Neutrophils are indispensable for hematopoietic stem cell mobilization induced by interleukin-8 in mice

Johannes F. M. Pruijt*†, Perry Verzaal*†, Ronald van Os*, Evert-Jan F. M. de Kruijf*, Marianke L. J. van Schie*, Alberto Mantovani‡, Annunciata Vecchi‡, Ivan J. D. Lindley§, Roel Willemze*, Sofie Starckx¶, Ghislain Opdenakker¶, and Willem E. Fibbe*‖

*Laboratory of Experimental Hematology, Department of Hematology, Leiden University Medical Center, 2300 RC, Leiden, The Netherlands; ¶Laboratory of Molecular Immunology, Rega Institute for Medical Research, University of Leuven, B-3000 Leuven, Belgium; ‡Department of Immunology and Cell Biology, Mario Negri Institute, 20157 Milan, Italy; and §Novartis Forschungsinstitut, Vienna A-1235, Austria

Communicated by Johannes van Rood, Europdonor Foundation, Leiden, The Netherlands, February 26, 2002 (received for review January 29, 2001)

The CXC chemokine interleukin-8 (IL-8/CXCL8) induces rapid mobilization of hematopoietic progenitor cells (HPCs). Previously we showed that mobilization could be prevented completely in mice by pretreatment with neutralizing antibodies against the β2-integrin LFA-1 (CD11a). In addition, murine HPCs do not express LFA-1, indicating that mobilization requires a population of accessory cells. Here we show that polymorphonuclear cells (PMNs) serve as key regulators in IL-8-induced HPC mobilization. The role of PMNs was studied in mice rendered neutropenic by administration of a single injection of antineutrophil antibodies. Absolute neutropenia was observed up to 3–5 days with a rebound neutrophilia at day 7. The IL-8-induced mobilizing capacity was reduced significantly during the neutropenic phase, reappeared with recurrence of the PMNs, and was increased proportionally during the neutrophilic phase. In neutropenic mice, the IL-8-induced mobilizing capacity was restored by the infusion of purified PMNs but not by infusion of mononuclear cells. Circulating metalloproteinase gelatinase B (MMP-9) levels were detectable only in neutropenic animals treated with PMNs in combination with IL-8, showing that *in vivo* activated PMNs are required for the restoration of mobilization. However, IL-8-induced mobilization was not affected in MMP-9-deficient mice, indicating that MMP-9 is not indispensable for mobilization. These data demonstrate that IL-8-induced mobilization of HPCs requires the *in vivo* activation of circulating PMNs.

metalloproteinases | MMP-9 | adhesion molecules | bone marrow | G-CSF

Despite the widespread clinical use of peripheral blood stem cells for transplantation, the mechanism(s) underlying stem cell mobilization are still largely unknown. Yet understanding of mechanisms of mobilization is critical in designing new strategies for mobilization. Adhesion molecules play an important role in the adherence of progenitor cells to the bone marrow (BM) microenvironment. In addition to the abundant expression of integrins and their ligands on hematopoietic progenitor cells (HPCs) and the BM microenvironment, adhesion-blocking experiments have indicated that the very late antigen (VLA)-4/vascular cell adhesion molecule (VCAM)-1, VLA-5/fibronectin, and β2-integrins/intercellular cell adhesion molecule-1 pathways play a role in the attachment of $CD34^+$ cells to stromal cells (1, 2). Furthermore, Papayannopoulou *et al.* (3, 4) demonstrated the prominent role of the β1-integrin, VLA-4 herein, because administration of antibodies against VLA-4 led to mobilization (3, 4). To delineate the mechanism(s) underlying cytokine-induced stem cell mobilization we have used the rapid mobilization of HPCs by IL-8 (5, 6). We have reported that the functional expression of the β2-integrin LFA-1 is required for IL-8-induced mobilization of HPCs in mice (7). The prevention of IL-8-induced mobilization by anti-LFA-1 antibodies was not caused by a direct effect of the antibodies on HPCs, because LFA-1 appeared not to be expressed on HPCs with colony-forming or radioprotective capacity (8–10). These data indicated the involvement of accessory cells, expressing both LFA-1 and IL-8 receptors. Subsequently, we showed that IL-8 induces the rapid systemic release of the metalloproteinase gelatinase B (MMP-9) with concurrent mobilization of HPCs in rhesus monkeys, which could be prevented by pretreatment of the monkeys with an inhibitory anti-MMP-9 antibody. These data indicated that MMP-9 is involved as a mediator of the IL-8-induced mobilization of HPCs in primates (11). Taken together, our data were in accordance with the hypothesis that polymorphonuclear cells (PMNs), which express LFA-1 (12) as well as high-affinity IL-8 receptors (13) and release MMP-9 upon activation by IL-8 (14), play a key role as accessory cells in mediating mobilization. Recently, Lévesque *et al.* (15) presented compelling evidence that PMNs accumulating in the BM during the course of granulocyte colony-stimulating factor (G-CSF)-induced mobilization release proteases that cleave VCAM-1, an important ligand of VLA-4.

In the present study we used a neutropenic model (16) to study further the role of PMNs in IL-8-induced mobilization. IL-8-induced mobilization of HPCs was reduced significantly in neutropenic animals and recovered simultaneously with the recurrence of circulating PMNs. Moreover, IL-8-induced mobilizing capacity could be restored by administration of PMNs to neutropenic mice. These results show that circulating PMNs are essential mediators of IL-8-induced stem cell mobilization.

Materials and Methods

Mice. BALB/c mice with ages ranging between 8 and 12 weeks were purchased from Broekman (Someren, The Netherlands). The animals were fed commercial rodent chow and acidified water *ad libitum*. Genetically homozygous mice, deficient for MMP-9 as well as littermate wild-type controls were bred at the Rega Institute for Medical Research and used for IL-8 mobilization studies (17). The genotypes of all animals were tested by Southern blot analysis, and the phenotypes were tested by zymography analysis of MMP-9, produced after overnight stimulation of peripheral blood cells with 100 ng/ml of phorbol 12-myristate 13-acetate. All experimental protocols were approved by the institutional ethical committee on animal experiments.

