

## Granulocyte-macrophage colony-stimulating factor (GM-CSF) but not granulocyte colony-stimulating factor (G-CSF) induces plasma membrane expression of proteinase 3 (PR3) on neutrophils *in vitro*

B. HELLMICH, E. CSERNOK, A. TRABANDT, W. L. GROSS & M. ERNST\* *Poliklinik für Rheumatologie, Medizinische Universität zu Lübeck, Lübeck, and \*Forschungszentrum Borstel, Zentrum für Medizin und Biowissenschaften, Borstel, Germany*

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### SUMMARY

The theoretical risk of triggering vasculitis resulting from administration of G-CSF and GM-CSF to patients with anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitides (AAV), such as Wegener's granulomatosis (WG), who develop agranulocytosis due to cytotoxic therapy, is unknown. Since there is strong evidence that activation of polymorphonuclear neutrophils (PMN) induced by binding of ANCA to PR3 or myeloperoxidase (MPO) expressed on their plasma membrane is involved in the pathogenesis of systemic vasculitides (SV), we studied the surface expression of PR3 and MPO on PMN from healthy donors in response to G-CSF and GM-CSF *in vitro* by flow cytometric analysis. Increasing doses of G-CSF did not alter PR3 expression on either untreated or tumour necrosis factor- $\alpha$  (TNF- $\alpha$ )-primed donor PMN significantly. In contrast, GM-CSF significantly increased PR3 membrane expression on both intact PMN and neutrophils primed with TNF- $\alpha$ . MPO expression was not significantly altered by either G-CSF or GM-CSF. In summary, these data demonstrate that GM-CSF, but not G-CSF, induces plasma membrane expression of PR3 on PMN *in vitro*. Since in AAV accessibility of the antigen (PR3 or MPO) to the antibody (ANCA) on the plasma membrane of PMN is thought to be essential for neutrophil activation by ANCA, the results of the present study suggest that administration of GM-CSF to patients with WG with neutropenia implies a definite theoretical risk of deterioration of vasculitis via this mechanism.

**Keywords** proteinase 3 granulocyte colony-stimulating factor  
granulocyte-macrophage colony-stimulating factor polymorphonuclear neutrophils

### INTRODUCTION

Administration of the recombinant protein form of human G-CSF (rhG-CSF) and human GM-CSF (rhGM-CSF) to patients with both congenital and acquired neutropenias is considered a safe and effective way to hasten recovery from neutropenia and curtail infectious complications [1]. rhG-CSF and rhGM-CSF not only stimulate the proliferation and differentiation of haematopoietic precursors, but also modulate functional features like microbicidal killing, chemotaxis or expression of adhesion molecules as well as survival of mature polymorphonuclear neutrophils (PMN) [1–5]. However, individual patients treated with rhG-CSF or rhGM-CSF for severe chronic neutropenia or Felty's syndrome developed

apparently treatment-induced cutaneous vasculitis or neutrophilic dermatoses like Sweet's syndrome [6–11]. Since rhGM-CSF and rhG-CSF also modulate various functions of mature neutrophils [1–5], which are considered the primary effector cells in vasculitis, a causal association of growth factor administration and development of vasculitis has been suggested and a cautious use of haematopoietic growth factors in autoimmune diseases has recently been proposed [11].

Since severe infections are common in patients with systemic vasculitides (SV) and are often related to severe neutropenia resulting from cytotoxic therapy [12–16], administration of G-CSF to SV patients with agranulocytosis might be a useful therapeutic strategy. We recently reported the results of a retrospective analysis of patients with Wegener's granulomatosis (WG) treated with low doses of rhG-CSF for cyclophosphamide (CYC)-induced agranulocytosis, showing that recombinant human G-CSF can shorten the duration of CYC-induced severe neutropenia in a clinically significant manner (reduction of infectious complications) without inducing a disease flare [17]. Recent experimental protocols using high-dose CYC as a potential therapy for patients

B.H. present address: Medizinische Klinik, Universitätsklinik Bergmannsheil, Ruhr-Universität Bochum, Bochum, Germany.

Correspondence: E. Csernok, Rheumaklinik Bad Bramstedt, Oskar-Alexander Straße 26, 24572 Bad Bramstedt, Germany.

