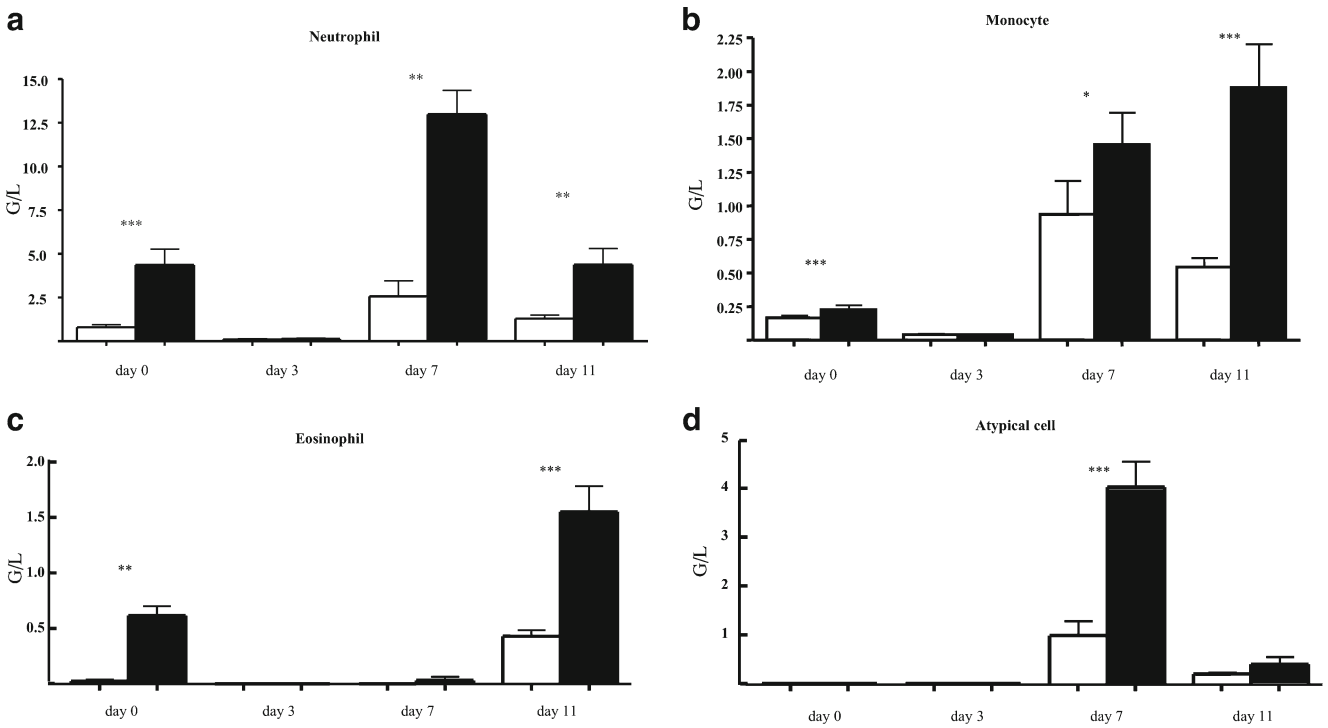

Fig. 2 In a series of experiments mice did not receive exogenous G-CSF after induced cytopenia ($n=7$). Neutophil (**a**), monocyte (**b**), eosinophil (**c**) and atypical cell counts (**d**) were expressed as 10^9/l. *Open bars* represent WT mice and *solid bars* represent KO mice. PSGL-1 $^{-/-}$ animals displayed significantly elevated values of all cell types, with a delay in eosinophil counts. *$p<0.05$, **$p<0.01$, ***$p<0.001$

mean neutrophil counts were 28.3 and 47.7 G/l, while the mean monocyte counts were 2.0 and 4.1 G/l, respectively. Neutrophil, monocyte and eosinophil absolute cell counts were significantly elevated ($p<0.05$ to $p<0.001$) in PSGL-$1^{-/-}$ compared to PSGL-$1^{+/+}$ mice (Fig. 3). The absolute number of neutrophils and monocytes but not eosinophils and atypical cells exceeded that of the results of the previous experiments without exogenous G-CSF treatment.