IL-8. Recombinant human IL-8 was purified from *Escherichia coli* expressing a synthetic gene (18) and provided by the Novartis

Abbreviations: BM, bone marrow; HPC, hematopoietic progenitor cell; VLA, very late antigen; VCAM-1, vascular cell adhesion molecule-1; MMP-9, metalloproteinase gelatinase B; PMN, polymorphonuclear cell (neutrophil); G-CSF, granulocyte colony-stimulating factor; MNC, mononuclear cell; CAFC, cobblestone area-forming cell; CFU-GM, colony-forming units/granulocyte macrophage.

†J.F.M.P. and P.V. contributed equally to this work.

‖To whom reprint requests should be addressed at: Department of Hematology, C2-R 140, Leiden University Medical Center, P.O. Box 9600, 2300 RC, Leiden, The Netherlands. E-mail: W.E.Fibbe@LUMC.nl.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Forschungsinstitut (Vienna, Austria). IL-8 had no colony-stimulating activity as reported previously (19). The concentration of endotoxin was less than 0.05 endotoxin units/ml as determined by the Limulus amoebocyte lysate assay. For *in vivo* experiments, IL-8 was diluted to the desired concentration in endotoxin-free PBS containing 0.1% BSA and administered as an i.p. injection.

Preparation of Cell Suspensions. Mice were killed by CO_2 asphyxiation. Blood was obtained by intracardiac puncture, and cell counts were performed on a Sysmex F800 (TOA Medical Electronics, Kobe, Japan). Manual PMN counts were performed after May Grünwald–Giemsa staining. Blood-derived mononuclear cell (MNC) suspensions were obtained by Ficoll separation as described earlier (20). BM cells were harvested by flushing the femur under sterile conditions with RPMI medium 1640 containing 500 μg/ml penicillin, 250 μg/ml streptomycin, and 2% FBS (GIBCO).

For PMN transfusion experiments, donor mice were treated with cyclophosphamide (200 mg/kg i.p.) on day 0 and recombinant human G-CSF (5 μg per mouse daily i.p.) (filgrastim, Amgen Biologicals) on days 2–5. On day 6, the mice were killed, and blood was obtained by cardiac puncture. To prevent activation and degranulation of the PMNs as much as possible, whole blood was centrifuged at 100 $\times$ *g* for 15 min at room temperature. The buffy coat was harvested, and cells were counted. On average, PMN transfusions contained 75–90% (range) PMNs. Because the transfused PMN suspension also contained HPCs, it was irradiated (6.5 Gy) and diluted with PBS containing 0.1% BSA to a volume of 250 μl. A sample was taken before and after irradiation to perform progenitor cell assays and showed less than 5% residual colony growth after irradiation. For further purification of PMNs, cell suspensions were derived through magnetic cell sorting with AutoMACS (Miltenyi Biotec, Auburn, CA). The buffy coat was harvested and resuspended in washing buffer (2 mM EDTA/PBS supplemented with 0.5% BSA). Then 10 μl of MACS CD45R (B220) (B cells) Microbeads, 10 μl of MACS CD90 (Thy1.2) (T cells) Microbeads, and 10 μl of MACS MHC class II (monocytes) Microbeads were added per 10^7 cells and incubated for 15 min at 4°C. Then the cells were washed (100 $\times$ *g*, 15 min) and resuspended in 500 μl of buffer per 10^8 cells. The unlabeled PMNs were separated and collected in washing buffer. Twenty-five percent of normal pressure was used to prevent neutrophil activation. The magnetic labeled MNCs (B cells, T cells, and monocytes) then were eluted with washing buffer. Both cell suspensions were diluted to ± 6–7×10^6 cells per 250 μl of PBS supplemented with 0.5% BSA.

mAbs and Cell Labeling. The anti-PMN hybridoma (RB6–8C5) was a kind gift from R. Coffman (DNAX; ref. 21). Antibodies were prepared and measured from ascites fluid as described (22). The concentration of endotoxin was less than 0.05 endotoxin units/ml. For *in vivo* experiments, antibodies were diluted to the desired concentration in PBS containing 0.1% BSA and administered as a single i.p. injection. Immunophenotyping of blood and BM samples was performed after erythrocyte lysis and incubation with phycoerythrin-conjugated CD3ε and CD45R/B220 (PharMingen) and FITC-conjugated GR-1 (PharMingen) antibodies. The cells then were incubated with Cy-chrome-conjugated anti-Ly-5 antibody for life cell staining. The fluorescence intensity was measured by FACScan analysis (Becton Dickinson). To detect circulating free antibody, plasma was obtained from mice at various time intervals after a single i.p. injection of 250 μg of anti-GR-1 antibody. A volume of 50 μl of plasma was incubated with 2×10^5 peripheral blood leukocytes of untreated mice and labeled with phycoerythrin-conjugated goat-anti-Rat IgG (GaRa-phycoerythrin, Caltag, South San Francisco, CA), and the fluorescence intensity was analyzed.

Progenitor Cell, Cobblestone Area-Forming Cell (CAFC), and Radioprotection Assays. Colony-forming units/granulocyte macrophages (CFU-GMs) were cultured as described (20). To determine the number of radioprotective stem cells mobilized in neutropenic versus control animals, 5×10^5 MNCs from IL-8-mobilized mice were transplanted into lethally irradiated (8 Gy) recipients. The radioprotective ability of the graft depends on the number of primitive stem cells (23). *In vitro* determination of HPC and stem cell frequencies was performed by limited dilution analysis of CAFCs in microcultures according to the method described (24–26).

Determination of Plasma MMP-9. The presence of MMP-9 in plasma of (neutropenic) mice given IL-8 was determined by using polyacrylamide gel electrophoresis procedures. With the use of gelatin zymography (14), MMP-9 was identified among other gelatinolytic enzymes by the comparison of samples from wild-type versus MMP-9-deficient mice. MMP-9 was identified further by Western blot analysis. First, equal sample volumes of plasma were prepurified by using gelatin-Sepharose followed by electrophoresis and transferred to a solid membrane support. Second, the transferred proteins were reacted with two highly specific antibodies against mouse MMP-9 (CDEM-ABA and CDEM-CIA, G.O., C. Dillen, E. Martens, P.E. Van den Steen, I. Van Aelst, and B. Arnold, unpublished data). Visualization of immune complexes in the Western blot was achieved by using a peroxidase-labeled anti-mouse IgG antibody.