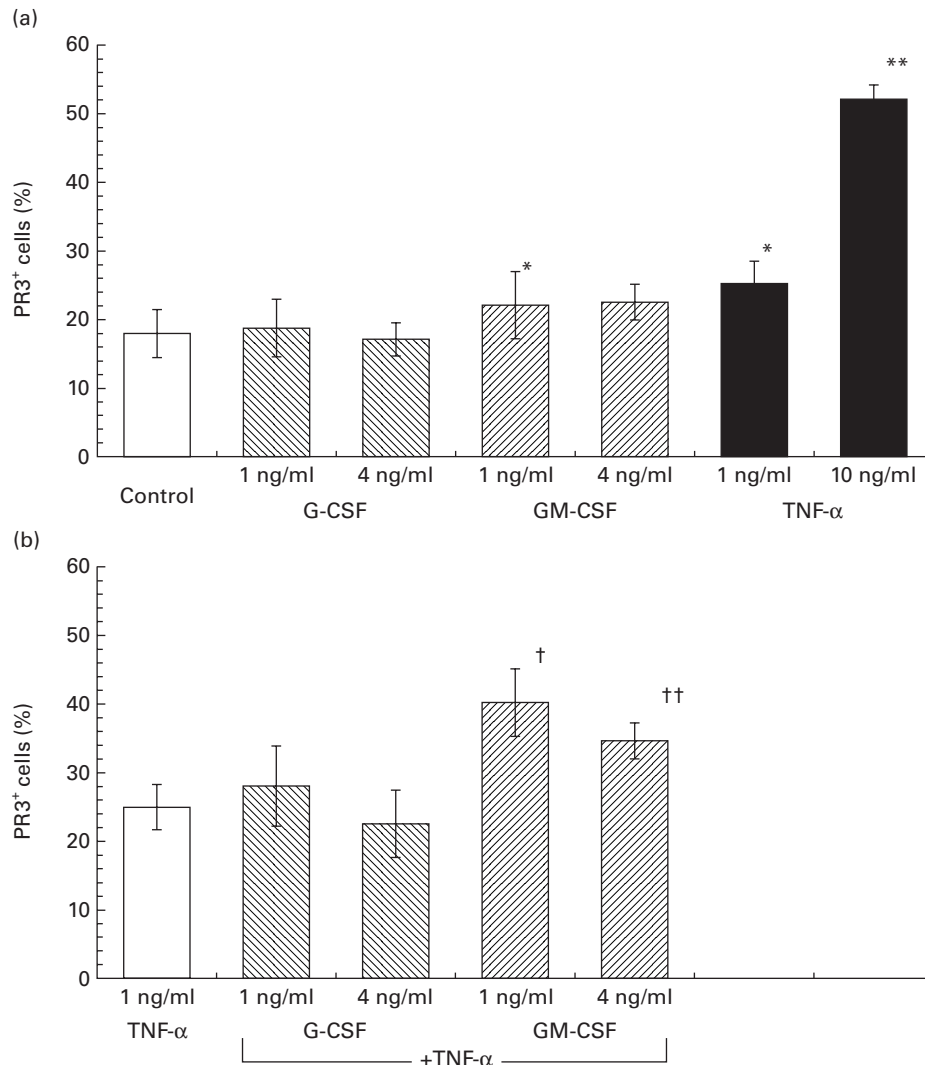
E-mail: c.moeck@rheuma-zentrum.de or Bernhard.Hellmich@ruhr-uni-bochum.de

with severe autoimmune disease, including vasculitis, involve the administration of rhG-CSF for stem cell mobilization, which also may cause a flare up of the underlying rheumatic disease [18]. Despite the growing importance of haematopoietic growth factors for the treatment of patients' vasculitides, the specific risk of administration of these powerful agents in view of the pathogenesis and pathophysiology of vasculitides like WG has not been defined.

Anti-neutrophil cytoplasmic antibodies (ANCA), which are directed against granule proteins translocated on the surface of PMN, are thought to play an important pathogenic role in primary small vessel vasculitides via activation of PMN [18–23]. Cytoplasmic (classic) C-ANCA and perinuclear P-ANCA, directed against PR3 or myeloperoxidase (MPO), respectively, are strongly associated with SV like WG (mostly C-ANCA directed against PR3), microscopic polyangiitis (mostly P-ANCA directed

against MPO) or Churg–Strauss syndrome (CSS) [19,20]. We and others demonstrated the presence of PR3 and MPO on the plasma membrane of PMN from patients with active WG [21,23,24]. Since certain cytokines like tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and IL-8 can induce a translocation of PR3 from the intragranular loci to the cell surface of PMN [23], we wondered whether G-CSF and/or GM-CSF could also induce the membrane surface expression of PR3 and MPO on PMN. In this case, GM-CSF/G-CSF administration to patients with ANCA-associated vasculitides would indeed bear a definite risk of exacerbating vasculitis by binding of G-CSF/GM-CSF-induced PR3 to C-ANCA, as leucocyte membrane expression of PR3 correlates with disease activity in patients with WG [24].

