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Modulation of granulocyte kinetics by GM-CSF/IFN-γ in a human LPS rechallenge model

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ABSTRACT

Inflammation in response to infection or trauma can lead to CARS, which is characterized by leukocyte dysfunction. In this study, we used a human model system for CARS to study the effect of GM-CSF and IFN-γ treatment on this immunoparalyzed state. Healthy human volunteers were treated with GM-CSF (4 μg/kg), IFN-γ (100 μg), or placebo in between two challenges with *Escherichia coli* LPS/endotoxin (2 ng/kg). Serial leukocyte blood counts were measured. Neutrophil subsets were discriminated using CD16 and CD62L expression. LPS rechallenge resulted in increased mobilization of mature neutrophils, whereas banded neutrophils decreased. GM-CSF and IFN-γ treatment did not restore these changes. GM-CSF treatment did, however, increase the number of $CD16^{bright}/CD62L^{dim}$ neutrophils that were previously shown be able to suppress T cell proliferation. IFN-γ treatment decreased neutrophilia seen after LPS rechallenge. Our study shows that LPS rechallenge was associated with changes in the distribution of neutrophil subsets, whereas no additional changes in kinetics of other granulocyte populations were observed. GM-CSF and IFN-γ treatment induced a shift in granulocyte composition toward an anti-inflammatory direction by increasing $CD16^{bright}/CD62L^{dim}$ cells or decreasing neutrophil counts, respectively. *J. Leukoc. Biol.* 94: 513–520; 2013.

Introduction

Inflammation, as a result of an infection or (surgical) trauma, can lead to the CARS [1–4]. Complications, such as secondary infections and sepsis, are common during this state of immunoparalysis [1] and are a major cause of late mortality [2]. Similar immune suppression is evoked by experimental human endotoxemia in healthy volunteers [5]. Administration of LPS induces endotoxin tolerance, characterized by a blunted production of inflammatory mediators upon LPS rechallenge [6]. One of the best-described markers of the endotoxin tolerance is a significant reduction of systemic TNF-α release [7]. Other cytokines with a decreased systemic production upon rechallenge include IL-6, IL-10, IL-8, TGF-β, and G-CSF [8, 9]. Tissue macrophages or more specifically, the Kupffer cells in the liver, mainly mediate this tolerance upon rechallenge with endotoxin [7, 9, 10]. Several studies have shown that IFN-γ and GM-CSF can restore cytokine responses in vivo in mice and ex vivo in humans [8, 11–13]. Leentjens et al. [14] have shown recently in in vivo human LPS rechallenge that TNF-α and IL-6 responses were restored partly by GM-CSF and IFN-γ.

Adequate granulocyte responsiveness is of critical importance for clearance of infections. As CARS often results in secondary infections and sepsis, it is of interest to study the functionality of granulocytes during this anti-inflammatory state. Little data are available regarding consequences of LPS rechallenges on the kinetics of the different granulocyte types and their subsets. A few studies described the absence of LPS tolerance on total leukocyte numbers with neutrophils as the main constituent [9, 15]. However, these data are difficult to interpret in terms of leukocyte kinetics, as neither data on leukocyte subsets nor their different phenotypes were assessed. For neutrophils, we recently described the occurrence of three phenotypes in the blood of volunteers challenged with LPS, defined by differences in FcγRIII (CD16) and L-selectin (CD62L) expression [16]: $CD16^{dim}/CD62L^{bright}$ young neutrophils with a banded nucleus, $CD16^{bright}/CD62L^{bright}$ mature, normal neutrophils, and $CD16^{bright}/CD62L^{dim}$ neutrophils with a hypersegmented nucleus. The latter $CD16^{bright}/CD62L^{dim}$ subset was found to suppress T cell proliferation [16]. Shifts in the distribution of neutrophil subsets are of great importance, as this can result in changes in anti-inflammatory (suppressive neutrophils) or proinflammatory (banded and mature neutrophils) responses.

Abbreviations: CARS=compensatory anti-inflammatory response syndrome, CD62L=CD62 leukocyte

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GM-CSF is known to induce an increase in neutrophil and eosinophil counts in peripheral blood [17, 18]. IFN-γ controls neutrophil recruitment in inflammation by the IL-1β-dependent mechanism [19]. Therefore, a boost in granulocytic responses after GM-CSF and IFN-γ treatment might be expected.

This study was conducted to investigate a putative effect of LPS rechallenge in humans in vivo on granulocyte kinetics—changes in neutrophil subsets. In addition, the modulating effect of GM-CSF and IFN-γ treatment—two of the putative, adjunctive, immunotherapeutic agents proposed to be used in sepsis on these parameters—will be assessed.