Experimental Design. Neutropenia was induced by a single i.p. injection of 250 μg of anti-GR-1 antibodies (RB6–8C5; ref. 16 and 27). To study the kinetics of the induced neutropenia, mice were treated with anti-GR-1 antibodies or saline and killed at various time intervals after injection. In mobilization experiments, 30 μg of IL-8 was administered i.p. to steady-state mice, anti-GR-1 antibody-treated neutropenic mice (days 1–3 after injection), and anti-GR-1 antibody-treated neutrophilic mice (days 7–8 after injection). Twenty minutes after IL-8 administration, mice were killed, and blood was obtained for progenitor cell assays.

In a final set of experiments an attempt was made to restore the IL-8-induced mobilizing capacity by reinfusion of PMNs into anti-GR-1 antibody-treated neutropenic mice. Because mice treated with anti-GR-1 antibodies were neutropenic up to 3–5 days after antibody administration and no free circulating antibody could be detected at day 3 after injection, recipient mice were rendered neutropenic with a single injection of 300 μg of anti-GR-1 3 days before PMN transfusion. In the first experiment, recipient mice were divided into three groups (A–C). Groups A (PMNs + saline) and C (PMNs + IL-8) received a transfusion by tail-vein injection of $\pm 7 \times 10^6$ PMNs. Group B (saline + IL-8) was injected with saline. Two hours after injection, 30 μg of IL-8 (groups B and C) or saline (group A) was administered i.p. Twenty minutes later the mice were killed, and blood was obtained to perform progenitor cell assays. Because the transfusions contained on average 75–90% PMNs, a possible role of other cell types could not be excluded. We therefore repeated the experiment with purified cell suspensions through magnetic cell sorting with the AutoMACS, which resulted in cell suspensions containing 98% PMNs. In addition to the first experiment, a fourth group (MNCs + IL-8) was treated with a positive-selected neutrophil-depleted transfusion consisting of $\pm 6 \times 10^6$ peripheral blood MNCs. To test whether the transfused PMNs were active *in vivo*, plasma samples of mice treated with PMN transfusions were assayed for MMP-9 before and after the administration of IL-8.

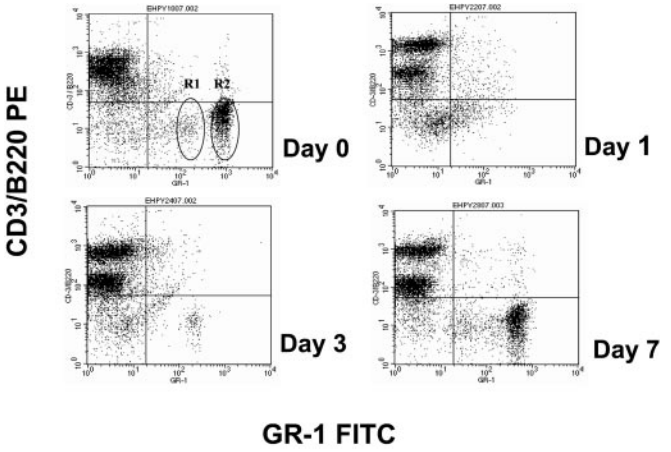

Fig. 1. Fluorescence-activated cell sorter analysis of peripheral blood using FITC-labeled anti-GR-1 antibodies and phycoerythrin (PE)-labeled anti-CD3/B220 in the appropriate life gate (total population of Ly-5 Cy-chrome-positive cells). R2 is the gate containing mature PMNs, whereas R1 contains more immature myeloid cells. Day 0 represents a steady-state control mouse. Days 1, 3, and 7 represent the peripheral blood of a mouse treated with a single injection of 250 μg of anti-GR-1 antibodies at day 0.

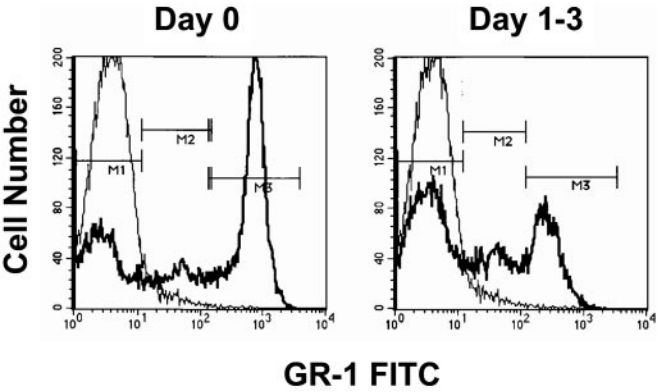

Fig. 2. The proportion of PMNs in the BM was assessed by fluorescence-activated cell sorter analysis using FITC-labeled anti-GR-1 antibodies in the appropriate life gate (total population of Ly-5 Cy-chrome-positive cells). M1 represents FITC-conjugated control antibody. The RB6–8C5-negative population (M1) consists of mainly lymphocytes/erythroid precursors and immature progenitor cells (34). The RB6–8C5 low/intermediate population in M2 contains mainly immature progenitor cells and myelocytes, whereas the RB6–8C5 high population in gate M3 contains almost only mature PMNs (38). Day 0 represents a steady-state control mouse, and days 1–3 represent the BM of a mouse treated on day 0 with a single dose of 250 μg of anti-GR-1 antibodies. A decrease was observed from 52 to 26% of mature PMNs and a relative increase of immature myeloid cells from 17 to 24%.

Statistical Analysis. Differences between base-line data and data collected after antibody and/or IL-8 treatment were evaluated by using the Student's *t* test. Correlations between the number of circulating PMNs and CFU-GMs were performed by regression analysis with Microsoft EXCEL software. CAFC frequencies were calculated by using Poisson distribution-based limited dilution analysis as described (24, 26). *P* values of <0.05 were considered statistically significant.

