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## Neutrophil functions in patients with myelodysplasia

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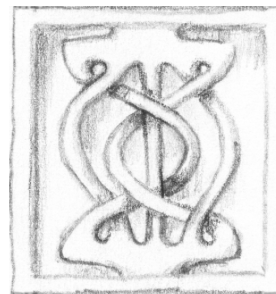
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# CHAPTER 4

## **Reduced expression of flavocytochrome $b_{558}$ , a component of the NADPH oxidase complex, in neutrophils from patients with myelodysplasia**

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## **Abstract**

*Objective.* Patients with myelodysplasia (MDS) show a disturbed production ROS in response to N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) in granulocyte-macrophage colony-stimulating factor (GM-CSF)-primed neutrophils. Because generation of ROS is mediated by the NADPH oxidase complex, a component of which is flavocytochrome b<sub>558</sub>, we investigated whether the expression of flavocytochrome b<sub>558</sub> in neutrophils from MDS patients is affected.

*Material and methods.* Neutrophils were stimulated with fMLP and GM-CSF, and plasma membrane expression of flavocytochrome b<sub>558</sub> and specific granule markers were assessed by fluorescence-activated cell sorting analysis. Protein levels of the flavocytochrome b<sub>558</sub> subunits gp91<sup>phox</sup> and p22<sup>phox</sup> in whole neutrophil lysates were detected by Western blotting.

*Results.* Stimulation of neutrophils with GM-CSF and fMLP increased the flavocytochrome b<sub>558</sub> plasma membrane expression. The fMLP-induced translocation of flavocytochrome b<sub>558</sub> was reduced in neutrophils from MDS patients (140±9% vs 180±13%, p<0.05). Analysis of cell surface expression of markers of flavocytochrome b<sub>558</sub> containing granules (CD35 and CD66b) indicated that exocytosis of these granules in response to fMLP stimulation was not affected in MDS patients. Western blot analysis demonstrated a decreased protein expression level of the flavocytochrome b<sub>558</sub> subunits gp91<sup>phox</sup> and p22<sup>phox</sup> in neutrophils from MDS patients.

*Conclusion.* Our results indicate both a lower basal protein level and a disturbed fMLP-induced increase in plasma membrane expression of flavocytochrome b<sub>558</sub> in neutrophils from MDS patients, which together might play a role in decreased ROS production.

## Introduction

The myelodysplastic syndromes (MDS) are a group of clonal stem cell disorders characterised by a disturbed differentiation of the myeloid, erythroid or megakaryocytic lineage <sup>1</sup>. In approximately 30% of cases, transformation to acute myeloid leukaemia (AML) occurs <sup>2</sup>. However, bacterial infections pose an even greater threat to MDS patients, as shown by the high incidence of bacterial infection-related deaths, indicating an important role for granulocytes in the pathology of the disease <sup>3</sup>. Indeed, the differentiation defect in the MDS multipotent stem cell compartment not only leads to neutropenia, but also results in aberrant neutrophil functioning. For instance, deficiencies in the contents of primary and specific granules have been reported in neutrophils from MDS patients <sup>4;5</sup>. Furthermore, we and others have shown that the production of reactive oxygen species (ROS) is affected in MDS patients <sup>6-8</sup>.

ROS production during the respiratory burst is essential for neutrophil bactericidal activity, and can be stimulated by chemoattractants such as the bacterial peptide analogue N-formyl-L-Methionyl-L-Leucyl-L-Phenylalanine (fMLP) <sup>9;10</sup>. Pro-inflammatory agents such as granulocyte-macrophage colony-stimulating factor (GM-CSF) are capable of enhancing the fMLP-mediated respiratory burst, but do not elicit ROS production on their own <sup>11</sup>. This process is known as *priming*.

The generation of ROS is mediated by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. This enzyme consists of a membrane bound component, flavocytochrome  $b_{558}$ , and the cytosolic factors  $p47^{phox}$ ,  $p40^{phox}$ ,  $p67^{phox}$  and Rac2, which exist in the cytosol as a complex <sup>12</sup>. In unstimulated neutrophils, 10-20% of the flavocytochrome  $b_{558}$ , which is composed of the subunits  $gp91^{phox}$  and  $p22^{phox}$ , is anchored in the plasma membrane, whereas the remaining 80-90% resides in the membrane of secretory vesicles, specific granules and tertiary granules <sup>13;14</sup>. Upon activation of the cells with a variety of stimuli, exocytosis of these granules results in an increase of flavocytochrome  $b_{558}$  present at the plasma membrane <sup>13</sup>. Furthermore, stimulation of the cells results in phosphorylation of  $p47^{phox}$  on multiple serine residues, activation of the GTP-ase Rac, and subsequent translocation of the entire cytosolic complex to the membrane, where it associates with flavocytochrome  $b_{558}$  to form the active oxidase <sup>15-20</sup>.

Several signal transduction pathways have been implicated in NADPH oxidase activation <sup>21-23</sup>. For example, the involvement of phosphatidylinositol 3-kinase (PI3K) in fMLP-stimulated ROS production has been established by using



specific inhibitors<sup>24-26</sup>. Furthermore, lipid products of PI3K associate with the cytosolic oxidase proteins p47<sup>phox</sup> and p40<sup>phox</sup><sup>27</sup>. The association of one of these lipids with p40<sup>phox</sup> has been shown to have a regulatory effect on ROS production<sup>28;29</sup>. In addition to PI3K, extracellular signal-regulated protein kinase 1 and 2 (ERK1/2), a key member of the mitogen-activated protein kinase (MAPK) signalling route, is involved in the neutrophil respiratory burst<sup>30</sup>. Inhibition of ERK activation by chemical inhibitors resulted in a reduction of fMLP-induced ROS production in GM-CSF-primed human neutrophils<sup>26;31</sup>.