MATERIALS AND METHODS

Subjects and study design

The study protocol was approved by the Ethics Committee of the Radboud University Nijmegen Medical Centre and complies with the Declaration of Helsinki and Good Clinical Practice guidelines. Healthy male volunteers gave written, informed consent. Experiments were part of a larger endotoxin trial (NCT01374711 at www.clinicaltrials.gov). Subjects were enrolled after screening [20] and were prehydrated with 1500 ml glucose/saline infusion [21]. U.S. Reference *E. coli* endotoxin (Lot Ec-5; Centre for Biologic Evaluation and Research, U.S. Food and Drug Administration, Bethesda, MD, USA) was used in this study. Endotoxin was reconstituted in 5 ml saline and injected as single i.v. bolus during 1 min at t = 0. Blood samples, anticoagulated with sodium heparin, were taken from the arterial catheter. Endotoxin rechallenge was performed 7 days after the first endotoxin challenge. An overview of the experimental design is drawn in **Fig. 1**. Healthy volunteers were challenged with LPS on Days 1 and 7 (time-point, 0 h). Blood was drawn at five time-points during these days, plus 24 h after challenge. Placebo, GM-CSF, or IFN-γ was administered at Days 2, 4, and 6; blood was drawn during these visits. We performed leukocyte flow cytometry analysis at time-points 0, 3, and 4 h after endotoxin administration on Days 1 and 7.

Study treatments

Following the first LPS administration, subjects were randomized in a double-blind fashion to receive a s.c. injection of 4 μg/kg GM-CSF (Leukine/Sargramostim; Bayer Healthcare Pharmaceuticals, Seattle, WA, USA), 100 μg IFN-γ (Immukine; Boehringer Ingelheim Alkmaar, The Netherlands), or placebo (0.9% NaCl) on Days 2, 4, and 6, using the sealed-envelope method. The dosages were based on previous studies [12, 22]. As GM-CSF and IFN-γ had different administration volumes, a double-dummy was used to ensure adequate blinding.

Reagents

Pasteurized plasma solution was purchased from the Central Laboratory of The Netherlands (Sanquin, Amsterdam, The Netherlands). Isolation buffer contained PBS supplemented with pasteurized plasma solution (10%) and trisodium citrate [0.4% (w/v)]. mAb used for flow cytometry included CD16 Alexa 647 (Clone 3G8) and CD62L FITC (Clone DREG56), purchased from Becton Dickenson (San Jose, CA USA). All other materials were reagent grade.

Cell counts

Measurement of white blood cell counts was performed by flow cytometry using a semiconductor laser to detect forward- and side-scattered light information (Sysmex XE-2100; Meyvis, Etten Leur, The Netherlands).

Flow cytometry

Erythrocytes were lysed in isotonic ice-cold NH_4Cl solution, followed by centrifugation at 4°C. After lysis, total leukocytes were stained with antibodies for 30 min at 4°C in isolation buffer. Cells were washed before analysis on FACSCalibur (Becton Dickenson). Percentage neutrophil subsets were calculated by gating, as described previously [16]. With the use of the percentages and the absolute neutrophil count, absolute numbers of circulating neutrophil subsets could be calculated.

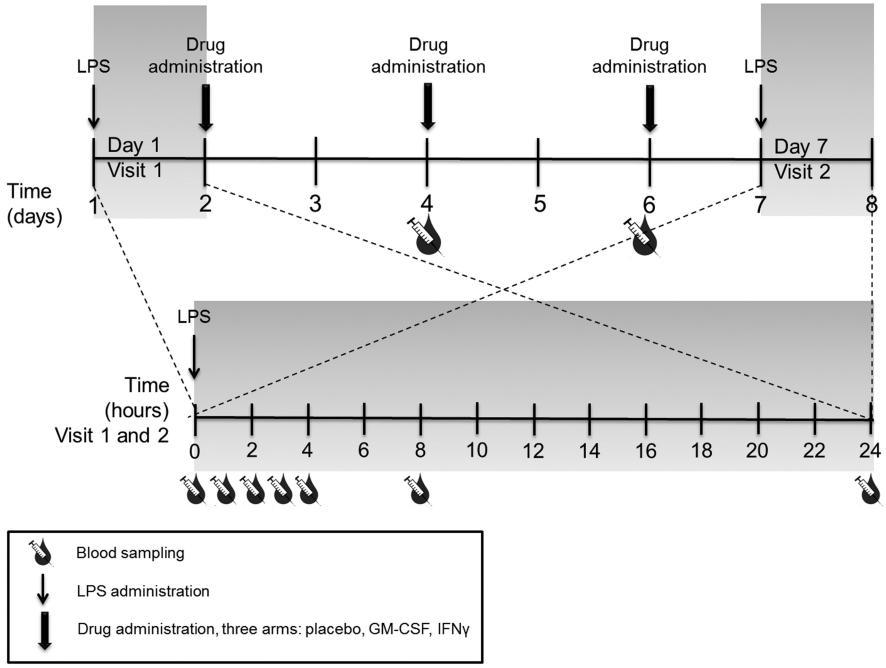

Figure 1. Study design and outline of treatment schedule. Male healthy volunteers received a dose of 2 ng/kg LPS at Days 1 and 7. On Days 2, 4, and 6, subjects received placebo, GM-CSF (4 μg/kg), or IFN-γ (100 μg), six volunteers/group. Blood was drawn at 0, 1, 2, 3, 4, 8, and 24 h after LPS administration on both days, and time-point 0 was just before LPS administration. Also, at Days 4 and 6, blood was drawn. Cell counts were measured on all time-points except 3 h after LPS. Flow cytometric analyses were performed at time-points 0, 3, and 4 h after LPS.