Thus, we studied the *in vitro* effect of rhG-CSF and rhGM-CSF on the surface expression of PR3 and MPO on neutrophils.



**Fig. 1.** Flow cytometric analysis of the membrane expression of PR3 on polymorphonuclear neutrophils (PMN) derived from healthy donors. Cells were incubated in the presence of increasing doses of tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), G-CSF and GM-CSF as described in Materials and Methods. (a) Unstimulated intact PMN. (b) Primed PMN, re-incubated with TNF- $\alpha$  (1 ng/ml) for 10 min. Antigen expression is given as the percentage of total PMN with fluorescence intensities above the threshold fluorescence determined as the upper level of fluorescence intensity of PMN treated with the isotype control antibodies. Results represent means  $\pm$  s.e.m. of nine individual experiments, by using cells from nine different donors. Statistically significant difference of \* $P$  < 0.05 or \*\* $P$  < 0.005 versus control PMN (unstimulated). Statistically significant difference of † $P$  < 0.05 or †† $P$  < 0.001 versus PMN primed with TNF- $\alpha$  (1 ng/ml for 10 min) alone.

## MATERIALS AND METHODS

### Volunteers

Blood samples were obtained from nine healthy donors (four males, five females, mean age  $36.0 \pm 5.1$  years (range 22–58 years)). Donors were working in departments of the Forschungsinstitut Borstel other than the laboratory where the *in vitro* stimulation experiments were performed. None of the donors had contact with PR3, to avoid contamination. There was no evidence of an inflammatory or malignant disorder in any of the subjects. None of the donors took any medication.

### Cytokines and monoclonal antibodies

For *in vitro* studies rhTNF- $\alpha$ , rhG-CSF, and rhGM-CSF purchased from TEBU GmbH (Frankfurt, Germany) were used. MoAbs against PR3 (designated WGM1 and WGM2) and MPO were raised in our laboratory as previously described [25].

### Preparation of human PMN

Heparinized blood samples (20 U heparin/ml blood) were diluted 1:2 with Hanks' balanced salt solution (HBSS) and the peripheral blood mononuclear cells (PBMC) were separated in a first step from erythrocytes and polymorphonuclear granulocytes (PMN) by Ficoll-Hypaque density gradient centrifugation. Then the PMN-containing erythrocyte sediments were mixed with two volumes of polyvinylalcohol. The PMN-containing supernatant was collected after sedimentation for 20 min. Contaminating erythrocytes were removed by hypotonic lysis. Cell purity was  $>98\%$  as determined by Pappenheim staining and microscopical examination at  $\times 630$  magnification using oil immersion. PMN viability was  $>95\%$  as determined by trypan blue exclusion. All media and buffers used for the neutrophil separation were free from endotoxin in order to avoid any contamination which might affect PR3 expression.

### In vitro stimulation experiments

PMN freshly isolated as described above were resuspended in PBS with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  at a final concentration of  $2 \times 10^6$  cells/ml. The cell suspension was then incubated with supraphysiological doses of rhG-CSF (0.03, 0.1, 0.3, 1 and 4 ng/ml), rhGM-CSF (0.03, 0.1, 0.3, 1 and 4 ng/ml), TNF- $\alpha$  (1, 3 and 10 ng/ml) or buffer in a shaking water bath at  $37^\circ\text{C}$  for 10 min. The concentrations of the haematopoietic growth factors used in all experiments were chosen according to the range of serum levels of rhG-CSF/rhGM-CSF observed during treatment with these cytokines [26]. To assess the effect of these cytokines on reactivated PMN, the cell suspension was preincubated with TNF- $\alpha$  (1 ng/ml) as the priming agent for 10 min. Subsequently, G-CSF (1 and 4 ng/ml), GM-CSF (1 and 4 ng/ml), or buffer alone were added and the incubation was continued for a further 10 min at  $37^\circ\text{C}$ . After rapid cooling to  $4^\circ\text{C}$  with excess of ice-cold PBS the cells were centrifuged at  $300 g$  for 10 min and the cells were used for subsequent immunofluorescence staining and flow cytometric analysis.