Results

Effect of Treatment with Anti-GR-1 Antibodies on the Number of PMNs in Peripheral Blood and BM. In accordance with earlier reports, injection of a single dose of 250 μg of anti-GR-1 mAb resulted in severe neutropenia up to 5 days after antibody injection (ref. 16; Fig. 1). After PMN recovery, antibody-treated mice developed a profound neutrophilia up to 3-fold at 7–8 days after the administration of the anti-GR-1 antibody (Fig. 1). In addition, we observed a substantial reduction in the number of GR-1 strongly positive cells in the BM after treatment with anti-GR-1 mAb together with an increase of GR-1 weakly positive cells (Fig. 2). To exclude interference of *in vivo* anti-GR-1 treatment with the use of FITC-labeled anti-GR-1 in fluorescence-activated cell sorter analysis, serial manual counts of peripheral blood were performed after May Grünwald–Giemsa staining. All samples tested showed a significant decrease of circulating PMNs (1.5 ± 0.4 PMNs per ml at base line and 0.1 ± 0.1 PMNs per ml at day 1 after *in vivo* anti-GR-1 treatment; mean $\pm$ SD, $n = 20$).

Effect of Treatment with Anti-GR-1 Antibodies on the Number of HPCs in the BM. Because our goal was to induce neutropenia without interfering with the number of HPCs in the BM, we assessed the effect of anti-GR-1 antibodies on the number of HPCs in the BM. Antibody-treated neutropenic mice did not reveal a reduction in the number of HPCs in the BM (steady-state BM $15{,}240 \pm 5{,}160$, $n = 12$ versus $15{,}178 \pm 5{,}897$ at days 1–3, $n = 16$, and $20{,}916 \pm 5{,}901$ CFU-GMs per femur at day 7, $n = 5$; mean $\pm$ SD, $P = 0.1$; Fig. 3).

Effect of Pretreatment with Anti-GR-1 Antibodies on IL-8-Induced Mobilization of HPCs. In neutropenic mice, the IL-8-induced mobilization of circulating HPCs was reduced significantly (saline + IL-8, 404 ± 196, $n = 10$, versus anti-GR-1 + IL-8, 114 ± 85 CFU-GMs per ml of blood, $n = 13$; mean $\pm$ SD, $P < 0.001$; Fig. 4). Previously we showed that an IgG2b antibody to VLA-4 did not block IL-8-induced mobilization, showing that IgG2b is not nonspecifically inhibiting mobilization (7). Free circulating antibodies could be detected up to 72 h after a single injection of antibody (data not shown). The mobilizing capacity was restored completely and even increased significantly with the reappearance of PMNs at day 7 or 8 after antibody injection (404 ± 96, $n = 10$ at day 0, versus $1{,}032 \pm 317$ CFU-GMs per ml of blood, $n = 10$ at day 7 or 8; mean $\pm$ SD, $P < 0.001$; Fig. 4). The addition of anti-GR-1 mAb to colony cultures in semisolid medium of steady-state BM MNCs had no inhibitory activity, indicating that the lack of mobilization after pretreatment with anti-PMN antibodies was not caused by interference of the antibody with colony growth *in vitro* (data not shown). To investigate whether the inhibition of mobilization in neutropenic mice includes also primitive stem cells, MNCs from IL-8-

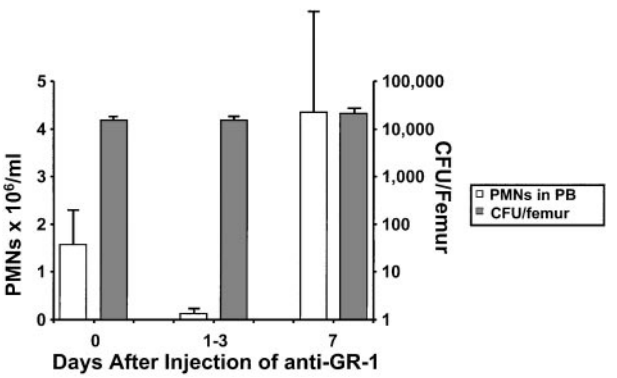

Fig. 3. Effect of anti-GR-1 antibodies on the number of HPCs in the BM. The number of peripheral blood PMNs and CFU-GMs per femur were assessed in control mice (day 0) and after 1–3 or 7 days after a single injection of 250 μg of anti-GR-1 antibodies. The results are expressed as mean $\pm$ SD of 12 experiments.

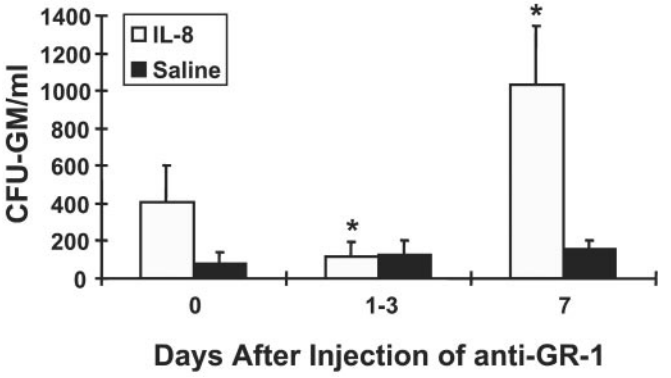

Fig. 4. Effect of anti-GR-1 antibodies on the IL-8-induced mobilization of HPCs. Control mice were treated with a single injection of 30 μg of IL-8 or saline with harvesting of peripheral blood after 20 min for assessment of circulating CFU-GMs (day 0, $n = 20$). Other mice were treated with a single injection of 250 μg of anti-GR-1 antibodies at day 0 and subsequently treated with 30 μg of IL-8 or saline with the assessment of circulating CFU-GMs at 1–3 ($n = 13$) or 7 ($n = 10$) days after the administration of anti-GR-1. The results are expressed as mean ± SD. *, $P < 0.001$.