Statistical analysis

Data were analyzed using Graphpad Prism 5 (GraphPad Software, La Jolla, CA, USA), using *t*-test, one-way ANOVA or two-way ANOVA, as indicated in figure legends. $P < 0.05$ was considered significant.

RESULTS

Endotoxin rechallenge does not influence the leukocyte kinetics

Repeated endotoxin administrations in humans can be used to study leukocyte responses upon a second challenge of the innate-immune system, as the human endotoxemia model is known to induce an, albeit mild, immunoparalyzed state [5]. Differences in kinetics of leukocyte populations were observed upon LPS challenge (**Fig. 2**). Neutrophil counts declined within the first hour after LPS, followed by an increase in total numbers. Neutrophil egress from the bone marrow upon acute inflammation is thought to be one of the main contributors of this neutrophilia [23]. The number of neutrophils was almost restored to baseline conditions after 24 h (Fig. 2A). During the LPS-induced neutrophilia, eosinophil counts markedly dropped. Lowest counts were measured within 2 h and stayed low until at least 8 h after LPS administration. Also, eosinophil counts restored within 24 h after LPS administration (Fig. 2B). Monocytes decreased in the first 2 h after LPS. However, after 2 h, monocyte numbers increased, and at 24 h after LPS challenge, more circulating monocytes were seen compared with baseline (Fig. 2C). Although basophil counts were quite variable as a result of the low numbers present in peripheral blood, their numbers decreased within 2 h upon endotoxin administration and returned to normal values after 24 h (Fig. 2D). All leukocytes decreased in number within the first hour after LPS challenge. Interestingly, only neutrophil counts increased sharply after this initial neutropenia. The early decrease that is seen for all leukocyte types is thought to be a result of sequestration as a consequence of increased expression of adhesion molecules on the endothelium [24]. This leukocyte decrease is not influenced or caused by the prehydration before LPS challenge [21]. Also, hemoglobin and hematocrit stayed relatively stable after challenge; they even showed a slight increase in the first hour after endotoxin (data not shown). This indicates that prehydration does not influence the cell counts after challenge.

Upon rechallenge, the production of TNF-α, IL-6, and IL-1R antagonist was decreased [14] compared with the first challenge. However, leukocyte kinetics were similar after initial and rechallenge with endotoxin. None of the leukocyte types showed significant differences in total numbers in response