### Flow cytometric analysis

Quantitative flow cytometric analysis of surface antigens was performed as previously described [27]. Cytokine-treated or control PMN ( $1 \times 10^6$ /ml) were incubated with MoAbs against PR3 and MPO, respectively, or with isotype control antibodies for 20 min. After washing, the cells were incubated with the second antibody

(dichlorotriazinyl aminofluorescein (DTAF)-conjugated F(ab) $_2$  goat anti-mouse IgG $_{\text{Fc}}$  or DTAF-conjugated F(ab) $_2$  goat anti-mouse IgM $_{\text{Fc}}$ ; Dianova, Hamburg, Germany). The labelled cells were washed and then fixed with PBS containing 1.5% paraformaldehyde. In order to minimize any activation of cells all procedures were performed on ice. The flow cytometric analysis was performed within the subsequent 24 h using a Cytofluorograf System 50 (Ortho Diagnostic Systems, Raritan, NJ). The expression of PR3 is given as the percentage of PMN with fluorescence intensities above the threshold fluorescence determined as the upper level of fluorescence intensity of PMN treated with isotype control antibodies. In addition, median fluorescence intensities are shown.

### Statistical analysis

Data are expressed as mean  $\pm$  s.e.m. The Kolmogorov–Smirnov Z-test was used to analyse the distribution of the samples. The *t*-test for paired samples was used to compare values between two groups.  $P < 0.05$  was considered significant. Statistical analysis was performed using SPSS for Windows, version 7.5 (SPSS Inc., Chicago, IL).

## RESULTS

Flow cytometric analysis revealed native membrane expression of PR3 on  $18.0 \pm 3.5\%$  of untreated human neutrophils (Fig. 1). Addition of supraphysiological (therapeutic) concentrations of rhG-CSF (0.03–4 ng/ml) to untreated PMN *in vitro* did not alter membrane expression of PR3 significantly ( $P > 0.2$ ) (Fig. 1a, Table 1). In contrast, a higher percentage of PR3-expressing PMN was found in response to GM-CSF, with a statistically significant difference at a GM-CSF dose of 1 ng/ml ( $18.0 \pm 3.5\%$  versus  $22.2 \pm 4.9\%$ ;  $P = 0.049$ ) (Fig. 1a, Table 1). Dose–response analyses demonstrated no significant stimulating effect of either G-CSF or GM-CSF at doses lower than 1 ng/ml (Fig. 2).

**Table 1.** Flow cytofluorometric analysis of the membrane expression of PR3 on polymorphonuclear neutrophils (PMN) from healthy donors after incubation with G-CSF and GM-CSF *in vitro*

Condition (concentration of cytokine)	Percentage of PMN expressing PR3		
	G-CSF	GM-CSF	<i>P</i>
Basal (1 ng/ml)	$18.0 \pm 3.5$	$22.2 \pm 4.9$	0.003*
Basal (4 ng/ml)	$17.18 \pm 1.9$	$22.7 \pm 2.6$	0.04*
Primed (1 ng/ml)	$28.1 \pm 5.2$	$40.2 \pm 7.5$	0.004*
Primed (4 ng/ml)	$22.6 \pm 2.8$	$34.7 \pm 3.8$	0.001*

PMN pretreated with tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) (1 ng/ml for 10 min) and PMN without prior priming (basal) were incubated with G-CSF and GM-CSF in different concentrations for 10 min. Subsequently, PMN ( $1 \times 10^6$ /ml) were incubated with MoAbs to PR3 (WGM2) for 20 min. After washing, the second antibody (fluorescein (DTAF)-conjugated F(ab) $_2$  goat anti-mouse (IgG) was added. Subsequently the labelled cells were fixed with PBS containing 1.5% paraformaldehyde. The surface expression of PR3 is given as the percentage of PMN showing PR3 expression on flow cytometric analysis. Data are means  $\pm$  s.e.m. from nine experiments using cells from nine different donors.