Our previous studies demonstrated that the fMLP-induced ROS production in GM-CSF-primed neutrophils was significantly reduced in neutrophils from MDS patients, which coincided with a decreased level of phosphorylation of ERK1/2 in response to fMLP in these cells<sup>6</sup>. In this study, we set out to further investigate the mechanisms underlying the defects in ROS generation observed in MDS patients. We demonstrate that in normal neutrophils both fMLP and GM-CSF induce an increase in flavocytochrome b<sub>558</sub> expression at the plasma membrane, and that the fMLP-stimulated expression is reduced in MDS patients. Furthermore, we show that the decreased expression of flavocytochrome at the plasma membrane of MDS neutrophils is most likely due to reduced protein levels of the p91<sup>phox</sup> and p22<sup>phox</sup> subunits, rather than a disturbed exocytosis of the flavocytochrome b<sub>558</sub> containing secretory vesicles and specific granules.

## **Materials and methods**

### **Reagents**

N-formyl-L-Methionyl-L-Leucyl-L-Phenylalanine (fMLP), ferricytochrome c and superoxide dismutase (SOD) were obtained from Sigma (St. Louis, MO). Recombinant human GM-CSF was from Novartis (Basel, Switzerland). Antibody 7D5 was a kind gift from Prof. Dr. D. Roos (Central Laboratory of the Netherlands Blood Transfusion Service (CLB), Amsterdam, The Netherlands) and Dr. M. Nakamura (Institute of Tropical Medicine, Nagasaki, Japan). Antibodies against gp91<sup>phox</sup> (Ab 48) and p22<sup>phox</sup> (Ab 449) were also kindly provided by Prof. Dr. D. Roos.

### **Patients**

Twelve patients with MDS were studied. MDS was classified as refractory anemia (RA), RA with ringed sideroblasts (RARS) or RA with excess of blasts (RAEB) according to FAB cooperative group criteria<sup>32</sup> (Table 1). None of the

patients suffered from recurring infections during the course of this study. Informed consent was obtained from all patients. The protocol was approved by the Human Subject Review Board of the University Hospital Groningen.

### **Isolation of neutrophils**

Peripheral blood was obtained from healthy volunteers and MDS patients. Neutrophils were isolated as described previously<sup>6,33</sup>. In all cases, the cell population isolated consisted of >95% neutrophils as determined by May-Grünwald Giemsa staining.

### **Respiratory burst measurement**

O<sub>2</sub><sup>-</sup> generation was measured by the superoxide dismutase-inhibitable reduction of ferricytochrome c, using an automated microplate reader (Thermomax, Molecular Devices, Sunnyvale, CA, USA), as described previously<sup>6,34</sup>. The superoxide production was expressed as the difference in OD<sub>550</sub> nm between the ferricytochrome c reduction test in the absence and in the presence of SOD. Each test was performed in quadruplicate.

### **Expression of plasma membrane proteins**

The plasma membrane expression of flavocytochrome b<sub>558</sub>, CD35 and CD66b were determined by flow cytometry. Isolated neutrophils were stimulated as indicated in the figure legends. Plasma membrane expression of flavocytochrome b<sub>558</sub> was determined using a monoclonal antibody against its extracellular domain, 7D5<sup>35</sup>, as described<sup>36</sup>. In short, neutrophils were blocked by incubation with 2% goat serum for 10 min at 4°C, and subsequently incubated with 7D5 for 30 min at 4°C. After washing of the cells with ice-cold PBS, the neutrophils were incubated for an additional 30 min at 4°C with FITC-conjugated anti-mouse IgG. Plasma membrane expression of CD35 and CD66b were determined as described by Ward et al.<sup>37</sup>. Briefly, stimulated neutrophils were washed with ice-cold PBS and samples were incubated with FITC-conjugated monoclonal anti-CD35 or FITC-conjugated monoclonal anti-CD66b for 45 min at 4°C. FITC-conjugated IgG1 and FITC-conjugated IgM, were used as isotype controls, respectively. Fluorescence intensities were measured by FACS analysis (FACScalibur, Becton Dickinson Medical Systems, Sharon, MA).

### **Western blotting**

The protein expression levels of the flavocytochrome b<sub>558</sub> subunits present in whole cell lysates were determined by Western blotting. Proteins were separated



on 10% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (Protran; Schleicher & Schuel, Dassel, Germany). Membranes were probed with Ab 48 and Ab 449<sup>38</sup>. Proteins were detected by enhanced chemiluminescence. To confirm equal loading, blots were subsequently reprobed with antibodies against ERK (K23). Quantification of protein levels was performed by densitometry of the films, using imagemaster1D Elite (Pharmacia, Woerden, the Netherlands).

### Statistical analysis

For comparison of O<sub>2</sub><sup>-</sup> generation and expression of plasma membrane proteins, differences between stimulations with or without inhibitors were calculated using the Student *t*-test for paired samples. Differences in values between healthy donors and MDS patients were calculated using the Student *t*-test for unpaired samples. For quantification of p22<sup>phox</sup> protein levels, p22<sup>phox</sup> densitometry values were divided by the densitometry values of ERK1/2 protein present in the same samples and differences between the normalised values of healthy controls and MDS patients were calculated using the Student *t*-test for unpaired samples. Data were expressed as mean ± SEM. P values <0.05 were considered significant.

**Table 1.**

FAB: French-American-British Classification (RA = refractory anemia; RARS = refractory anemia with ringed sideroblasts; RAEB = refractory anemia with excess of blasts); Hb = hemoglobin; Transf = blood transfusion after collection of peripheral blood for this study; Plat = platelets; Leuko = leukocytes; Granulo = granulocytes; Mono = monocytes; Chrom. abn = presence (abn.) or absence (norm.) of chromosomal abnormalities; ND = not done.