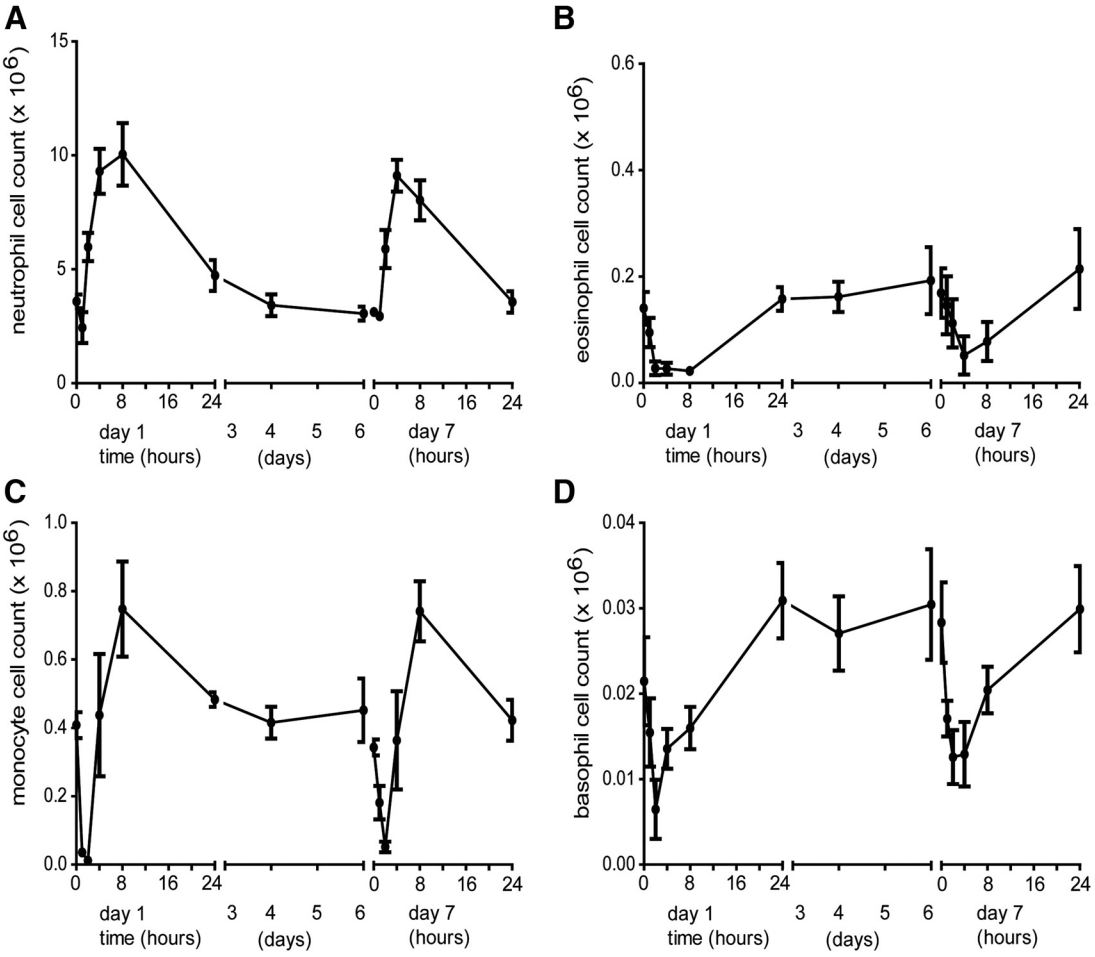

Figure 2. Leukocyte kinetics during experiment. Absolute number of neutrophils (A), eosinophils (B), monocytes (C), and basophils (D) was measured during the experiment. Results are plotted as mean ± SEM; n = 3–6. One-way ANOVA with Bonferroni multicomparison post-test.

upon LPS rechallenge compared with the initial challenge (Fig. 2A–D). These results are in agreement with previous studies showing that circulating concentrations of TNF-α, IL-6, IL-10 (human) [9], or CSF (mouse) [15], after endotoxin rechallenge, do not correlate with the mobilization of leukocytes. These data suggest that systemically increased levels of inflammatory cytokines TNF-α, IL-6, and IL-10 are not responsible for leukopenia and neutrophil egress in this model.

Eosinophils and neutrophils recruited by GM-CSF do not return to the blood after endotoxin challenge

Next, we analyzed the potential of GM-CSF to modulate the cellular immune response during the rechallenge. GM-CSF is known to recruit neutrophils and eosinophils [17], and therefore, differences were expected in cellular responses upon treatment with this cytokine. GM-CSF-mediated induction of neutrophil and eosinophil counts in peripheral blood was also observed in this study (**Fig. 3A** and **B**), which is in line with the hypothesis and previous studies [17, 18]. Following administration of three dosages of GM-CSF, the neutrophil count before rechallenge with LPS increased to 5.2 ± 0.4 × 10^6/ml, which was significantly higher than levels found in the placebo group 3.1 ± 0.2 × 10^6/ml ($P<0.05$; Fig. 3A). Rechallenge with LPS increased neutrophils even more, and the differences between placebo and GM-CSF-treated individuals disappeared. This indicates that GM-CSF has no additional effect on neutrophil recruitment following the second LPS administration. The underlying mechanisms remain to be established, but LPS and GM-CSF are known to recruit neutrophils by accelerating the release of mature neutrophils from the bone marrow [25], followed by increased granulopoiesis [25, 26]. This neutrophil recruitment upon GM-CSF or LPS is likely to be regulated by a similar mechanism, as there is no additional neutrophil egress after combined treatment.

GM-CSF also raised the number of eosinophils from 1.7 ± 0.4 × 10^5/ml in the placebo-treated group to 5.0 ± 0.7 × 10^5/ml ($P<0.01$) in the GM-CSF-treated group (Fig. 3B). This increase in eosinophils, however, disappeared within 4 h after endotoxin challenge.

Surprisingly, 24 h after rechallenge, neutrophil and eosinophil numbers did not return to their elevated levels seen before rechallenge (Fig. 3A and B) but became similar to the counts seen in the placebo group. The fact that neutrophil