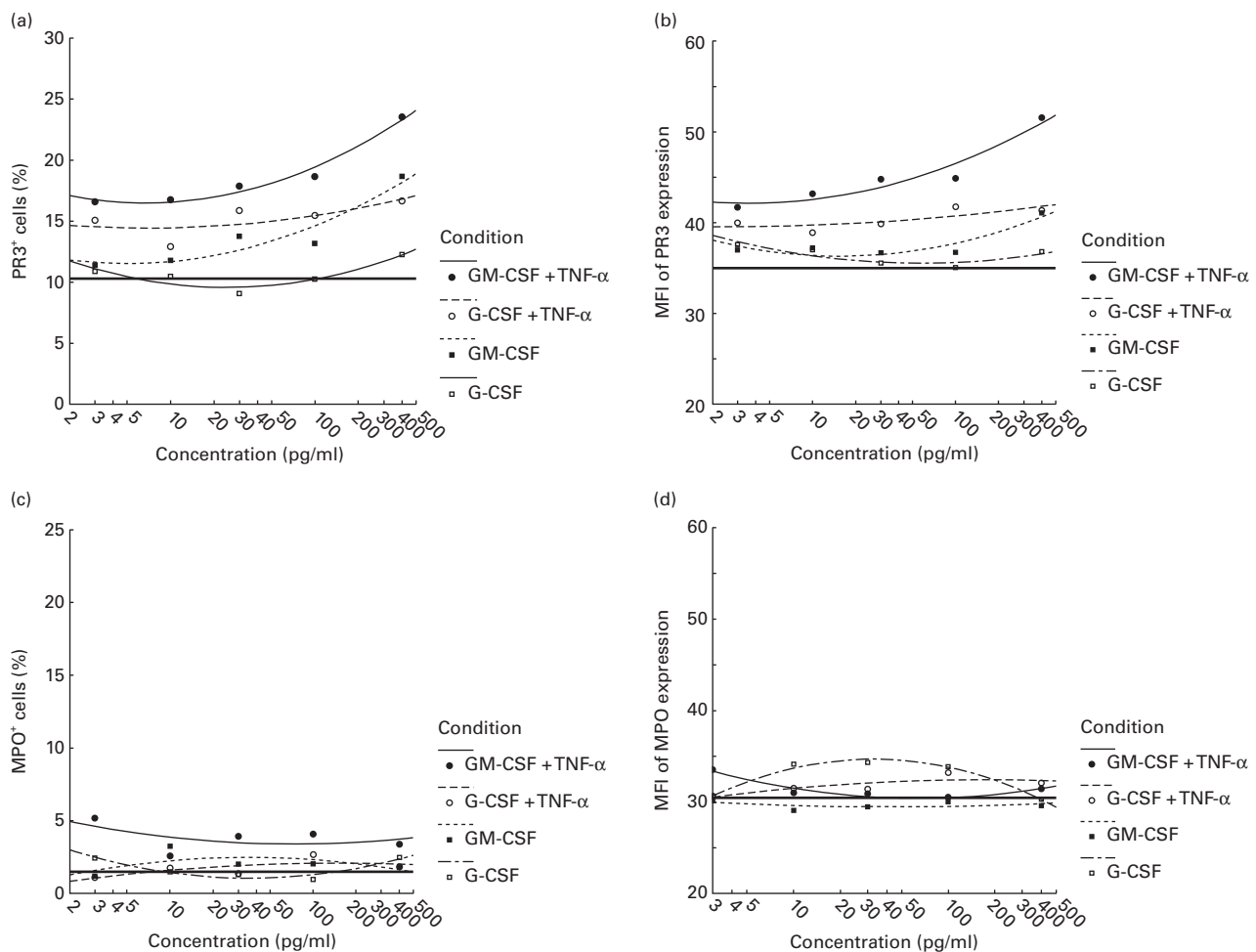
\*Statistically significant difference between G-CSF- and GM-CSF-stimulated PMN.

Incubation of PMN in the presence of increasing doses of TNF- $\alpha$  (1–10 ng/ml) caused a dose-dependent increase of total PR3 surface expression (Table 2). After treatment with TNF- $\alpha$  at a dose of 10 ng/ml the percentage of PMN expressing PR3 ( $52.3 \pm 2.2\%$ ) was significantly higher than after stimulation with G-CSF at doses of 1 ng/ml ( $18.8 \pm 4.2\%$ ;  $P = 0.005$ ) and 4 ng/ml ( $17.18 \pm 2.4\%$ ;  $P = 0.016$ ), and GM-CSF at a dose of 1 ng/ml ( $22.2 \pm 4.9\%$ ;  $P = 0.001$ ) and 4 ng/ml ( $22.6 \pm 2.6\%$ ;  $P = 0.001$ ), each without prior priming with TNF- $\alpha$  (Fig. 1a).