No.	FAB	Hb (mmol/L)	Plat. (10 <sup>9</sup> /L)	Leuko. (10 <sup>9</sup> /L)	Granulo. (%)	Mono. (%)	Chrom. Abn.
1	RA	78/F	117	11	63	7	Abn.
2	RA	78/M	130	3.3	74	6	Abn.
3	RA	83/M	63	3.3	72	10	ND
4	RA	29/F	80	3.8	19	1	Abn.
5	RA	68/M	30	2.0	42	16	ND
6	RA	33/M	70	3.1	80	11	Abn.
7	MDS	30M	30	4.0	60	4	Norm.
8	RARS	64/M	150	2.9	40	8	Abn.
9	RARS	71/F	225	2.5	14	6	ND
10	REAB	65/M	32	2.2	52	10	Abn.
11	REAB	29/F	56	6.7	40	10	Abn.
12	RAEB	76/F	25	2.3	45	6	Norm.

## Results

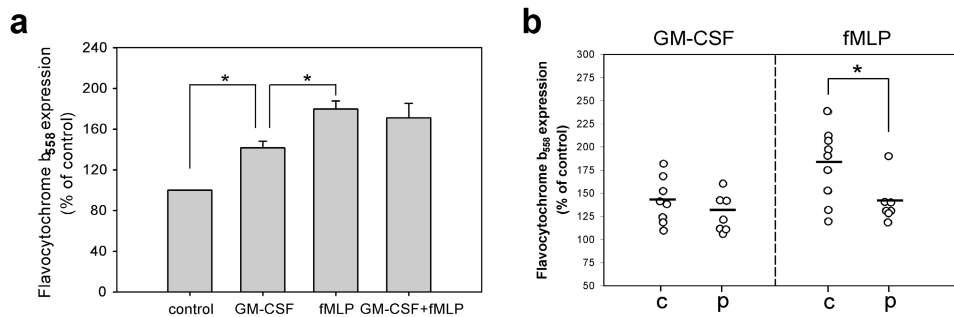
### ***fMLP and GM-CSF induce plasma membrane expression of flavocytochrome $b_{558}$ on neutrophils from healthy donors***

GM-CSF priming of the fMLP-triggered neutrophil respiratory burst is significantly decreased in MDS patients (6). As extracellular  $O_2^-$  is generated by the active NADPH oxidase complex at the plasma membrane of the neutrophil<sup>39</sup>, we examined the plasma membrane expression of flavocytochrome  $b_{558}$ , the membrane-bound component of the NADPH oxidase complex. As demonstrated in Figure 1A, stimulation of neutrophils with 5 ng/mL GM-CSF resulted in a significant increase of flavocytochrome  $b_{558}$  present at the plasma membrane ( $142 \pm 9\%$ ,  $p < 0.05$ ,  $n=8$ ). Stimulation of neutrophils with 1  $\mu$ M fMLP for 10 minutes resulted in a higher membrane expression of flavocytochrome  $b_{558}$  ( $180 \pm 13\%$ ,  $p < 0.05$ ). Pretreatment of neutrophils with GM-CSF prior to fMLP stimulation led to a flavocytochrome  $b_{558}$  membrane expression of  $171 \pm 11\%$  of that of unstimulated cells ( $p < 0.05$ ), which was not significantly different from that of fMLP-stimulated, unprimed neutrophils ( $p=0.64$ ). These results indicate that both GM-CSF and fMLP are capable of increasing the levels of flavocytochrome  $b_{558}$  present at the plasma membrane. However, in contrast to  $O_2^-$  generation, GM-CSF has no priming effect on the fMLP-triggered flavocytochrome plasma membrane expression.



### ***Decreased fMLP-mediated flavocytochrome $b_{558}$ expression on plasma membrane of neutrophils from MDS patients.***

Next we studied the fMLP- and GM-CSF-stimulated increase in flavocytochrome  $b_{558}$  translocation to the plasma membrane, by comparing the expression of this NADPH oxidase component at the plasma membrane of neutrophils from 7 MDS patients and 8 healthy donors. When we compared the basal expression of flavocytochrome on the plasma membrane of unstimulated neutrophils, no significant difference was observed between MDS patients ( $28 \pm 5$  [mean fluorescence intensity]) versus healthy volunteers ( $26 \pm 3$  [mfi]) ( $p=0.73$ ). Stimulation of MDS neutrophils with 5 ng/mL GM-CSF or 1  $\mu$ M fMLP resulted in a significant increase in flavocytochrome  $b_{558}$  membrane expression to  $128 \pm 8\%$  and  $140 \pm 9\%$  of control, respectively (Figure 1B,  $p < 0.05$ ).



**Figure 1. Flavocytochrome  $b_{558}$  plasma membrane expression on neutrophils from healthy donors and MDS patients.** (A) Isolated neutrophils were stimulated with 1  $\mu$ M fMLP for 10 minutes, either with or without prior priming with 5 ng/mL GM-CSF for 15 minutes. Alternatively, neutrophils were stimulated for 15 minutes with GM-CSF alone. Stimulation was stopped by washing the cells with ice-cold PBS. Neutrophils were blocked by incubation with 2% goat serum for 10 minutes at 4°C, and incubated with 7D5 antibody for 30 minutes. After washing of the cells with ice-cold PBS, the neutrophils were incubated for an additional 30 minutes with FITC-conjugated anti-mouse IgG. Fluorescence intensities were measured by FACS analysis. Results represent the mean increase in flavocytochrome  $b_{558}$  plasma membrane expression after stimulation, compared to unstimulated cells, on neutrophils from eight healthy donors. Significant differences between stimulated groups are indicated by asterisks ( $P < 0.05$ ). (B) Freshly isolated neutrophils from 7 MDS patients and 8 healthy donors were stimulated with either 5 ng/mL GM-CSF for 15 minutes or 1  $\mu$ M fMLP. Flavocytochrome  $b_{558}$  plasma membrane expression was measured as described above. Significant differences between MDS patients and healthy donors are indicated by an asterisk ( $P < 0.05$ ).