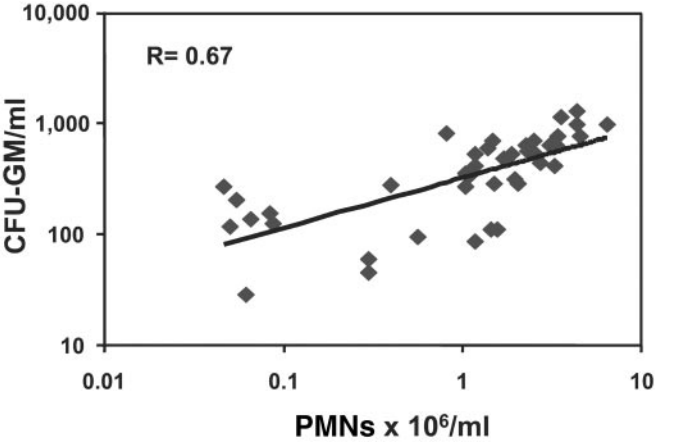

Fig. 5. Correlation between the number of circulating PMNs and IL-8-induced mobilization of HPCs. All mice were treated with 30 μg of IL-8 and killed after 20 min for the assessment of circulating PMNs and circulating CFU-GMs. The mice were pretreated either with anti-GR-1 antibodies (1–7 days before IL-8 injection) or saline. Each diamond represents a single mouse.

mobilized neutropenic and control mice were tested in the CAFC and radioprotection assays. All control animals transplanted with MNCs derived from IL-8-mobilized mice were radioprotected (9/9 animals survived until 30 days after stem cell transplantation), whereas 20% (2/10) of animals transplanted with MNCs from neutropenic mice mobilized with IL-8 survived. In addition, the number of CAFCs on day 28, a measure for repopulating stem cells, was 6-fold higher in control mobilized mice than in neutropenic mobilized mice (5.4, 95% confidence interval of 3.5–8.3 versus 0.82, 95% confidence interval of 0.2–3.3).

Correlation Between the Number of Circulating PMNs and IL-8-Induced Mobilization of HPCs. We studied the relation between the number of circulating PMNs and the IL-8-induced mobilizing capacity in three groups of mice: steady-state control mice (1), anti-GR-1-treated neutropenic mice (2), and anti-GR-1-treated neutrophilic mice (day 7 or 8 after antibody administration) (3). The number of circulating PMNs was $1.5 \pm 0.4 \times 10^6$ per ml in group 1, $0.1 \pm 0.1 \times 10^6$ per ml in group 2, and $4.3 \pm 1.3 \times 10^6$ per ml in group 3, respectively (Table 1). The number of circulating HPCs in IL-8-treated mice was 404 ± 196 in group 1 ($n = 10$), 114 ± 85 in group 2 ($n = 13$), and 1,032 ± 317 CFU-GMs per ml in group 3 ($n = 10$) (mean ± SD; Table 1). A clear correlation was observed between the number of circulating PMNs and the number of circulating HPCs. This correlation was observed throughout all treatment groups (steady-state control mice, antibody-treated neutropenic mice, and neutrophilic mice; $R = 0.67$, $P < 0.001$; Fig. 5).

IL-8-Induced Mobilization of HPCs Can Be Restored by Administration of PMNs into Anti-GR-1-Treated Neutropenic Mice. To demonstrate further the central role of PMNs in IL-8-induced stem cell mobilization, we studied the effect of readministration of PMNs on IL-8-induced mobilizing capacity. As shown in Fig. 6 (experiment 1), IL-8-induced mobilizing capacity was restored in mice receiving a transfusion of PMNs in combination with IL-8 (353 ± 61 CFU-GMs per ml, $n = 4$, PMN + IL-8) but not in control mice receiving PMNs and saline (87 ± 30 CFU-GMs per ml, $n = 4$, PMN + saline, $P < 0.01$) or IL-8 but no PMN transfusion (69 ± 20 CFU-GMs per ml, $n = 4$, saline + IL-8, $P < 0.01$). Because the transfusions contained on average 75–90% PMNs, an important role for other cells in the restoration of the mobilizing capacity could not be excluded. For this purpose, we repeated the experiment with a purified PMN suspension depleted of monocytes, T cells, and B cells. As shown in Fig. 6 (experiment 2), IL-8-induced mobilizing capacity was restored in mice receiving a purified PMN transfusion in combination with IL-8 (404 ± 82 CFU-GMs per ml, $n = 3$, PMN + IL-8) but not in control mice receiving purified PMNs and saline (120 ± 32 CFU-GMs per ml, $n = 3$, PMN + saline, $P < 0.02$) or IL-8 but no neutrophil transfusion (55 ± 29 CFU-GMs per ml, $n = 3$, saline + IL-8, $P < 0.01$). In addition, a transfusion with PMN-depleted MNCs followed after 2 h by IL-8 did not restore

Table 1. Relation between absolute PMN counts and IL-8-induced mobilizing capacity in anti-GR-1 antibody-treated mice

	Days after anti-GR-1 mAb		
	0 (Base line)	1–3	7
PMNs $\times 10^6$ per ml (PB)*	1.5 ± 0.4	0.1 ± 0.1	4.3 ± 1.3
PMNs per femur ($\times 10^6$)†	3.2 ± 1.0	2.8 ± 0.5	5.5 ± 1.9
CFU-GMs per ml after injection of PBS	75 ± 64 ($n = 20$)	125 ± 78 ($n = 25$)	153 ± 50 ($n = 6$)
CFU-GMs per ml after injection of IL-8	404 ± 196 ($n = 10$)	114 ± 85 ($n = 13$)‡	1,032 ± 317 ($n = 10$)‡

Data are expressed as mean ± SD.
*PMNs determined by automatic cell counter and May Grünwald–Giemsa staining. PB, peripheral blood.
†PMNs determined by automatic cell counter.
‡, $P < 0.001$.