The effect of G-CSF on PSGL-1 expression

In four human and four mouse blood samples the analysis of PSGL-1 expression was carried out on neutrophils after various incubation periods with G-CSF in vitro. Results were expressed in terms of ABC that directly related to receptor copy numbers on the surface of neutrophil leukocytes. Although there was a considerable spontaneous shedding of PSGL-1, we confirmed previous reports (Jilma et al. 2002) that PSGL-1 is downregulated in human (data not shown) as well as in mouse neutrophils (Fig. 4). The amount of PSGL-1 on PSGL-$1^{+/+}$ mouse neutrophils was in the range described previously for humans (Ushiyama et al. 1993; Kappelmayer et al. 2001).

CFU-GM analysis in the peripheral blood and bone marrow

Based on the results of the colony-forming assays, we found a continuously higher CFU-GM count in the peripheral blood in the PSGL-$1^{-/-}$ mice even after returning to the likely baseline values on day 5. They released four times as much CFU-GM into the circulation than PSGL-$1^{+/+}$ mice (Fig. 5a). A considerable reduction was seen by day 5, but the number of the mobilized CFU-GM remained significantly higher ($p<0.01$) compared to wild-type mice.

In the bone marrow, cellularity was investigated as total mononucleated cell number, that decreased on the first days after cyclophosphamide-induced damage as a consequence of the death of the proliferating progenitor cells (Fig. 5b). The cellularity mirrored the whole haemopoiesis; however, the CFU-GM participated in the regeneration of cellularity of bone marrow that paralleled with expansion of the CFU-GM pool. In the bone marrow, the number of CFU-GM colonies per 10^5 mononuclear cells increased in parallel in both PSGL-$1^{-/-}$ and PSGL-$1^{+/+}$ mice after induced cytopenia, but their number became significantly higher in PSGL-$1^{-/-}$ animals on day 3 ($p<0.05$ on Fig. 5c). As a result, the expansion of the CFU-GM population was significantly higher in PSGL-$1^{-/-}$ than in wild-type mice on days 4 and 5 in the bone marrow (Fig. 5d).

Stem cell analysis in the peripheral blood and the bone marrow

These changes were in agreement with the peripheral blood CD34+/CD117+ mouse stem cell numbers, since on day 5 these numbers were also significantly higher in PSGL-$1^{-/-}$