However, although one patient exhibited an fMLP-induced translocation that was more than 2 SD higher than the mean of that group, the fMLP-induced flavocytochrome  $b_{558}$  translocation was significantly lower in neutrophils from MDS patients than in those from healthy volunteers ( $140 \pm 9\%$  vs  $180 \pm 13\%$ ,  $p < 0.05$ ). The GM-CSF-induced flavocytochrome  $b_{558}$  plasma membrane expression also seemed to be slightly lower compared to healthy donors, although this difference was not significant ( $128 \pm 8\%$  vs  $141 \pm 9\%$ ,  $p = 0.27$ ). Our data indicate that, upon stimulation with fMLP, the increase in expression levels of flavocytochrome  $b_{558}$  at the plasma membrane of neutrophils from MDS patients is significantly disturbed.

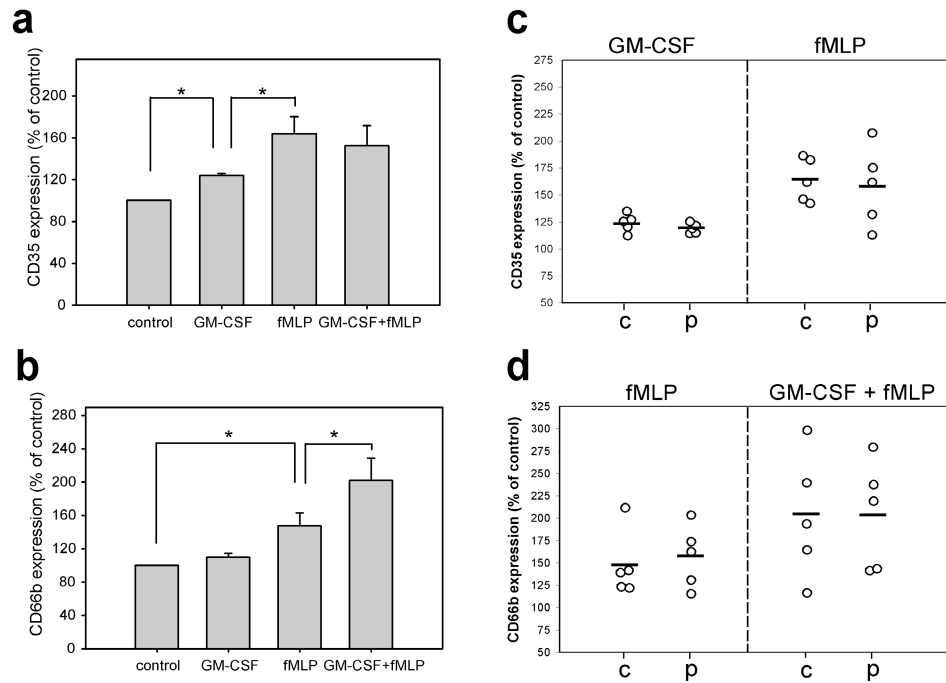
**Normal exocytosis of secretory vesicles and specific granules in neutrophils from MDS patients**

Previous studies have indicated that in the resting neutrophil, most flavocytochrome  $b_{558}$  is anchored in the membrane of specific granules and secretory vesicles. Upon stimulation of the cell, these granules fuse with the plasma membrane, leading to an increased expression of flavocytochrome at the plasma membrane. To determine whether the decreased flavocytochrome  $b_{558}$  expression at the plasma membrane of MDS neutrophils could be attributed to a decreased exocytosis of flavocytochrome-containing granules, we studied the membrane expression of CD35 and CD66b, which are specific markers of secretory vesicles and specific granules, respectively<sup>40</sup>. Similar to flavocytochrome  $b_{558}$  expression, stimulation of healthy neutrophils with 5 ng/mL GM-CSF lead to an enhanced membrane expression of the secretory vesicle marker, CD35 ( $124 \pm 4\%$ ,  $p > 0.05$ ,  $n = 5$ ). Similarly, 1  $\mu$ M fMLP induced an enhancement of CD35 membrane expression of  $164 \pm 9\%$  ( $p < 0.05$ ), which could not be further elevated by pretreatment of the neutrophils with GM-CSF (Figure 2A). The expression pattern of CD66b was different from that of CD35 and flavocytochrome  $b_{558}$ . GM-CSF stimulation alone was not capable of significantly increasing the amount of CD66b present at the plasma membrane ( $109 \pm 6\%$ ,  $p = 0.20$ ,  $n = 5$ ; Figure 2B). fMLP alone increased the CD66b membrane expression up to  $147 \pm 16\%$  ( $p < 0.05$ ). However, unlike either CD35 or flavocytochrome  $b_{558}$  expression, CD66b expression could be significantly increased by priming the neutrophils with GM-CSF prior to fMLP stimulation ( $202 \pm 31\%$ ,  $p = 0.05$ ).