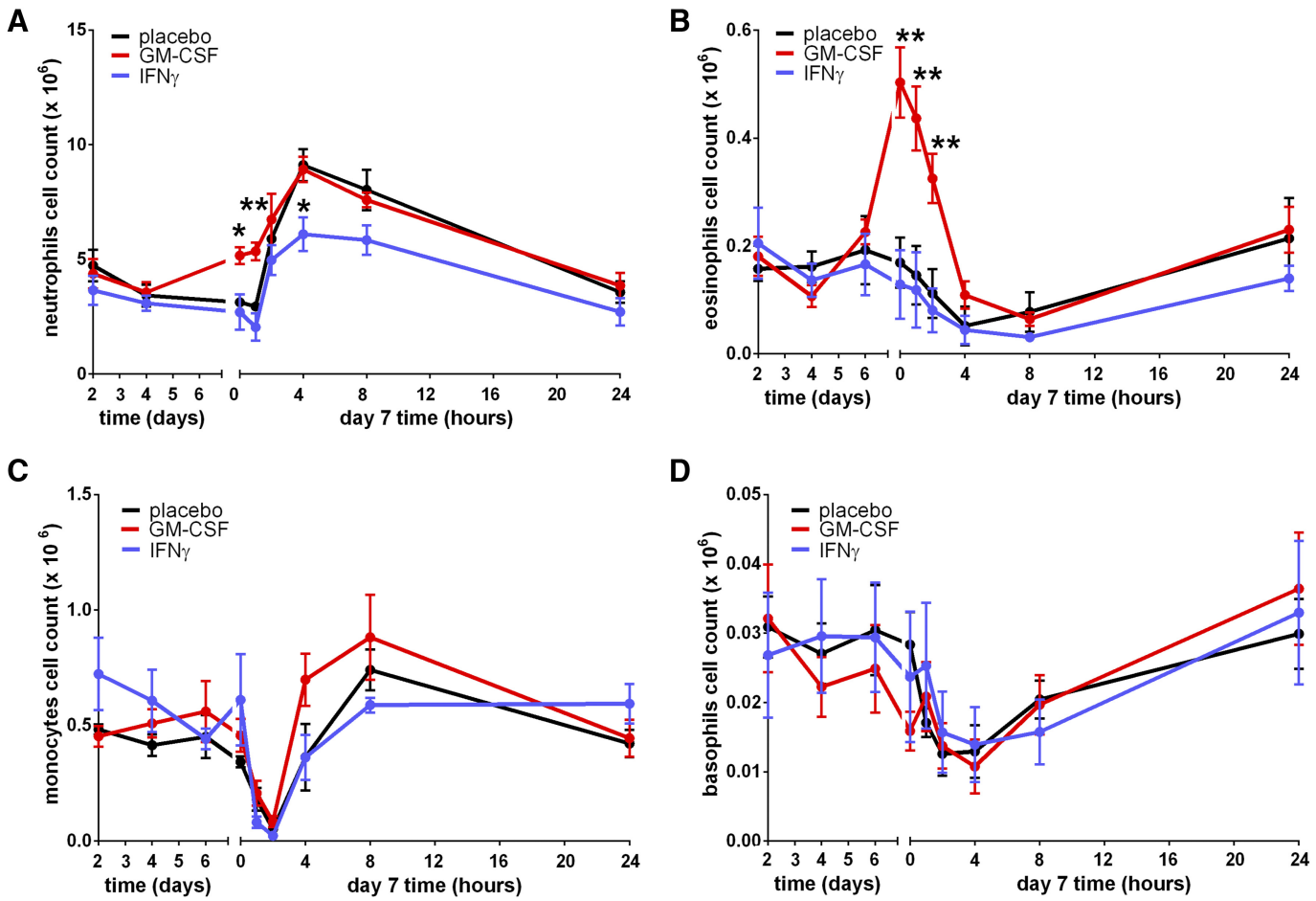

Figure 3. Neutrophil kinetics, Days 2–7, during GM-CSF and IFN-γ treatment. Absolute number of neutrophils (A), eosinophils (B), monocytes (C), and basophils (D) was measured during GM-CSF and IFN-γ treatment and then following LPS challenge. Results are plotted as mean ± SEM; n = 3–6. One-way ANOVA was performed for every time-point with Tukey post-test. *$P < 0.05$; **$P < 0.01$.

and eosinophil numbers after challenge were not restored to the levels observed before LPS rechallenge suggested that these cells homed to other compartments of the body. The nature and localization of these compartments remain to be established, but data have suggested that inflammatory neutrophils can migrate to liver, bone marrow, and spleen with a preference for the liver [27].

Monocyte and basophil cell counts were not changed by GM-CSF treatment before or during endotoxin challenge (Fig. 3C and D).

IFN-γ decreases the number of neutrophils in the blood upon LPS challenge

Just as GM-CSF, IFN-γ is known to modulate the cellular immune response, as it increases neutrophil trafficking [19]. IFN-γ treatment did not influence the leukocyte counts before rechallenge (**Fig. 3A–D**). However, the increase in neutrophils after LPS rechallenge was significantly lower in IFN-γ-treated individuals, namely, $6.1 \times 10^6 \pm 0.7$ neutrophils/ml in IFN-γ-treated individuals compared with $9.1 \times 10^6 \pm 0.7$ neutrophils/ml ($P<0.05$) in the placebo group (Fig. 3A).Two hypotheses can explain this phenomenon. First, experiments in murine models showed that IFN-γ production is normally down-regulated upon emergency granulopoiesis [26]. In addition, IFN-γ inhibited granulopoiesis and induced monopoiesis [28]. These findings lead to the hypothesis that IFN-γ treatment will result in decreased neutrophil production and therefore, also in decreased neutrophil egress. This could explain the decreased number of neutrophils in the IFN-γ-treated group. However, the expected increased egress of monocytes from the bone marrow upon LPS rechallenge was not detected (Fig. 3C). The second hypothesis is that IFN-γ induced neutrophil infiltration into the tissues [19], resulting in less neutrophils present in the peripheral blood.

