Immunity in atherosclerosis: novel assays, biomarkers and therapeutic approaches

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CHAPTER 3

In vitro and *in vivo* lipopolysaccharide-driven activation of human neutrophils in healthy volunteers

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Graphical abstract

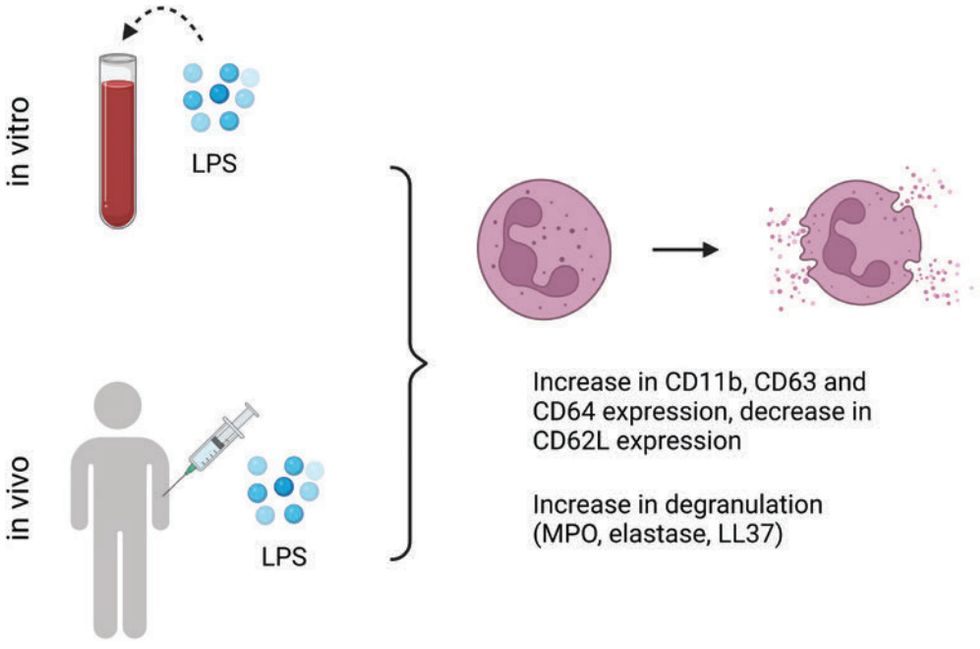


Figure is created using biorender.com.

Abstract

Neutrophils are an emerging target for therapeutical intervention in both autoimmune diseases as well as cancer. However, evaluating investigational compounds in healthy humans remains challenging, since this population lacks constitutive neutrophil activation. Induction of neutrophil activation via intravenous administration of lipopolysaccharides (LPS) can be a potent strategy to overcome this challenge. Furthermore, LPS stimulation can be performed *ex vivo* during clinical trials, and *in vitro* for pre-clinical analysis. Therefore, we aimed to provide a time course of the neutrophil response after *in vivo* LPS administration using samples from human endotoxemia clinical studies and compared this to *in vitro* LPS stimulated whole blood cultures. We performed shotgun proteomics on *in vivo* stimulated neutrophils, and measured neutrophil activation by flow cytometry using CD11b, CD62L, CD63 and CD64 as activation markers and elastase, MPO and LL37 levels as degranulation markers. The numbers of neutrophils rapidly increased after LPS administration, while monocyte and lymphocyte numbers significantly decreased. In line, we found significant increases in neutrophil activation and degranulation markers both *in vitro* as well as *in vivo*, which all returned to baseline within 24 hours. Degranulation proteins rapidly increase after LPS administration (1 hour after exposure) *in vivo*, while higher concentrations of LPS were necessary *in vitro*. Lastly, shotgun proteomics revealed little but significant differences in the neutrophil proteome after *in vivo* LPS administration, pointing to degranulation after LPS stimulation. In general, neutrophils show similar activation *in vitro* and *in vivo*. Both, the *in vitro* whole blood LPS stimulation assay and the human endotoxemia model, could be valuable tools for evaluation of the effects of future drugs modulating neutrophil responses during preclinical and clinical development.