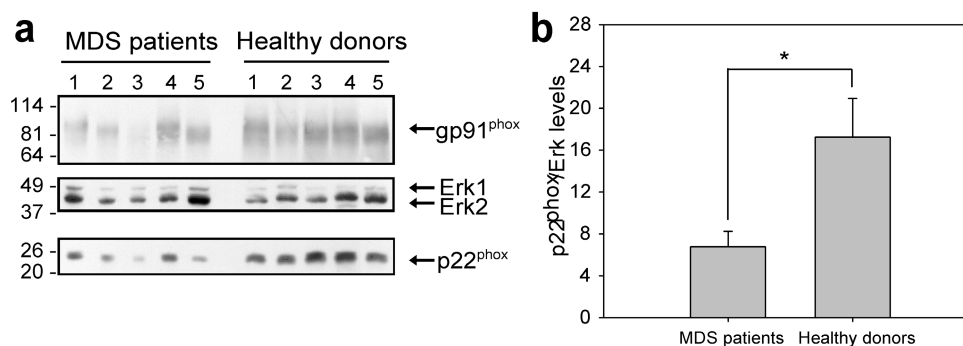
We next analysed the capacity of fMLP and GM-CSF to induce exocytosis of secretory vesicles and specific granules in neutrophils from 5 MDS patients compared to 5 healthy donors. Figure 2C shows that both the GM-CSF- and the fMLP-stimulated increase in CD35 membrane expression were not altered in MDS neutrophils. The basal expression of CD35 on unstimulated neutrophils of MDS patients and healthy donors was similar ( $21 \pm 3$  [mfi] vs  $19 \pm 1$  [mfi],  $p = 0.50$ , data not shown). Likewise, the fMLP-stimulated increase in CD66b membrane expression in GM-CSF-primed and unprimed neutrophils was comparable between MDS patients and healthy donors (Fig 2D). Again, basal CD66b membrane expression on unstimulated cells was equal between neutrophils from MDS patients and healthy volunteers ( $34 \pm 5$  [mfi] vs  $39 \pm 4$  [mfi],  $p = 0.40$ , not shown). Taken together, these results indicate that the decreased fMLP-induced plasma membrane expression of flavocytochrome  $b_{558}$



on MDS neutrophils is not due to a decreased fusion of secretory vesicles or specific granules with the neutrophil plasma membrane.



**Figure 2. CD35 and CD66b plasma membrane expression on neutrophils from healthy donors and MDS patients.** (A, B) Isolated neutrophils were stimulated with 1  $\mu$ M fMLP for 10 minutes, either with or without prior priming with 5 ng/mL GM-CSF for 15 minutes. Alternatively, neutrophils were stimulated for 15 minutes with GM-CSF alone. Stimulation was terminated by washing the neutrophils with ice-cold PBS and placing them on ice. The samples were incubated with FITC-conjugated monoclonal anti-CD35 (A) or FITC-conjugated monoclonal anti-CD66b (B) for 45 minutes at 4°C. FITC-conjugated IgG1 and FITC-conjugated IgM, were used as isotype controls, respectively. Fluorescence intensities were measured by FACS analysis. The mean increase in CD35 and CD66b plasma membrane expression after stimulation, compared to unstimulated cells, is shown for 5 healthy donors. Significant differences between stimulated groups are indicated by an asterisks ( $P < 0.05$ ). (C) Isolated neutrophils from 5 MDS patients and 5 healthy volunteers were stimulated with either 5 ng/mL GM-CSF for 15 minutes, or 1  $\mu$ M fMLP. CD35 expression at the cell surface was measured as described above. (D) Isolated neutrophils from 5 MDS patients and 5 healthy volunteers were stimulated with 1  $\mu$ M fMLP for 10 minutes, either with or without prior priming with 5 ng/mL GM-CSF for 15 minutes. CD66b plasma membrane expression was measured as described earlier.



**Figure 3. Decreased protein levels of the flavocytochrome  $b_{558}$  subunits, gp91<sup>phox</sup> and p22<sup>phox</sup>, in neutrophils from MDS patients.** (A) Neutrophils from healthy donors and MDS patients were resuspended in 1 X Laemmli buffer and samples were boiled. Proteins were separated by SDS-PAGE, and Western blotting was performed using antibodies directed against gp91<sup>phox</sup> (Ab 48) (upper panel), p22<sup>phox</sup> (Ab449) (lower panel). Blots were reprobed with antibodies against ERK1/2 (middle panel). Proteins were detected by enhanced chemiluminescence. Whole neutrophil lysates of 5 out of 9 MDS patients and 5 out of 8 healthy volunteers are shown. (B) Protein levels of the flavocytochrome  $b_{558}$  subunit p22<sup>phox</sup> and ERK1/2 were quantified by densitometry of the films. ERK1/2 was used as control for equal loading, as we have previously shown that equal numbers of neutrophils from different donors (patients or healthy donors) contains equal amounts of the ERK1/2 protein (6). The p22<sup>phox</sup> protein levels in the samples were corrected for the ERK levels in those same samples, and differences between the means of the normalised values were calculated between MDS neutrophils (n=9) and healthy donors (n=8) using the Student *t*-test for unpaired samples. Significant differences are indicated with an asterisk ( $P < 0.05$ ).



### **Decreased expression of flavocytochrome $b_{558}$ subunits in neutrophils from MDS patients**

To further examine the possible cause of the lower flavocytochrome  $b_{558}$  expression in response to fMLP stimulation on neutrophils from MDS patients, we next investigated the total amount of flavocytochrome  $b_{558}$  protein present in the neutrophils of MDS patients compared to healthy donors. Western blot analysis of whole neutrophil lysates probed with antibodies directed against the flavocytochrome  $b_{558}$  subunits gp91<sup>phox</sup> and p22<sup>phox</sup> indicated that these NADPH oxidase subunits were present in neutrophils from both MDS patients and healthy volunteers (Figure 3A). However, quantification of the protein levels by densitometry indicated that the protein levels of p22<sup>phox</sup> present in the neutrophil lysates were significantly lower in MDS patients ( $7 \pm 1$ , n=9) than in the healthy controls ( $17 \pm 4$ , n=8) (Figure 3B,  $p < 0.05$ ). The amount of gp91<sup>phox</sup> could not be

quantified by densitometry because the monoclonal antibody detecting this protein results in a wide band with high background<sup>38</sup>. However, the protein levels of gp91<sup>phox</sup> present in the MDS neutrophil lysates did appear to be lower than the levels of gp91<sup>phox</sup> present in healthy neutrophils (Figure 3A, upper panel). Our results indicate that the total amount of flavocytochrome b<sub>558</sub> present in the neutrophils from MDS patients is lower than that in healthy neutrophils. This might partially explain the reduced fMLP-stimulated increase in flavocytochrome b<sub>558</sub> plasma membrane expression observed in these patients.