To assess the effect of rhG-CSF and rhGM-CSF on activated neutrophils PMN were pretreated with TNF- $\alpha$  (1 ng/ml) as the priming agent. After pretreatment with TNF- $\alpha$ , incubation of the primed PMN with rhG-CSF (1 ng/ml) did not alter PR3 expression compared with PMN treated with the same concentration of TNF- $\alpha$  only ( $28.1 \pm 5.2\%$  versus  $25.3 \pm 3.3\%$ ;  $P > 0.2$ ). Higher doses of G-CSF (4 ng/ml) showed no additional effect ( $22.6 \pm 2.8\%$  versus  $25.3 \pm 1.4\%$ ;  $P = 0.1$ ) (Fig. 1b). In contrast, rhGM-CSF significantly increased PR3 membrane expression on activated PMN, resulting in  $40.3 \pm 7.5\%$  (1 ng/ml GM-CSF;

$P = 0.04$ ) and  $34.7 \pm 3.8\%$  (4 ng/ml GM-CSF;  $P = 0.001$ ) PR3<sup>+</sup> cells compared with  $25.3 \pm 3.3$  PR3<sup>+</sup> cells after stimulation with TNF- $\alpha$  alone (Fig. 1b). Direct comparison between the stimulatory effect of both haematopoietic cytokines on PR3 expression on primed PMN demonstrated a significantly higher percentage of PR3<sup>+</sup> cells in response to GM-CSF compared with G-CSF at both 1 and 4 ng/ml of each cytokine, respectively (Table 1). In contrast to the observations made for TNF- $\alpha$ , higher doses of rhG-CSF (up to 4 ng/ml), and rhGM-CSF (up to 4 ng/ml) showed no additional stimulating effect on membrane expression of PR3 on either native or primed PMN (Fig. 1). Dose–response analyses revealed also that with G-CSF and GM-CSF doses  $< 1$  ng/ml significant stimulation of PR3 expression on primed PMN could not be achieved (Fig. 2). Analysis of the median fluorescence intensity of PR3 expression (Fig. 2b) demonstrated that the higher total PR3 expression was mainly a result of an increased subset of PR-expressing PMN (% PR3<sup>+</sup> PMN) than a result of enhanced surface expression on individual cells.

Surface expression of MPO was found in  $< 4\%$  of untreated



**Fig. 2.** Flow cytometric analysis of the membrane expression of PR3 and myeloperoxidase (MPO) on polymorphonuclear neutrophils (PMN) derived from three healthy donors. Both intact and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ )-primed (1 ng/ml for 10 min) PMN were incubated in the presence of increasing doses (0.03–4 ng/ml, respectively) of G-CSF and GM-CSF as described in Materials and Methods. Antigen expression is given as the percentage of total PMN (a,c) with fluorescence intensities above the threshold fluorescence determined as the upper level of fluorescence intensity of PMN treated with the isotype control antibodies, and the median fluorescence intensity (MFI) (b,d). The solid horizontal line represents the basal level of PR3 or MPO expression without any stimulation. Results represent means  $\pm$  s.e.m. of three individual experiments, by using cells from three different donors.

**Table 2.** Flow cytometric analysis of the membrane expression of PR3 on polymorphonuclear neutrophils (PMN) from healthy donors after incubation with tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) *in vitro*

Condition (concentration of TNF- $\alpha$ )	Percentage of PMN expressing PR3	<i>P</i>
Basal	18.0 $\pm$ 3.5	–
TNF- $\alpha$ (1 ng/ml)	25.3 $\pm$ 3.3	0.044*
TNF- $\alpha$ (3 ng/ml)	36.0 $\pm$ 0.8	0.014*
TNF- $\alpha$ (10 ng/ml)	52.3 $\pm$ 2.2	0.002*

PMN were incubated with TNF- $\alpha$  in increasing concentrations ranging from 1 to 10 ng/ml for 10 min. Subsequently, PMN ( $1 \times 10^6$ /ml) were incubated with MoAbs to PR3 (WGM2) for 20 min. After washing, the second antibody (fluorescein (DTAF)-conjugated F(ab)<sub>2</sub> goat anti-mouse (IgG) was added. Subsequently the labelled cells were fixed with PBS containing 1.5% paraformaldehyde. The surface expression of PR3 is given as the percentage of PMN showing PR3 expression on flow cytometric analysis. Data are means  $\pm$  s.e.m. from nine experiments using cells from nine different donors.