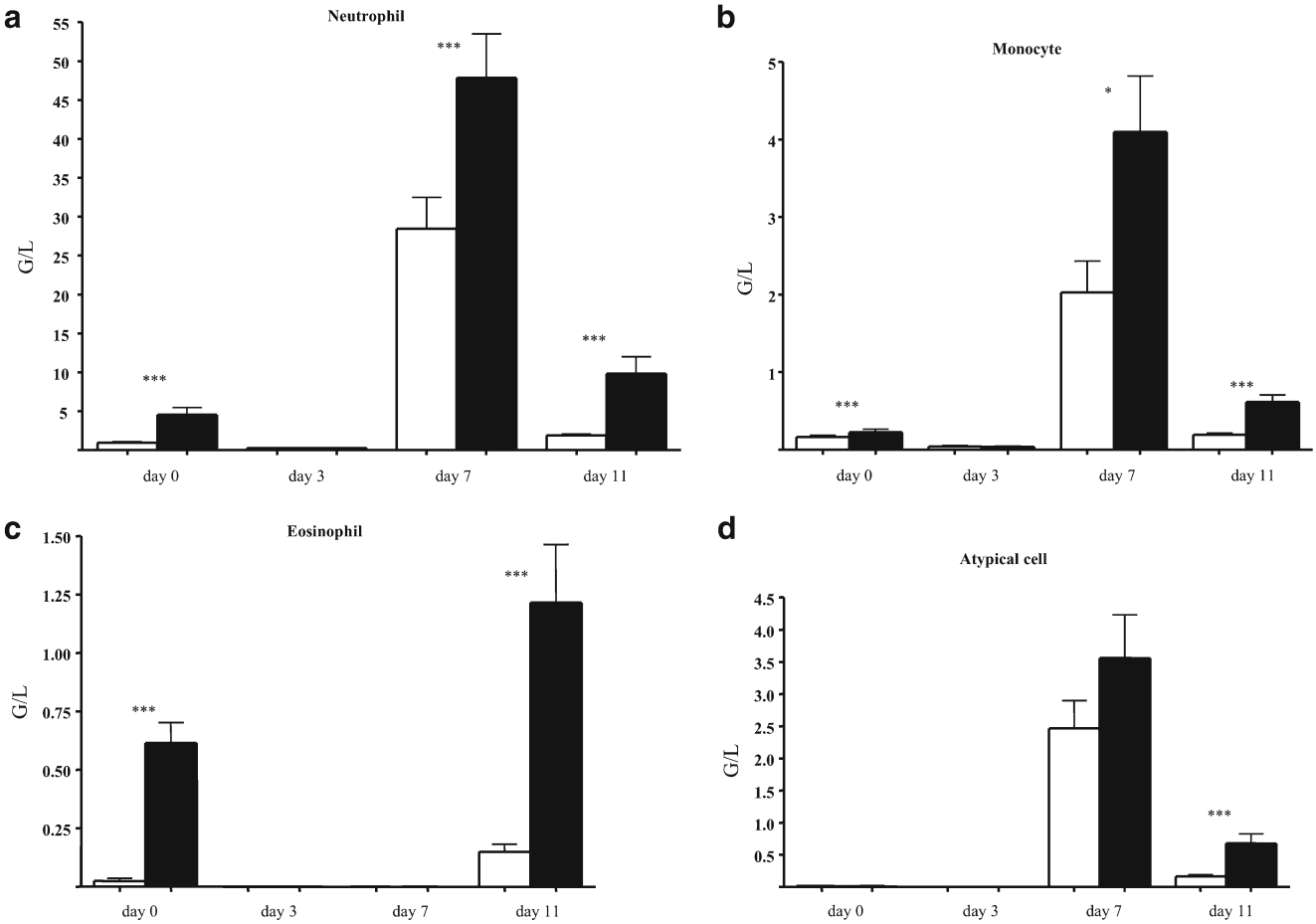

Fig. 3 The effect of exogenous G-CSF after induced cytopenia, resulted in similar differences with much larger absolute counts for neutrophils and monocytes. Cell counts after treating mice with G-CSF (7.8 μg/kg) ($n=15$). Neutophil (**a**), monocyte (**b**), eosinophil (**c**) and atypical cell counts (**d**) expressed as 10^9/l. *Open bars* represent WT mice and *solid bars* represent KO mice. *$p<0.05$, **$p<0.01$, ***$p<0.001$

mice compared to wild-type animals as detected by two-color flow cytometry (Fig. 6). By day 7, however, the stem cell numbers considerably increased in the peripheral blood in the PSGL-$1^{+/+}$ mice, that could also be verified in the bone marrow by immunohistochemistry (Fig. 7).