Effect of rechallenge with endotoxin on neutrophil subsets: increased numbers of mature neutrophils and decreased numbers of banded neutrophils

Whereas there were no significant differences in total neutrophil numbers after initial and rechallenge with LPS (Fig. 2A), differences in the kinetics of the three subsets of neutrophils were apparent (**Fig. 4**). The number of mature neutrophils was increased significantly during the second LPS challenge ($5.4\pm0.7\times10^6$ cells/ml first challenge; $7.1\pm1.0\times10^6$ cells/ml rechallenge; $P<0.05$; Fig. 4A), whereas the number of banded neutrophils decreased ($2.1\pm0.3\times10^6$ cells/ml first challenge; $1.2\pm0.3\times10^6$ cells/ml rechallenge; $P<0.05$; Fig. 4B). The attenuated increase in the number of $CD16^{bright}/CD62L^{dim}$ neutrophils did not reach statistical significance ($2.1\pm0.5\times10^6$ cells/ml first challenge; $1.1\pm0.2\times10^6$ cells/ml rechallenge; $P=0.059$; Fig. 4C). Thus, we found that the total number of neutrophils did not differ after rechallenge with LPS, but the distribution shifted toward a more mature neutrophil population.

This effect might be mediated by attenuation of the cytokine response during the LPS rechallenge, which can be restored partly by IFN-γ and GM-CSF treatment [14]. Therefore, we tested whether IFN-γ and GM-CSF could restore the distribution of neutrophil subsets. Treatment with IFN-γ and GM-CSF, however, did not restore the number of normal, banded, and $CD16^{bright}/CD62L^{dim}$ neutrophils (**Fig. 5**).

In mice, it is known that neutrophil release from the bone marrow evokes emergency granulopoiesis. This resulted in an increased number of mature neutrophils in the bone marrow for a short period of time (~6 days after the mobilization of the initial neutrophils) [26]. Such a mechanism might explain the enhanced numbers of mature neutrophils after a rechallenge with LPS after 7 days in man.

GM-CSF treatment increases the percentage of $CD62L^{dim}$ (suppressive) neutrophils; treatment with IFN-γ and GM-CSF modulates L-selectin (CD62L) expression on leukocytes

During analysis of the neutrophil subsets, differences in overall CD62L expression were observed (**Fig. 6A**). Upon GM-CSF treatment, two patterns could be distinguished: (1) the increase in the number of $CD16^{bright}/CD62L^{dim}$ neutrophils (Figs. 5C and 6A) and (2) a decreased CD62L expression on the whole neutrophil population (Fig. 6A). To ensure that the cell counts of neutrophil subsets were not based on differences in overall expression in CD62L expression, the different neutrophil subsets were gated separately for every patient (Figs. 5 and 6). The increase in the number of $CD16^{bright}/CD62L^{dim}$ neutrophils upon GM-CSF stimulation was apparent before and during the LPS rechallenge after GM-CSF treatment (Fig. 5C).

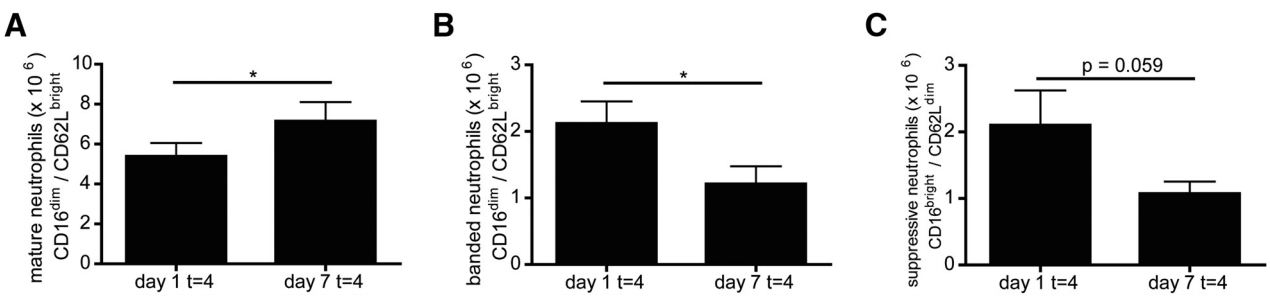

Figure 4. Different distribution of neutrophil subsets after first and second LPS challenge. Leukocytes were stained with CD62L and CD16 to discriminate neutrophil subsets by flow cytometry. Number of mature (A), banded (B), and $CD16^{bright}/CD62L^{dim}$ neutrophils (C) was calculated. Mean ± SEM; $n = 6$. For statistics, a paired *t*-test was performed. $^*P < 0.05$.