Introduction

Neutrophils are the first responders upon infection. They are professional phagocytes, with short life-spans of multiple days.[1] Neutrophils carry granules, which can be released upon activation and contain proteolytic enzymes including neutrophil elastase and myeloperoxidase (MPO), reactive oxygen species, and anti-microbial peptides such as LL37. To prevent tissue damage by excessive neutrophil activity, apoptosis of neutrophils is tightly regulated.[2] Besides their role in acute responses against pathogens, neutrophil activation has been found to contribute to several auto-immune diseases, amongst others rheumatoid arthritis[3] and systemic lupus erythematosus,[4] by inducing tissue damage via production of cytokines and chemokines and via the formation of NETs.[5] In atherosclerosis, neutrophils are involved in the onset of disease, mainly by accumulating in the vessel wall and attracting monocytes to the lesion site.[6] They also play a role in destabilization of the atherosclerotic plaque and atherothrombosis, as is illustrated bythe presence of neutrophils in unstable plaques.[7-9] Besides their role in auto-immune diseases, neutrophils have recently been implicated to play a role in cancer development and progression.[10] They can have both protumoural and antitumoural effects, indicating that a balance in neutrophil activation is required for homeostasis.[10] Because of their role in autoimmune diseases and in cancer progression, neutrophils have emerged as potential therapeutic target.[11] Inhibition of neutrophil activity can be accomplished at several levels, for example by blocking neutrophil migration or activation and thereby interfering with neutrophil accumulation at the site of inflammation. Furthermore, interference of neutrophil-specific cell death via neutrophil extracellular traps (NETosis), and blocking of neutrophil-specific proteins can be used to inhibit neutrophil activity.[11] Indeed, several drugs are in clinical development, such as a CXCR2 antagonist, a neutrophil elastase inhibitor and NETosis inhibitors.[11]

For novel drug candidates targeting neutrophils, specific (pre)clinical tests are required. Evaluation of the pharmacological activity of neutrophil-targeted investigational compounds in healthy humans is however challenging since this population does not have constitutive neutrophil activation. Induction of neutrophil activation via intravenous administration of lipopolysaccharides (LPS) can be a potent strategy to be able to test drug candidates. LPS activates neutrophils *in vivo*, leads to increasing neutrophil numbers in the circulation[12,13] and shifts the neutrophil population towards CD16^{dim} (banded) neutrophils and CD62L^{low} (hypersegmented) neutrophils, besides the CD16^{high}CD62L^{high} neutrophils found in homeostasis.[14] CD62L (L-selectin) is an adhesion molecule that is quickly downregulated by activated neutrophils via ectodomain shedding.[15] CD11b (integrin alpha M), a constitutively expressed adhesion molecule on myeloid cells,[16,17] is another marker for neutrophil activation, known to be upregulated by for instance LPS exposure.[18,19] As such, the human endotoxemia model may be a useful tool to study the pharmacodynamic effects of novel drugs on neutrophils in an early stage of drug development. However, a clear overview of the effects of *in vivo* LPS exposure on neutrophils is currently lacking.

Additionally, LPS stimulation of whole blood *ex vivo* during clinical trials or of isolated neutrophils *in vitro* in pre-clinical analysis are important approaches to be explored to assess drug effects on neutrophils. To be able to compare *in vivo* LPS effects with *in vitro* LPS effects on neutrophils, we aimed to provide a time course of the neutrophil response after *in vivo* LPS administration and compared this to the effects of LPS stimulation on neutrophil activation status *in vitro*. We used samples from a clinical trial using the human endotoxemia model and performed *in vitro* whole blood stimulations using different concentrations of LPS. In the endotoxemia samples, we performed shotgun proteomics to identify changes in the neutrophil proteome. Furthermore, we assessed neutrophil activation by flow cytometry using CD11b, CD62L, CD63 and CD64 as activation markers and measured neutrophil degranulation by measuring myeloperoxidase (MPO), elastase and LL37 levels.