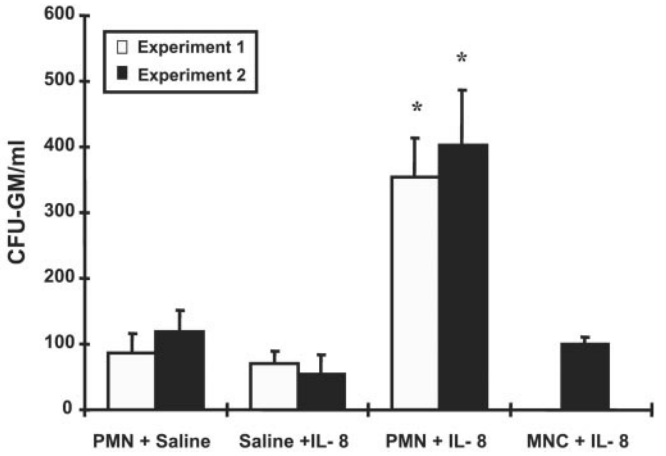

Fig. 6. Effect of the administration of PMNs into anti-GR-1-induced neutropenic mice on the mobilization of HPCs by IL-8. Mice were rendered neutropenic by a single injection of 300 μg of anti-GR-1. Three days after antibody injection (when the mice were still neutropenic without free circulating antibody), irradiated, donor-derived, cyclophosphamide- and G-CSF-mobilized PMNs were administered in the tail vein. Two hours later, 30 μg of IL-8 was administered, and the number of circulating CFU-GMs was assessed (PMN + IL-8). Neutropenic control mice received either PMNs and saline instead of IL-8 (PMN + saline) or saline instead of PMNs and IL-8 (saline + IL-8, Experiment 1, 75–90% PMNs). To exclude the possible role of accessory cells, we repeated the experiment with purified cell suspensions through magnetic cell sorting and added an additional control group receiving neutrophil-depleted peripheral blood MNCs and IL-8 (MNC + IL-8, Experiment 2, 98% neutrophils). The results are expressed as mean ± SD. $n = 4$ (experiment 1) or $n = 3$ (experiment 2) per group. *, $P < 0.05$.

IL-8-inducing mobilizing capacity (101 ± 11 CFU-GMs per ml, $n = 3$, MNC + IL-8, $P < 0.01$).

Induction of MMP-9 by IL-8 in the Mouse. As shown in Fig. 7, MMP-9 is constitutively expressed at very low levels in steady-state mice and clearly is induced in IL-8-treated control mice but not in anti-GR-1-treated neutropenic mice stimulated with IL-8. Furthermore, neutropenic mice receiving a PMN transfusion and then injected with IL-8 showed an increase in circulating MMP-9 in contrast to neutropenic mice treated with PMN transfusions only.

IL-8-Induced Mobilization of HPCs in MMP-9-Deficient Mice. MMP-9-deficient mice did not show a significant difference in mobilizing capacity in response to IL-8 in comparison with wild-type control animals. In MMP-9-deficient mice circulating CFU-GMs increased from 42 to 332 ± 132 per ml of blood after IL-8 administration (fold increase was 8.0 ± 3.2, $n = 6$) compared with an increase from 98 to 535 ± 250 CFU-GMs per ml of blood (fold increase was 6.0 ± 2.5, $n = 4$) in steady-state control mice.

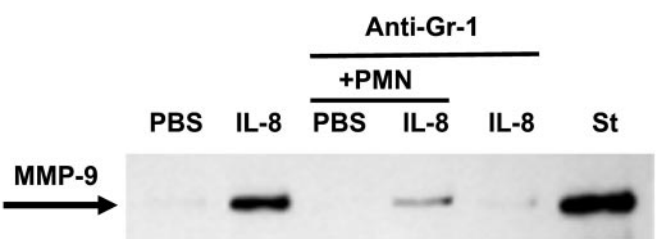

Fig. 7. MMP-9 Western blot of plasma samples taken from mice given PBS, IL-8, or antibodies against GR-1 on day 0 followed by purified PMNs on day 2 and PBS 2 h later, antibodies against GR-1 on day 0 followed by purified PMNs on day 2 and IL-8 2 h later, or antibodies against GR-1 on day 0 followed by IL-8 on day 2 as indicated in the figure and purified murine MMP-9 as standard (St). The arrow indicates the position of MMP-9. The used mAbs against mouse MMP-9 did not cross-react with mouse gelatinase A/MMP-2.

Discussion

In this study, we showed that IL-8-induced mobilization of both progenitors and primitive stem cells was reduced significantly in neutropenic mice, whereas the number of HPCs in the BM was not reduced. Moreover, the IL-8-induced mobilizing capacity was enhanced during the neutrophilic phase and correlated with the number of circulating PMNs. Finally, we showed that IL-8-induced mobilization could be restored by the administration of PMNs to neutropenic mice. Reintroduction of the mobilizing capacity of IL-8 coincided with increased MMP-9 levels, showing that *in vivo* activation of mature PMNs is required for this effect. Experiments in MMP-9-deficient mice revealed normal mobilization, indicating that MMP-9 serves as a marker for PMN activation but does not mediate mobilization as such. Our results therefore demonstrate that circulating PMNs are essential mediators of IL-8-induced stem cell mobilization. In accordance with our findings, Liu *et al.* (28) found that G-CSF receptor-deficient mice that are characterized by a defect in granulopoiesis show markedly impaired HPC mobilization in response to IL-8.

Several mechanisms can be proposed to explain this major role for PMNs, which comprise the main target cell population for IL-8. IL-8 induces a cascade of events, leading finally to the attraction and accumulation of activated PMNs at sites of inflammation. Recent evidence shows that degranulation of PMNs by activating agents such as IL-8 occurs more efficiently in PMNs that are attached as opposed to nonadherent PMNs (29). In addition, elastase release was reduced by prevention of PMN attachment by anti-LFA-1 or anti-CD18 antibodies (29). We found that both anti-LFA-1 and anti-Mac-1 blocking antibodies prevented IL-8-induced mobilization of HPCs in mice (7, 30) and propose that the blocking effect of anti-LFA-1 and anti-Mac-1 antibodies results from interference with PMN attachment to intercellular cell adhesion molecule-1 expressed on endothelial cells (31). Indeed, mobilization could be prevented also by pretreatment with anti-intercellular cell adhesion molecule-1 antibodies (7). In accordance, patients with the leukocyte adhesion deficiency syndrome (LAD) and CD18 (β2-integrin)-deficient mice exhibit a persistent granulocytosis (32, 33). Thus, activation of PMNs by IL-8 results in firm adhesion to the endothelium and subsequent degranulation and release of proteases.