## **Discussion**

The generation of extracellular O<sub>2</sub><sup>-</sup>, as measured by cytochrome c reduction by neutrophils, is mediated by the NADPH oxidase enzyme complex at the plasma membrane of these cells<sup>39</sup>. The plasma membrane expression of flavocytochrome b<sub>558</sub>, the membrane-bound component of the oxidase complex, can be increased after stimulation of the neutrophil with either fMLP or GM-CSF. In contrast to the data reported for ROS production in neutrophils, we show here that the membrane expression of flavocytochrome b<sub>558</sub> induced by fMLP cannot be primed by GM-CSF pretreatment. This indicates that GM-CSF priming of fMLP-induced O<sub>2</sub><sup>-</sup> generation does not occur through enhancement of the fMLP-induced plasma membrane expression of the membrane-bound component of the NADPH oxidase complex. Recent studies have suggested that stimulation of neutrophils with priming agents (e.g. granulocyte colony-stimulating factor, tumour necrosis factor- $\alpha$ , lipopolysaccharide) results in priming of the respiratory burst by increasing the flavocytochrome b<sub>558</sub> plasma membrane expression, irrespective of the fMLP-induced increase of flavocytochrome present at the membrane<sup>37,41</sup>. In neutrophils from MDS patients, GM-CSF priming of the fMLP-triggered respiratory burst is significantly decreased. However, whereas in this study we demonstrated that the fMLP-stimulated translocation of flavocytochrome to the plasma membrane of MDS neutrophils was significantly reduced, the GM-CSF-mediated expression was not significantly affected. This suggests that, in neutrophils from MDS patients, the lower fMLP-induced increase in flavocytochrome b<sub>558</sub> is limiting the amount of O<sub>2</sub><sup>-</sup> that can be produced in GM-CSF-primed cells. This presumption is further corroborated by the finding that both the GM-CSF-primed ROS production and the fMLP-triggered flavocytochrome b<sub>558</sub> expression can be partially attenuated with the chemical

inhibitor U0126, indicating a role for the MEK/ERK pathway in both these processes (unpublished results, GM Fuhler).

It was previously reported that the translocation of flavocytochrome to the plasma membrane of neutrophils upon stimulation occurs through exocytosis of flavocytochrome  $b_{558}$  containing secretory vesicles and specific granules<sup>37</sup>. In this study we demonstrated that the degranulation of these vesicles, as measured by the plasma membrane expression of CD35 and CD66b, was not affected in neutrophils from MDS patients. Interestingly, GM-CSF alone did not increase the expression levels of CD66b at the plasma membrane, but was capable of enhancing the fMLP-induced CD66b expression. This is in distinct contrast to the effect of GM-CSF on membrane expression of CD35, suggesting that the fusion of secretory vesicles and specific granules are differentially regulated. The expression patterns of flavocytochrome  $b_{558}$  and CD35 at the neutrophil plasma membrane correlated with each other (compare Figure 1A to Figure 2A), indicating that the flavocytochrome  $b_{558}$  expression measured at the plasma membrane after fMLP stimulation might be caused by the degranulation of secretory vesicles. However, whereas the fMLP-induced flavocytochrome  $b_{558}$  membrane expression was decreased in MDS neutrophils, the fMLP-induced exocytosis of secretory vesicles was not. These data indicate that degranulation of other types of flavocytochrome  $b_{558}$ -containing granules, such as tertiary granules, might be affected in MDS patients. Indeed, flavocytochrome  $b_{558}$  translocation to the plasma membrane in response to G-CSF stimulation has been attributed to the exocytosis of such tertiary granules<sup>41</sup>.

Another explanation for the decreased fMLP-induced flavocytochrome  $b_{558}$  membrane expression could be a reduced presence of flavocytochrome  $b_{558}$  in the membrane of intracellular granules. Indeed, we demonstrated that the amount of  $p22^{phox}$  and  $gp91^{phox}$  were significantly reduced in neutrophils from MDS patients. However, this could not constitute the sole reason for the decreased ROS production found in MDS patients, because phorbol 12-myristate 13-acetate (PMA) induces a high  $O_2^-$  release in neutrophils from MDS patients<sup>6,7</sup>. Furthermore, GM-CSF-triggered flavocytochrome  $b_{558}$  membrane expression was not affected in MDS patients. Therefore, the defect in translocation of flavocytochrome to the plasma membrane is at least partially stimulant dependent. Indeed, it has been suggested that decreased protein levels of  $gp91^{phox}$  and  $p22^{phox}$  by themselves do not necessarily lead to reduced NADPH activity<sup>42;43</sup>. On the other hand, studies performed in patients suffering from X-linked type of chronic granulomatous disease, in which neutrophils are defective in the  $gp91^{phox}$  subunit of the NADPH complex, indicate that defects in



superoxide production might be related to the absence of flavocytochrome b<sub>558</sub><sup>44</sup>. Furthermore, Itoh et al, who spectrometrically measured the basal levels of flavocytochrome b<sub>558</sub> present in neutrophils from MDS patients, found a correlation between the levels of flavocytochrome b<sub>558</sub> present in resting cells and the amount of O<sub>2</sub><sup>-</sup> that could be produced by these neutrophils in response to fMLP<sup>45</sup>.