Discussion

Cellular release from the bone marrow is orchestrated mostly by receptor pair interactions, that are all required to maintain adequate release of precursor and mature myeloid cells upon

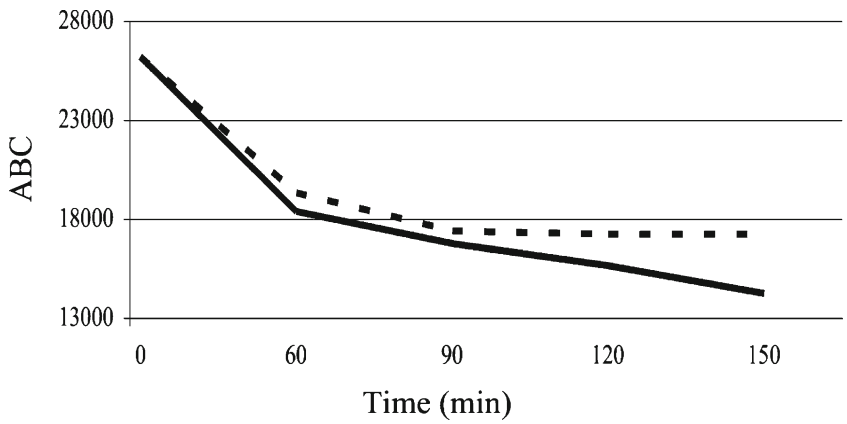

Fig. 4 ACD-anticoagulated retro-orbital blood samples from PSGL-$1^{+/+}$ mice were incubated in the presence (*continuous line*) or absence (*dashed line*) of 60 μg/l G-CSF. Mouse neutrophils downregulated surface PSGL-1 spontaneously and even more in the presence of G-CSF. *ABC* antibody binding capacity (representative of four similar experiments)

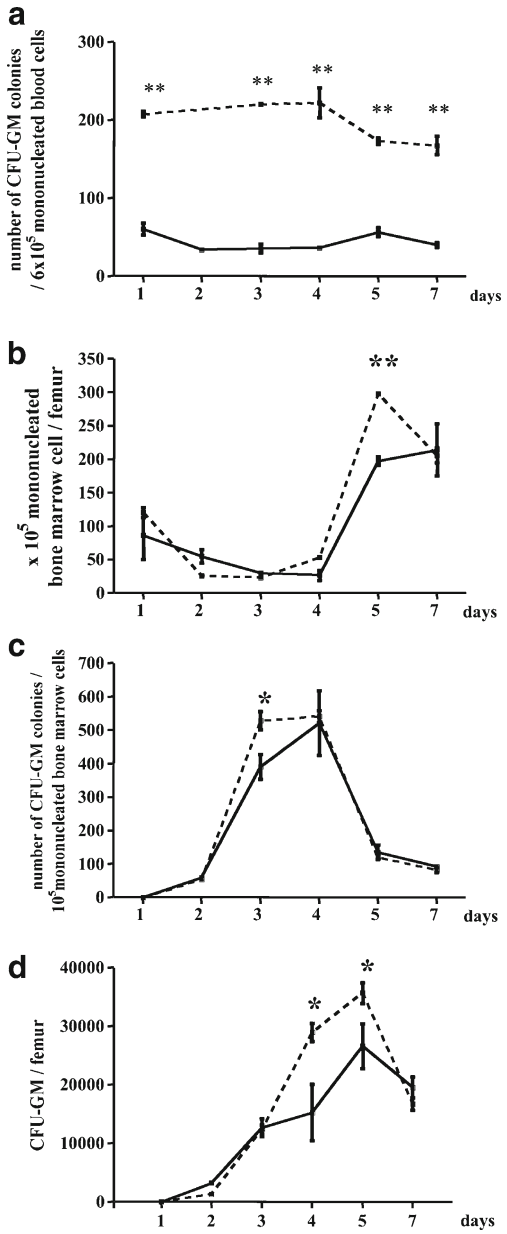

Fig. 5 Colony-forming units and mononuclear cells were evaluated in the bone marrow and peripheral blood after cyclophosphamide treatement, with no G-CSF added. **a** Peripheral blood CFU-GM numbers. The *dashed line* shows the results of PSGL-$1^{-/-}$ and the *continuous line* displays the values of PSGL-$1^{+/+}$ animals. Differences at all time points were highly significant. In the bone marrow the mononuclear cell numbers and the number of CFU-GM colonies were determined (**b** and **c**), and based on these values the CFU-GM content in the femurs were established (**d**). The number of CFU-GM colonies was significantly elevated on day 3 in the PSGL-$1^{-/-}$ strain that resulted in a significantly higher mononuclear cell count and CFU-GM content in the femurs that were observed on day 4 and day 5. *$p<0.05$, **$p<0.01$