\*Statistically significant difference between stimulated (TNF- $\alpha$ ) and unstimulated PMN (basal).

PMN. Addition of G-CSF and GM-CSF at five different doses (0.03–4 ng/ml) did not result in significant stimulation of MPO expression (percentage of MPO-expressing cells and median fluorescence intensity), either in native PMN or in TNF- $\alpha$ -primed neutrophils (Fig. 2c,d).

## DISCUSSION

ANCA-induced activation of primed neutrophils is thought to be the initiating event in the development of vasculitis in WG [19,21–25,28–30]. We previously reported that certain cytokines like TNF- $\alpha$  can induce a translocation of PR3 to the plasma membrane of PMN [23]. Other cytokines, like IL-8 and transforming growth factor- $\beta$  (TGF- $\beta$ ), which alone do not increase PR3 expression, can stimulate PR3 expression significantly if PMN are primed [23,31]. The results of the present study demonstrate a divergent effect of the two haematopoietic growth factors G-CSF and GM-CSF on PR3 expression *in vitro*. While even high concentrations of rhG-CSF given as single agent or after priming with TNF- $\alpha$  did not alter the expression of PR3 on the plasma membrane of PMN *in vitro*, rhGM-CSF, but not rhG-CSF, induced significant expression of PR3 on plasma membranes of both intact and preactivated PMN primed with TNF- $\alpha$ . In agreement with our observation, it has been reported that the subset of PMN expressing PMN on their surface membrane largely varies among healthy probands, but stays relatively stable over time in each individual [21]. The results of the present study indicate that GM-CSF can shift this bimodal expression pattern of PR3 towards a higher percentage of PMN expressing PR3 which may become possible targets of ANCA-induced neutrophil activation. The reasons for the differential effect of rhGM-CSF and rhG-CSF on PR3 surface expression on neutrophils are unclear. Despite recent progress regarding structure and function of the human PR3 genome and protein [32–34], the factors modulating production, packaging and surface expression on PMN are poorly understood. Interestingly, the same divergent effect of G-CSF and GM-CSF for PR3 expression on neutrophils observed in the present study has been reported for the production

of MPO, another target antigen of ANCA [35]. Treweeke *et al.* not only demonstrated a five-fold increase in fMLP-induced MPO production in PMN in response to GM-CSF, which was not seen in the presence of G-CSF, but also found several other functions of mature PMN including chemiluminescence and O<sub>2</sub><sup>-</sup> production which were stimulated by GM-CSF, but not by G-CSF [35]. However, expression of MPO on plasma membranes of PMN was found not to be influenced by either G-CSF or GM-CSF in the present study. The different modes of stimulation (fMLP *versus* TNF- $\alpha$ ) and that the fact that increased MPO production not necessarily implies increased surface expression may explain the different findings of our study and that of Treweeke. While GM-CSF and G-CSF share many common properties regarding the production and function of PMN, several differential effects of these haematopoietic growth factors on mature PMN have been shown *in vitro* and *in vivo*, which have been extensively reviewed recently [2]. Different signal transduction mechanisms of GM-CSF (via tyrosine kinase) and G-CSF (involvement of G-proteins) have been demonstrated in PMN and may possibly account for the different functional pattern of the two factors [36].