Our data suggest that in neutrophils from MDS patients, the amount of O<sub>2</sub><sup>-</sup> produced in GM-CSF-primed cells is limited by the decreased amount of flavocytochrome present at the plasma membrane. However, it cannot be excluded that the cytosolic components of the NADPH oxidase complex, e.g. p47<sup>phox</sup>, p40<sup>phox</sup>, p67<sup>phox</sup> and Rac, are also affected in MDS neutrophils. Although treatment of GM-CSF-primed neutrophils with the PI3K inhibitor LY294002 reduces the amount of O<sub>2</sub><sup>-</sup> produced in response to fMLP<sup>6</sup>, it does not affect the fMLP-mediated translocation of flavocytochrome b<sub>558</sub> to the cell surface (unpublished results, GM Fuhler). This indicates the involvement of PI3K-dependent processes other than flavocytochrome translocation, in neutrophil ROS production.

In conclusion, the data presented here indicate that neutrophils from MDS patients demonstrate decreased flavocytochrome b<sub>558</sub> protein levels. Together with the reduced fMLP-induced translocation of flavocytochrome b<sub>558</sub> to the plasma membrane, this might account for the disturbed ROS production that is typically found in MDS patients.

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## Reference List

1. Kouides PA, Bennett JM. Morphology and classification of the Myelodysplastic Syndromes and their pathologic variants. *Seminars in Hematology*. 1996;33:95-100.
2. Willemze R, Fibbe WE, Falkenburg JH, Kluin-Nelemans JC, Kluin PM, Landegent JE. Biology and treatment of myelodysplastic syndromes--developments in the past decade. *Ann Hematol*. 1993;66:107-115.
3. Kouides PA, Bennett JM. Understanding the Myelodysplastic Syndromes. *Oncologist*. 1997;2:389-401.
4. Ito Y, Kawanishi Y, Shoji N, Ohyashiki K. Decline in antibiotic enzyme activity of neutrophils is a prognostic factor for infections in patients with myelodysplastic syndrome. *Clin Infect Dis*. 2000;31:1292-1295.
5. Elghetany MT, Peterson B, MacCallum J, et al. Deficiency of neutrophilic granule membrane glycoproteins in the myelodysplastic syndromes: a common deficiency in 216 patients studied by the Cancer and Leukemia Group B. *Leuk Res*. 1997;21:801-806.
6. Fuhler GM, Drayer AL, Vellenga E. Decreased phosphorylation of protein kinase B (PKB/Akt) and extracellular signal-regulated kinase (ERK) in neutrophils from patients with myelodysplasia. *Blood*. 2003;101:1172-1180.
7. Zabernigg A, Hilbe W, Eisterer W, Greil R, Ludescher C, Thaler J. Cytokine priming of the granulocyte respiratory burst in myelodysplastic syndromes. *Leuk Lymphoma*. 1997;27:137-143.
8. Ohsaka A, Kitagawa S, Yuo A, et al. Effects of granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor on respiratory burst activity of neutrophils in patients with myelodysplastic syndromes. *Clin Exp Immunol*. 1993;91:308-313.
9. Babior BM. Oxidants from phagocytes: agents of defense and destruction. *Blood*. 1984;64:959-966.
10. McPhail LC, Henson PM, Johnston RB, Jr. Respiratory burst enzyme in human neutrophils. Evidence for multiple mechanisms of activation. *J Clin Invest*. 1981;67:710-716.
11. Weisbart RH, Kwan L, Golde DW, Gasson JC. Human GM-CSF primes neutrophils for enhanced oxidative metabolism in response to the major physiological chemoattractants. *Blood*. 1987;69:18-21.
12. Babior BM. NADPH oxidase: an update. *Blood*. 1999;93:1464-1476.
13. DeLeo FR, Renee J, McCormick S, et al. Neutrophils exposed to bacterial lipopolysaccharide upregulate NADPH oxidase assembly. *J Clin Invest*. 1998;101:455-463.
14. Borregaard N, Cowland JB. Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood*. 1997;89:3503-3521.
15. Bokoch GM, Diebold BA. Current molecular models for NADPH oxidase regulation by Rac GTPase. *Blood*. 2002;100:2692-2695.
16. El Benna J, Faust LP, Babior BM. The phosphorylation of the respiratory burst oxidase component p47phox during neutrophil activation. Phosphorylation of sites recognized by protein kinase C and by proline-directed kinases. *J Biol Chem*. 1994;269:23431-23436.
17. El Benna J, Faust RP, Johnson JL, Babior BM. Phosphorylation of the respiratory burst oxidase subunit p47phox as determined by two-dimensional phosphopeptide mapping. Phosphorylation by protein kinase C, protein kinase A, and a mitogen-activated protein kinase. *J Biol Chem*. 1996;271:6374-6378.
18. Rotrosen D, Leto TL. Phosphorylation of neutrophil 47-kDa cytosolic oxidase factor. Translocation to membrane is associated with distinct phosphorylation events. *J Biol Chem*. 1990;265:19910-19915.
19. Abo A, Pick E, Hall A, Totty N, Teahan CG, Segal AW. Activation of the NADPH oxidase involves the small GTP-binding protein p21rac1. *Nature*. 1991;353:668-670.
20. Kalina U, Hofmann WK, Koschmieder S, et al. Alteration of c-mpl-mediated signal transduction in CD34(+) cells from patients with myelodysplastic syndromes. *Exp Hematol*. 2000;28:1158-1163.