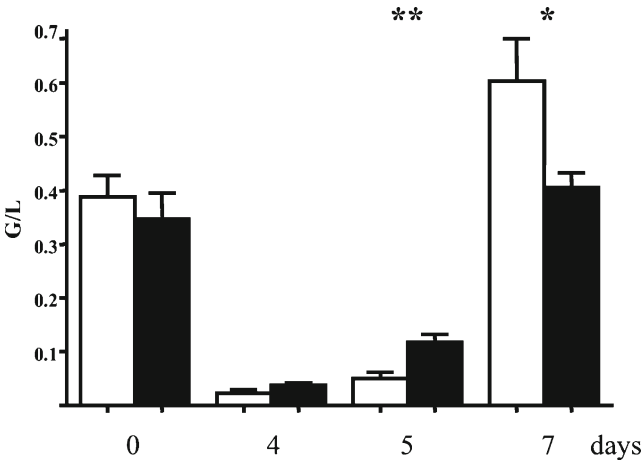

Fig. 6 The kinetics of CD34/CD117 double positive cells after cyclophosphamide treatment. Absolute count of CD 34 and CD117 double positive cells (10^9/l) on 0, 4, 5 and 7 days after cyclophosphamide treatment are displayed ($n=4$–7). *Open bars* represent WT mice and *solid bars* represent KO mice. *$p<0.05$, **$p<0.01$

appropriate signals. These interactions have been examined in detail (Wojciechowski et al. 2008; Zarbock et al. 2011); however, it is not fully clear as to what extent these receptorial interactions are redundant, or whether these bindings are all important for a timely cellular release. Therefore in the mouse model experiments presented here our aim was to provide data on the effect of PSGL-1 in mediating cellular anchorage. Lack of PSGL-1 may impair cellular interactions at two sites. On one hand, the receptor is expressed in the extracellular matrix (Nervi et al. 2006), and this mucin binds to the leukocyte L-selectin facilitating adhesion of haematopoietic stem and progenitor cells. Furthermore, this dimeric mucin is crucial in the extravasation of myeloid cells in the peripheral blood. Activated endothelial cells express P-selectin and E-selectin that are the primary determinants in short-lived interactions with rolling leukocytes (Laszik et al. 1996; Patel and McEver 1997; Sperandio et al. 2003). Leukocytes establish this connection via the constitutively expressed PSGL-1 molecule (Kappelmayer et al. 2004). Here, for the first time, we provided experimental evidence that in the absence of PSGL-1, neutrophils and monocytes extravasate considerably slower and thus are retained in the circulation for prolonged periods when released from the bone marrow after cyclophosphamide induced cytopenia.

The release of different myeloid cell types from the bone marrow was variable. Neutrophils and monocytes were released with quite similar kinetic pattern both with or without the use of exogenous G-CSF. The ratio of myeloid cell increase in both set of experiments were similar in the two strains, but this resulted in significantly higher absolute myeloid cell numbers in the PSGL-$1^{-/-}$ mice. The only difference between the two set of experiments was that without exogenous G-CSF, monocytes reached their peak count on day 11, while with exogenous G-CSF their number peaked already at day 7 similarly to neutrophils. This may be related to the slower release of monocytes from the bone marrow or much rather for their prolonged presence in the circulation in the absence of PSGL-1 but these differences disappeared by day 16 (data not shown).