Of the proteolytic enzymes released, MMP-9 is considered to be essential in PMN emigration, because it degrades collagen type IV, one of the main constituents of the basement membrane (34, 35). It is released as a proenzyme and activated by elastase and stromelysins that also are released after stimulation with IL-8 (34–36). Furthermore, PMN-derived MMP-9 boosts IL-8 10-fold by cleaving the NH_2 terminus of IL-8 (37). Experiments in rhesus monkeys showed that inhibitory anti-MMP-9 antibodies prevented IL-8-induced mobilization of HPCs (11). Although plasma levels of MMP-9 and HPC mobilization show similar patterns in mice and monkeys, MMP-9 does not seem to be essential in mice, because mice deficient for MMP-9 show normal mobilization in response to IL-8. Differences between antibody-mediated neutralization of an enzyme and inactivation of enzymes by a genetic knockout and also the involvement of other proteases therefore must be considered as possible explanations for the results in monkeys and mice. Degradation of the BM basement membrane would allow egress of HPCs from the BM into the blood (38). Proteolytic cleavage by MMP-9 or other proteases of adhesion molecules from the surface of HPCs and/or stromal cells also may contribute to their mobilization (15, 39, 40).

Treatment with anti-GR-1 antibodies resulted in an absolute peripheral neutropenia but only a modest reduction in the number of GR-1-positive BM cells with a proportional increase in immature myeloid cells. In addition, MMP-9 is expressed during the latest stages of PMN differentiation (41), and it is possible that immature PMNs in the BM release insufficient quantities of proteases to induce stem cell mobilization. The pool of PMNs residing in the BM venous sinusoids (42) still may be important in IL-8-induced stem cell mobilization, and anti-GR-1 treatment could have reduced their number substantially. The correlation between the number of circulating PMNs and mobilizing capacity and the restoration of mobilization by the i.v. administration of PMNs, however, points toward the pool of circulating PMNs as essential mediators of IL-8-induced mobilization.

It still is unknown whether PMNs play a similar role in hematopoietic stem cell mobilization induced by G-CSF. Watanabe *et al.* (43) showed a correlation between plasma levels of IL-8 and mobilization yield of HPCs after treatment of healthy donors with G-CSF. In addition, levels of MMP-9 and neutrophil elastase are increased in the plasma of healthy donors during G-CSF-induced stem cell mobilization (15, 44), both suggesting that activation of PMNs may represent a final common pathway in cytokine-induced stem cell mobilization. In accordance with these findings, it has been shown that expression of the G-CSF receptor on HPCs is not required for their mobilization by G-CSF (45). Finally, Lévesque *et al.* (15) recently found that after HPC mobilization by G-CSF, neutrophil-derived proteases are able to cleave VCAM-1, thereby suggesting that disruption of the adhesive interaction of VLA-4/VCAM-1 is a prerequisite for stem cell mobilization. Their *in vitro* experiments indicated that cleavage of VCAM-1 was mediated by the release of elastase and cathepsin-G from PMNs in the BM but not by MMP-9. In addition, we showed that MMP-9-deficient mice are able to mobilize HPCs in response to IL-8, suggesting that MMP-9 can be replaced by other proteases. Altogether, *in vivo* activation of PMNs is identified now as an essential step in mediating hematopoietic stem cell mobilization.

We thank Peter de Jong of the facilities for laboratory animals for his excellent animal care, Erik Martens for help with the Western blots, and Dr. Bernd Arnold (Deutsches Krebsforschungszentrum, Heidelberg) for the generation of the gelatinase B knockout mice. This work was supported by grants from the Dutch Cancer Society (UL 99-2029), the Belgian Federation against Cancer and the Fund for Scientific Research (FWO-Vlaanderen), the Associazone Italiana per la Ricerza sul Cancro, and the European Commission.