21. Ambruso DR, Knall C, Abell AN, et al. Human neutrophil immunodeficiency syndrome is associated with an inhibitory Rac2 mutation. *Proc Natl Acad Sci U S A.* 2000;97:4654-4659.
22. Williams DA, Tao W, Yang F, et al. Dominant negative mutation of the hematopoietic-specific Rho GTPase, Rac2, is associated with a human phagocyte immunodeficiency. *Blood.* 2000;96:1646-1654.
23. Dorseuil O, Reibel L, Bokoch GM, Camonis J, Gacon G. The Rac target NADPH oxidase p67phox interacts preferentially with Rac2 rather than Rac1. *J Biol Chem.* 1996;271:83-88.
24. Ding J, Vlahos CJ, Liu R, Brown RF, Badwey JA. Antagonists of phosphatidylinositol 3-kinase block activation of several novel protein kinases in neutrophils. *J Biol Chem.* 1995;270:11684-11691.
25. Coffey PJ, Geijsen N, M'rabet L, et al. Comparison of the roles of mitogen-activated protein kinase kinase and phosphatidylinositol 3-kinase signal transduction in neutrophil effector function. *Biochem J.* 1998;329 ( Pt 1):121-130.
26. McLeish KR, Knall C, Ward RA, et al. Activation of mitogen-activated protein kinase cascades during priming of human neutrophils by TNF-alpha and GM-CSF. *J Leukoc Biol.* 1998;64:537-545.
27. Zhan Y, Virbasius JV, Song X, Pomerleau DP, Zhou GW. The p40phox and p47phox PX domains of NADPH oxidase target cell membranes via direct and indirect recruitment by phosphoinositides. *J Biol Chem.* 2002;277:4512-4518.
28. Kanai F, Liu H, Field SJ, et al. The PX domains of p47phox and p40phox bind to lipid products of PI(3)K. *Nat Cell Biol.* 2001;3:675-678.
29. Ellson CD, Gobert-Gosse S, Anderson KE, et al. PtdIns(3)P regulates the neutrophil oxidase complex by binding to the PX domain of p40phox. *Nat Cell Biol.* 2001;3:679-682.
30. Dewas C, Fay M, Gougerot-Pocidalo MA, El Benna J. The mitogen-activated protein kinase extracellular signal-regulated kinase 1/2 pathway is involved in formyl-methionyl-leucyl-phenylalanine- induced p47phox phosphorylation in human neutrophils. *J Immunol.* 2000;165:5238-5244.
31. Djerdjouri B, Lenoir M, Giroud JP, Perianin A. Contribution of mitogen-activated protein kinase to stimulation of phospholipase D by the chemotactic peptide fMet-Leu-Phe in human neutrophils. *Biochem Biophys Res Commun.* 1999;264:371-375.
32. Bennett JM, Catovsky D, Daniel MT, et al. Proposals for the classification of the myelodysplastic syndromes. *Br J Haematol.* 1982;51:189-199.
33. Koenderman L, Kok PT, Hamelink ML, Verhoeven AJ, Bruijnzeel PL. An improved method for the isolation of eosinophilic granulocytes from peripheral blood of normal individuals. *J Leukoc Biol.* 1988;44:79-86.
34. Franssen CF, Huitema MG, Muller Kobold AC, et al. In vitro neutrophil activation by antibodies to proteinase 3 and myeloperoxidase from patients with crescentic glomerulonephritis. *J Am Soc Nephrol.* 1999;10:1506-1515.
35. Nakamura M, Murakami M, Koga T, Tanaka Y, Minakami S. Monoclonal antibody 7D5 raised to cytochrome b558 of human neutrophils: immunocytochemical detection of the antigen in peripheral phagocytes of normal subjects, patients with chronic granulomatous disease, and their carrier mothers. *Blood.* 1987;69:1404-1408.
36. Yamauchi A, Yu L, Potgens AJ, et al. Location of the epitope for 7D5, a monoclonal antibody raised against human flavocytochrome b558, to the extracellular peptide portion of primate gp91phox. *Microbiol Immunol.* 2001;45:249-257.
37. Ward RA, Nakamura M, McLeish KR. Priming of the neutrophil respiratory burst involves p38 mitogen- activated protein kinase-dependent exocytosis of flavocytochrome b558-containing granules. *J Biol Chem.* 2000;275:36713-36719.
38. Verhoeven AJ, Bolscher BG, Meerhof LJ, et al. Characterization of two monoclonal antibodies against cytochrome b558 of human neutrophils. *Blood.* 1989;73:1686-1694.
39. Hampton MB, Kettle AJ, Winterbourn CC. Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. *Blood.* 1998;92:3007-3017.
40. Kuijpers TW, Weening RS, Roos D. Clinical and laboratory work-up of patients with neutrophil shortage or dysfunction. *J Immunol Methods.* 1999;232:211-229.
41. Mansfield PJ, Hinkovska-Galcheva V, Shayman JA, Boxer LA. Granulocyte colony-stimulating factor primes NADPH oxidase in neutrophils through translocation of cytochrome b(558) by gelatinase- granule release. *J Lab Clin Med.* 2002;140:9-16.

42. Dusi S, Donini M, Lissandrini D, Mazzi P, Bianca VD, Rossi F. Mechanisms of expression of NADPH oxidase components in human cultured monocytes: role of cytokines and transcriptional regulators involved. *Eur J Immunol.* 2001;31:929-938.
43. Yagisawa M, Yuo A, Yonemaru M, et al. Superoxide release and NADPH oxidase components in mature human phagocytes: correlation between functional capacity and amount of functional proteins. *Biochem Biophys Res Commun.* 1996;228:510-516.
44. Geiszt M, Kapus A, Ligeti E. Chronic granulomatous disease: more than the lack of superoxide? *J Leukoc Biol.* 2001;69:191-196.
45. Itoh Y, Kuratsuji T, Aizawa S, Sai M, Ohyashiki K, Toyama K. Superoxide anion production and expression of cytochrome b 558 by neutrophils are impaired in some patients with myelodysplastic syndrome. *Ann Hematol.* 1991;63:270-275.