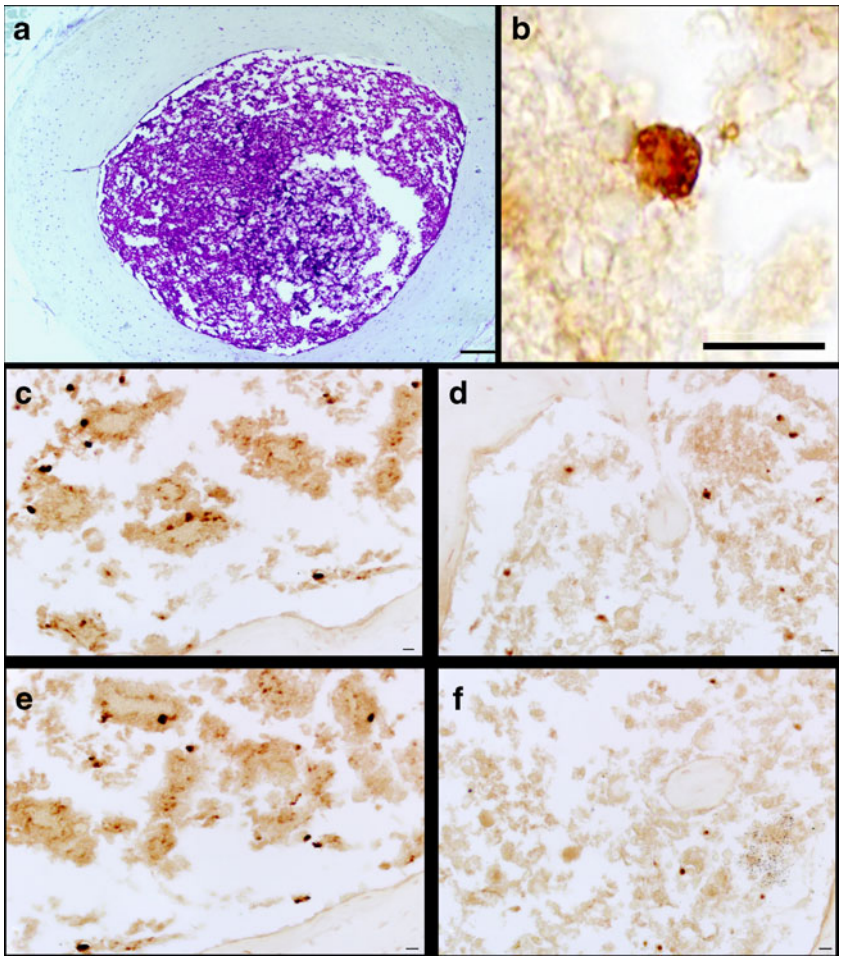

Fig. 7 Microscopic detection of CD 34 positive cells in the bone marrow. Mouse bone cross section from femur at small magnification (**a**), CD34 positivity immunoreaction enlarged (**b**), untreated WT (**c**) and KO (**d**) animals. Mouse bone marrow 7 days after cyclophosphamide treatment in WT (**e**) and KO (**f**) animals. Scale bar indicates 100 μm in panel **a**, and 10 μm in panels **b**–**f**