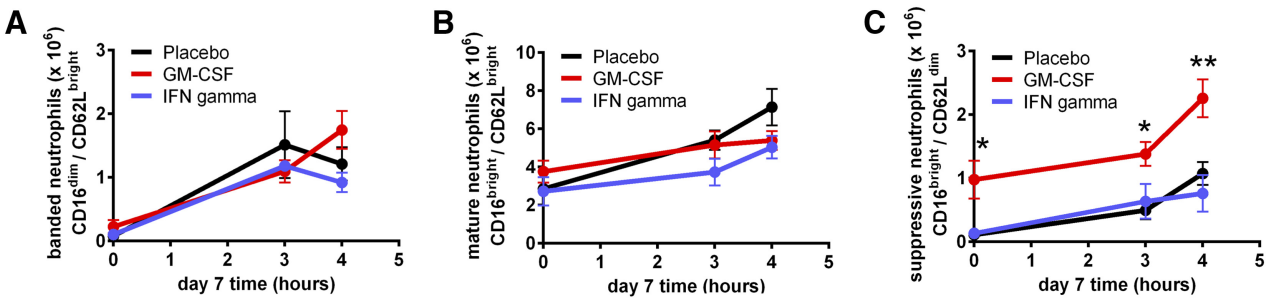

Figure 5. Neutrophil subsets after GM-CSF and IFN-γ treatment. Leukocytes from 3 h and 4 h after LPS challenge were stained with CD62L and CD16 to discriminate neutrophil subsets by flow cytometry. Number of mature (A), banded (B), and CD16^{bright}/CD62L^{dim} neutrophils (C) was calculated. Mean ± SEM; $n = 5–6$. Statistical analysis: one-way ANOVA with Tukey post-test. $^*P < 0.05$; $^{**}P < 0.01$.

Differences in CD62L expression were most apparent at baseline before the second LPS administration (Fig. 6B). GM-CSF down-regulated CD62L expression on neutrophils, whereas IFN-γ treatment led to up-regulation of CD62L. After LPS administration, different neutrophil subsets appeared, and the differences between CD62L expression became less clear on mature (Fig. 6C), banded (Fig. 6D), and CD16^{bright}/CD62L^{dim} (Fig. 6E) neutrophils. After LPS challenge, the dif-