1. Teixido, J., Hemler, M. E., Greenberger, J. S. & Anklesaria, P. (1992) *J. Clin. Invest.* **90,** 358–367.
2. Simmons, P. J., Masinovsky, B., Longenecker, B. M., Berenson, R., Torok-Storb, B. & Gallatin, W. M. (1992) *Blood* **80,** 388–395.
3. Papayannopoulou, T. & Nakamoto, B. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 9374–9378.
4. Papayannopoulou, T., Craddock, C., Nakamoto, B., Priestley, G. V. & Wolf, N. S. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 9647–9651.
5. Laterveer, L., Lindley, I. J. D., Hamilton, M. S., Willemze, R. & Fibbe, W. E. (1995) *Blood* **85,** 2269–2275.
6. Laterveer, L., Lindley, I. J. D., Heemskerk, D. P. M., Camps, J. A. J., Pauwels, E. K. J., Willemze, R. & Fibbe, W. E. (1996) *Blood* **87,** 781–788.
7. Pruijt, J. F. M., van Kooyk, Y., Figdor, C. G., Lindley, I. J. D., Willemze, R. & Fibbe W. E. (1998) *Blood* **91,** 4099–4105.
8. Gunji, Y., Nakamura, M., Hagiwara, T., Hayakawa, K., Matshushita, H., Osawa, H., Nagayoshi, K., Nakauchi, H., Yanagisawa, M., Miura, Y. & Suda T. (1992) *Blood* **80,** 429–436.
9. Torensma, R., Raymakers, R. A. P., van Kooyk, Y. & Figdor, C. G. (1996) *Blood* **87,** 4120–4128.
10. Pruijt, J. F. M., van Kooyk, Y., Figdor, C. G., Willemze, R. & Fibbe W. E. (1999) *Blood* **93,** 107–112.
11. Pruijt, J. F. M., Fibbe, W. E., Laterveer, L., Pieters, R. A., Lindley, I. J. D., Paemen, L., Masure S., Willemze, R. & Opdenakker, G. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 10863–10868.
12. Lund Johansen, F. & Terstappen, L. W. (1993) *J. Leukocyte Biol.* **54,** 47–55.
13. Samanta, A. K., Oppenheim, J. J. & Matsushima, K. (1989) *J. Exp. Med.* **169,** 1185–1189.
14. Masure, S., Proost, P., Van Damme, J. & Opdenakker, G. (1991) *Eur. J. Biochem.* **198,** 391–398.
15. Lévesque, J. P., Takamatsu, Y., Nilsson, S. K., Haylock, D. N. & Simmons, P. J. (2001) *Blood* **98,** 1289–1297.
16. Czuprynski, C. J., Brown, J. F., Maroushek, N., Doug Wagner, R. & Steinberg, H. (1994) *J. Immunol.* **152,** 1836–1846.
17. Dubois, B., Masure, S., Hurtenbach, U., Paemen, L., Heremans, H., van den Oord, J., Meinhardt, T., Hämmerling, G., Opdenakker, G. & Arnold, B. (1999) *J. Clin. Invest.* **104,** 1507–1515.
18. Lindley, I., Aschauer, H., Seifert, J. M., Lam, C., Brunowsky, W., Kownatzki, E., Thelen, M., Peveri, P., Dewald, B., von Tscharner, V., Walz, A. & Baggiolini, M. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 9199–9203.
19. Zwierzina, H., Holzinger, I., Gaggi, S., Wolf, H., Schollenberger, S., Lam, C., Bammer, T., Geissler, D. & Lindley, I. J. D. (1993) *Scand. J. Immunol.* **37,** 322–328.
20. Fibbe, W. E., Hamilton, M. S., Laterveer, L. L., Kibbelaar, R. E., Falkenburg, J. H. F., Visser, L. W. M. & Willemze, R. (1992) *J. Immunol.* **148,** 417–421.
21. Tepper, R. I., Coffman, R. & Leder, P. (1992) *Science* **257,** 548–551.
22. Fioretti, F., Fradelizi, D., Stoppacario, A., Ruco, L., Minty, A., Sozani, S., Vecchi, A. & Mantovani, A. (1998) *J. Immunol.* **161,** 342–346.
23. Zijlmans, J., Visser, J., Laterveer, L., Kleiverda, K., Heemskerk, D., Kluin, P., Willemze, R. & Fibbe, W. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 725–729.
24. Ploemacher, R.E. (1994) in *Culture of Hematopoietic Cells*, eds. Freshney R. I., Pragnell, I. B. & Freshney, M. G. (Wiley–Liss, New York) p. 1.
25. Van Os, R., Dawes, D., Mislow, J. M., Witsell, A. & Mauch, P. M. (1997) *Blood* **89,** 2376–2383.
26. Fazekas de St. Groth, S. (1982) *J. Immunol. Methods* **49,** R11–R23.
27. Hestdal, K., Ruscetti, F. W., Ihle, J. N., Jacobsen, S. E. W., Dubois, C. M., Kopp, W. C., Longo, D. L. & Keller, J. R. (1991) *J. Immunol.* **147,** 22–28.
28. Liu, F. L., Poursine-Laurent, J. & Link, D. C. (1997) *Blood* **90,** 2522–2528.
29. Rainger, G. E., Rowley, A. F. & Nash, G. B. (1998) *Blood* **92,** 4819–4827.
30. Velders, G. A., Pruijt, J. F. M., Verzaal, P., van Os, R., van Kooyk, Y., Figdor, C. G., de Kruijf, E.J.F.M., Willemze, R. & Fibbe, W. E. (2002) *Blood*, in press.
31. Smith, C. W., Marlin, S. D., Rothlein, R., Toman, C. & Anderson, D. C. (1989) *J. Clin. Invest.* **83,** 2008–2017.
32. Wilson, R. W., Ballantyne, C. M., Wayne Smith, C., Montgomery, C., Bradley, A., O'Brien, W. & Beaudet, A. L. (1993) *J. Immunol.* **151,** 1571–1578.
33. Harlan, J. M. (1993) *Clin. Immunol. Immunopathol.* **67,** S16–S24.
34. Delclaux, C., Delacourt, C., d'Ortho, M. P., Boyer, V., Lafuma, C. & Harf, A. (1996) *Am. J. Respir. Cell Mol. Biol.* **14,** 288–295.
35. Opdenakker, G., Van den Steen, P. E. & Van Damme, J. (2001) *Trends Immunol.* **22,** 571–579.
36. Shapiro, S. D., Fliszar, C. J., Broekelmann, T. H. J., Mecham, R. P., Senior, R. M. & Welgus, H. G. (1995) *J. Biol. Chem.* **270,** 6351–6356.
37. Van den Steen, P. E., Proost, P., Wuyts, A., Van Damme, J. & Opdenakker, G. (2000) *Blood* **96,** 2673–2681.
38. Opdenakker, G., Fibbe, W. E. & Van Damme, J. (1998) *Immunol. Today* **19,** 182–189.
39. Arribas, J., Coodly, L., Vollmer, P., Kishimoto, T. K., Rose-John, S. & Massague, J. (1996) *J. Biol. Chem.* **271,** 11376–11382.
40. Preece, G., Murphy, G. & Ager, A. (1996) *J. Biol. Chem.* **271,** 11634–11640.
41. Borregaard, N. & Cowland, J. B. (1997) *Blood* **89,** 3503–3521.
42. Terashima, T., English, D., Hogg, J. C. & van Eeden, S. F. (1998) *Blood* **92,** 1062–1069.
43. Watanabe, T., Kawano, Y., Kanamaru, S., Onishi, T., Kaneko, S., Wakata, Y., Nakagawa, R., Makimoto, A., Kuroda, Y., Takaue, Y. & Talmadge, J. E. (1999) *Blood* **93,** 1157–1181.
44. Van Os, R., van Schie, M. L. J., Willemze, R. & Fibbe, W. E. (2002) *J. Hematother. Stem Cell Res.*, in press.
45. Liu, F., Poursine-Laurent, J. & Link, D. C. (2000) *Blood* **95,** 3025–3031.