The release of eosinophils, however was delayed in both series of experiments and — unlike in case of neutrophils and monocytes — their absolute number was not augmented by the addition of exogenous G-CSF. Since eosinophils reside primarily in the gastrointestinal mucosa and normally constitute only 1–4 % of blood nucleated cells, their kinetic of appearance and disappearance may be much different than that of neutrophils. In addition, there are numerous factors that may contribute to this phenomenon. G-CSF does not act directly on eosinophils however, their release may be induced by several other cytokines (e.g., IL-2, IL-3, IL-5 and IL-17), thus resulting in a delayed indirect stimulatory effect. It has also been observed that fewer eosinophils than neutrophils accumulate at both P- and E-selectin surfaces (Patel and McEver 1997). Furthermore, eosinophils are also known to form fewer leukocyte–leukocyte interactions than neutrophils. For the proper interpretation of these findings, it is important to note that according to previous studies (Davenpeck et al. 2000; Jilma et al. 2002) G-CSF reduces surface PSGL-1 expression and adhesion to P-selectin in vitro. Thus, it is quite likely that in the G-CSF treated wild-type animals not only more cells were mobilized from the bone marrow but cells retained somewhat longer in the circulation due to the partial loss of their surface PSGL-1; hence, reduced tethering and rolling could occur. Indeed, we have also witnessed a decrease in PSGL-1 expression in both human and mouse samples. All myeloid cell types attenuated CD162 expression upon incubation with G-CSF, while PSGL-1 positive lymphocytes displayed no change. Extravasation may also be hampered in both strains due to the well-known effect of G-CSF to downregulate L-selectin — an important mediator of cell adhesion. In addition to its role on the selectin–selectin ligand interaction, G-CSF also causes SDF-1 decrease on the surface of osteoblasts (Nervi et al. 2006).

Atypical neutrophilic precursor cells were analysed and their number was found to be significantly elevated in knockout mice compared to wild-type animals. The number of these cells however was not different in the absence or presence of exogenous G-CSF. The release of precursor cells is of primary importance in G-CSF treatment thus we utilized colony forming assays in blood and bone marrow samples and similarly we identified stem cells at these locations by flow cytometry and immunohistochemistry. The majority of colony forming cells consisted of lineage-restricted colonies. In our assay we verified a much higher number of CFU-GM colonies in the peripheral blood throughout the investigated induced

cytopenia period. In the bone marrow of the femur an elevation was observed in the number of colonies during the induced cytopenia but with a different kinetics in the two strains. PSGL-$1^{-/-}$ mice displayed significantly more CFU-GM colonies in the femur. Although colony-forming assays are informative about the progenitor cell content, the colony forming cells can not be equalled to haemopoietic stem cells. Nevertheless, the bone marrow CFU-GM content parallelled with the number of stem cells as evaluated by double positivity for CD34 and CD117. In human studies stem cells are identified by their CD45+/CD34+ staining that excludes circulating endothelial cells. In mice it should be noted, that there may be a fluctuation in staining since the CD34 expression is reversible (Sato et al. 1999) and so the CD117+/CD34+ double positive event count more reliably identifies stem cells.

At the peak of the mobilization and also in agreement with previous studies (De Haan et al. 2000), we detected significantly elevated amounts of CD34/CD117 positive progenitors in wild-type but not in PSGL-1 deficient mice. These data were in agreement with the immunomorphological findings in the bone marrow where more CD34 positivity was observed in wild-type animals both in the untreated ones and also in the ones after induced cytopenia. It has been known for quite some time that PSGL-1 not only mediates the attachement of mature leukocytes to P-selectin, but is also the sole receptor of P-selectin on primitive CD34+ HPCs (Lévesque et al. 1999). These very immature cells contain considerably less copies of other adhesive receptors (e.g., CD44 and CD49d) and are probably more prone to be released in the absence of PSGL-1. Blasts and stem cells, on the other hand, express significantly more of these adhesive receptors (Lévesque et al. 1999; Elghetany 2002), which may result in different kinetic of stem cell release when the PSGL-1-selectin anchor is missing.

It should be noted that the dose of G-CSF was lower in our experiments than described previously for mouse studies (Lord et al. 2001; Ellefson et al. 2004) and was in the range of 5–10 μg/kg that were utilized in previous human studies. In our experiments both with and without exogenous G-CSF, the elevation in neutrophil and monocyte counts in PSGL-$1^{-/-}$ mice compared to wild-type animals was striking. Thus, when there is a lack of PSGL-1, G-CSF is more potent in mobilizing a larger number of myeloid cells and particularly myeloid precursors. This raises the possibility of applying PSGL-1 blockade for the enhancement of bone-marrow release of myeloid cells that may also be retained longer in peripheral blood.

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