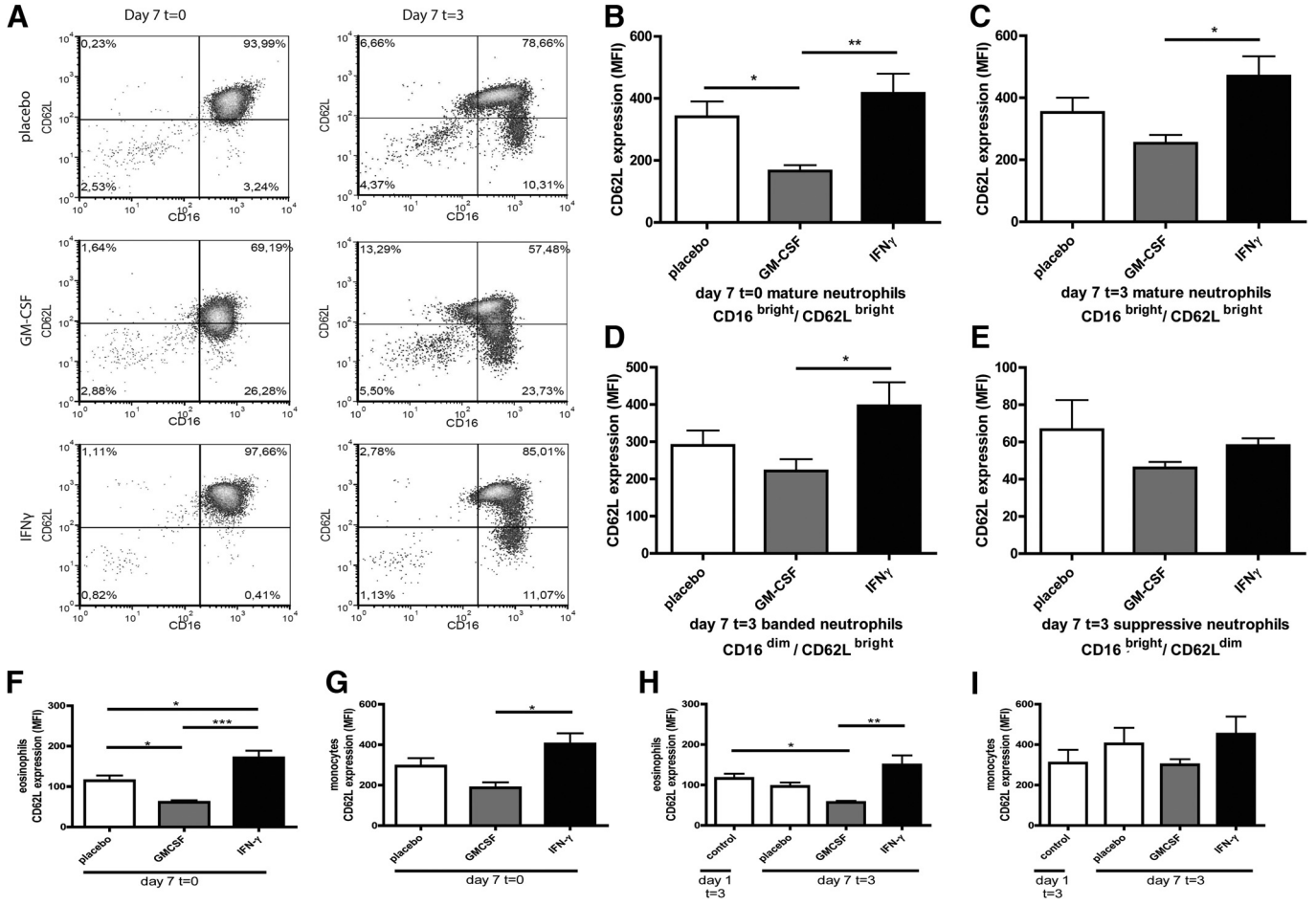

Figure 6. CD62L expression on leukocytes. Leukocytes (time-point 3 h) of both challenges were stained with CD62L and CD16. (A) Representative examples of the CD16/CD62L stains on neutrophils on Day 7 before and after LPS and with placebo, GM-CSF, or IFN-γ treatment. (B–E) Neutrophil subsets were gated, and CD62L expression was determined on the mature neutrophils before LPS administration (B) and and 3 h after LPS administration on mature (C), banded (D), and CD16^{bright}/CD62L^{dim} (E) neutrophils. Additionally, the CD62L expression before LPS was determined on eosinophils (F) and monocytes (G), as well as the CD62L expression on eosinophils (H) and monocytes (I), 3 h after LPS. MFI, Mean fluorescence intensity. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

ference in CD62L expression between the IFN-γ- and GM-CSF-treated group was only significant for mature and banded neutrophils (Fig. 6C and D).

CD62L expression on other leukocytes was also analyzed to investigate whether the increase (IFN-γ) and decrease (GM-CSF) in CD62L expression were overall effects on all leukocytes. Eosinophils and monocytes showed a similar phenotype as the neutrophils (Fig. 6F and G), decreased CD62L expression upon GM-CSF treatment, and increased expression upon IFN-γ treatment. Upon LPS challenge, these differences in CD62L expression became less apparent (Fig. 6H and I). The fact that there were no differences in CD62L expression on Day 1 compared with Day 7 excluded a LPS rechallenge effect on CD62L expression (Fig. 6H and I).

Whereas GM-CSF treatment could not restore the distribution of a neutrophil subset, it increased the number of $CD16^{bright}/CD62l^{dim}$ neutrophils. These cells have been shown previously to be immune-suppressive cells [16]. This might suggest that GM-CSF treatment increased the number of suppressive neutrophils, and consequently, this treatment might dampen, at least in part, the granulocytic immune response upon rechallenge. However, shedding of L-selectin can also be a sign of neutrophil activation [29], so interpretation of these results should be done with care. Unfortunately, it was impossible to study the effect of IFN-γ and GM-CSF on L-selectin expression in vitro, as L-selectin is shed upon culture rapidly, which does not necessarily correlate with the in vivo situation [29, 30].

DISCUSSION

Our study, using LPS rechallenge in healthy human volunteers, indicated that multiple challenges with an innate-immune stimulus led to subtle changes in neutrophil phenotypes, whereas the kinetics of the main leukocyte subsets were not influenced. Despite the finding that GM-CSF and IFN-γ partly restored the systemic TNF-α and IL-6 response upon a second LPS challenge [14], we could not find a relation between the response in inflammatory mediators and a change in the granulocyte responses. In fact, GM-CSF and IFN-γ treatment did not seem to boost the LPS-induced granulocytic response and might even dampen it by increasing the number of $CD16^{bright}/CD62L^{dim}$ neutrophils upon GM-CSF treatment or decreasing the overall neutrophil recruitment after IFN-γ treatment. Surprisingly, the increasing amount of eosinophils and neutrophils seen in the blood upon GM-CSF treatment did not return to the circulation 24 h after LPS challenge. Probably, these cells migrated to other compartments within the body.

AUTHORSHIP

V.M.K., J.D.L., and S.d.K. carried out the flow cytometry experiments and wrote the manuscript. J.L. performed the clinical experiments and provided the cell count data. M.K., J.P. and M.G.N. were involved in the design and management of the study. P.P. conceived of the study and participated in its design. L.K. conceived of the study and was involved in the writing of the manuscript. All authors read and approved the final manuscript.

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KEY WORDS:
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