Our results are of clinical relevance in view of recent observations describing an increased incidence of cutaneous vasculitides in patients treated with rhG-CSF or rhGM-CSF for severe neutropenia due to different disorders, including autoimmune neutropenia due to Felty's syndrome [6–11]. Thus, the question has been raised whether administration of rhG-CSF or rhGM-CSF to patients with SV for neutropenia due to cytotoxic medication like CYC might possibly trigger the inflammatory process and aggravate the vasculitic disease [11]. For rhGM-CSF, our data indicate a substantially higher theoretical risk of exacerbating the vasculitis when given to patients with active C-ANCA-associated vasculitides. In the present study we used (TNF- $\alpha$ ) primed PMN, which are also found in active C-ANCA-associated vasculitides, where activated PMN as well as increased levels of proinflammatory cytokines like TNF- $\alpha$  or TGF- $\beta$  are found [31,37]. In view of the general limitation that information gathered from *in vitro* studies is not always correlated with results obtained during administration of the drug *in vivo*, the results of the present study indicate that rhGM-CSF given to WG patients with drug-induced neutropenia is likely to increase the relative and absolute amount of PMN expressing PR3 on their plasma membrane. Since in C-ANCA-associated vasculitides accessibility of the antigen (PR3) to the antibody (ANCA) on the plasma membrane of PMN is thought to be essential for neutrophil activation by ANCA, the results of the present study suggest that deterioration of vasculitis appears possible, at least via this mechanism in patients with WG receiving rhGM-CSF. In contrast, our data make a clinically relevant C-ANCA-mediated neutrophil activation induced by rhG-CSF less likely. However, the short incubation times chosen for the present *in vitro* experiments may not necessarily reflect the *in vivo* situation where the concentrations of haematopoietic growth factors are elevated for a longer time during therapeutic administration. The data of the present study support the results of a recent clinical study showing that administration of rhG-CSF to WG patients with CYC-induced agranulocytosis was not associated with an increase of clinical and laboratory chemical signs of disease activity during up to 6 months of follow up [17].

However, it has to be pointed out that our data do not exclude the possibility that rhG-CSF given to patients with C-ANCA-associated vasculitides *in vivo* might aggravate vasculitic disease

by raising the neutrophil count far above normal or by modulating the function of mature PMN via other mechanisms. Clinical observations as well as data from comprehensive *in vitro* and *in vivo* studies investigating the influence of rhG-CSF and rhGM-CSF on mature neutrophils suggest that the first rather than the latter might be the case [6,38–43]. Mechanisms whereby rhG-CSF and GM-CSF theoretically could modulate neutrophil function in WG apart from direct stimulation of PR3 expression include direct stimulation of neutrophil function as well as indirect mechanisms such as release of proinflammatory mediators.

First, expression of Fc $\gamma$  receptor IIIb (CD16), which is involved in initiation of ANCA-induced neutrophil activation [41], has been shown to be markedly decreased after administration of rhG-CSF to neutropenic patients and healthy volunteers [2], suggesting inhibition rather than stimulation of ANCA-induced neutrophil activation via this mechanism. However, stimulation of ANCA-induced neutrophil activation by other mechanisms remains possible and merits further exploration. Second, a reduced expression of leucocyte adhesion molecule-1 (LAM-1) [37] and a reduction of directed migration and chemotaxis of PMN from healthy donors given rhG-CSF *in vivo* [2,39] argue against a significant promoting effect of rhG-CSF on leucocyte adherence and migration during the initial phase in the development of necrotizing vasculitis. Third, G-CSF induces a shift towards an anti-inflammatory cytokine response (reduced secretion of TNF- $\alpha$  and interferon-gamma (IFN- $\gamma$ ), increased expression of IL-8, IL-10 and soluble TNF- $\alpha$  receptors), making an indirect impact of G-CSF on proinflammatory properties of PMN from patients receiving G-CSF less likely [39,40]. Finally, G-CSF or GM-CSF alone is unable to initiate a respiratory burst in native PMN [2]. Recent *in vivo* studies however demonstrated that following administration of G-CSF *in vivo* superoxide anion production in response to several physiological stimuli can be different and depends on the stimulus used, resulting in enhanced (fMLP), normal (opsonized zymosan) or reduced (phorbol myristate acetate, platelet-activating factor/fMLP) respiratory burst activity [38]. Numerous *in vitro* studies describe many other pro- and anti-inflammatory capabilities of G-CSF [1–5], but data derived from several recent studies in healthy volunteers investigating the effect of G-CSF on functional parameters of mature PMN *in vivo* indicate that G-CSF administration imparts only modest effects on neutrophil function *in vivo* [38–40].

In conclusion, these data demonstrate that rhGM-CSF, but not rhG-CSF, induces plasma membrane expression of PR3 on activated PMN *in vitro*. Thus, patients with ANCA-associated vasculitis receiving GM-CSF instead of rhG-CSF for drug-induced agranulocytosis may be at higher risk of exacerbating vasculitic disease by increased binding of ANCA to PR3 and subsequent neutrophil activation. In view of the data shown here and our clinical experience reported earlier [17], we recommend the use of short courses of low-dose rhG-CSF in severely neutropenic patients with C-ANCA-associated vasculitides (if clinically indicated) or for progenitor cell rescue in experimental protocols of stem cell transplantation, until greater experience with the use of haematopoietic growth factors in vasculitic disorders is available.

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