Materials and Methods

HUMAN SAMPLES

For the *in vivo* LPS challenge response evaluation, samples from 2 clinical trials were used. Both studies were conducted after obtaining written informed consent in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki. In one study, healthy male volunteers received LPS intravenously at a dosage of 2 ng/kg, in a study registered under ToetsingOnline number NL65264.056.18 and under ISRCTN number 13923422.[20] In the other study, healthy male and female volunteers

received i.v. LPS at 1 ng/kg. For both studies purified lipopolysaccharide prepared from *Escherichia coli*, 113:H10:K negative (U.S. Standard Reference Endotoxin), manufactured by List Biological Laboratories (Campbell, CA, USA) was used. Subjects were hydrated with glucose/saline (2.5% glucose/0.45% sodium chloride) from 2 hours prior to LPS administration until 6 hours afterwards. For the *in vitro* LPS whole blood challenges, blood from healthy male and female donors was collected by venipuncture after obtaining written informed consent in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki. Neutrophil activation was analyzed by flow cytometry in CTAD anti-coagulated blood. For elastase, MPO and LL37 measurements, sodium heparin plasma samples were used. For proteomic analysis, blood was collected into K_2EDTA tubes (Becton Dickinson). The demographics of the subjects are summarized in Table I.

WHOLE BLOOD STIMULATION

Whole blood was incubated with 2 ng/mL, 10 ng/mL or 100 µg/mL LPS (from *E.coli* O111:B4, Sigma-Aldrich, Deisenhofen, Germany) for 1 hour at 37°C 5% CO_2.

FLOW CYTOMETRY

Whole blood was incubated with RBC lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) for 10 minutes to lyse red blood cells. Next, the cells were washed once with phosphate buffered saline (PBS, Gibco, Thermo Fisher Scientific) and stained with fluorochrome labeled antibodies for 30 minutes on ice (see supplemental table I for a list of antibodies used and Figure S1 for the gating strategy). After staining, the cells were washed and measured on a MACSQuant 16 analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany). Flow cytometry data was analyzed using Flowlogic software (Inivai, Melbourne, Australia).

ELISAS

Elastase, myeloperoxidase (MPO) and LL37 were measured by ELISA according to the user's manuals. Elastase and LL37 ELISAS were obtained from Hycult Biotech (Uden, the Netherlands), MPO ELISA from Bio-Techne (Abingdon, UK). ELISAS were read on a Varioskan LUX reader and analyzed using the SkanIt software (both Thermo Fisher Scientific).

NEUTROPHIL ISOLATION

Neutrophils were isolated directly from whole blood by negative selection using the EasySep™ direct human neutrophil isolation kit (Stemcell, Vancouver, Canada) according to user's manual. After isolation, the neutrophils were pelleted and snap frozen in liquid nitrogen until analysis.

SHOTGUN PROTEOMICS

Samples were mixed with loading buffer and loaded on an 8 % pre-cast RunBlue gel (Expedeon), and run at 100 V for 5 min. Large gel spots with unseparated proteins were stained with InstantBlue (Expedeon) and excised in one gel slice and destained using 70% 50 mM NH_4HCO_3 and 30% acetonitrile. Reduction was performed using 10 mM dithiotreitol dissolved in 50 mM NH_4HCO_3 for 30 min at 55°C. Next, the samples were alkylated using 55 mM chloroacetamide in 50 mM NH_4HCO_3 for 30 min at room temperature. Subsequently, samples were washed for 10 min with 50 mM NH_4HCO_3 and for 15 min with 100% acetonitrile. Protein digestion was performed by addition of sequencing-grade modified trypsin (Promega; 25 µL of 10 ng/mL in 50 mM NH_4HCO_3) and overnight incubation at 37°C. Peptides were extracted using 5% formic acid in water followed by a second elution with 5% formic acid in 75% acetonitrile. Samples were dried in a SpeedVac centrifuge and dissolved in 20 µL 5% formic acid in water for analysis with LC-MS/MS.

The samples were analyzed on a nanoLC-MS/MS consisting of an Ultimate 3000 LC system (Thermo Fisher Scientific, USA) interfaced with a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific). Peptide mixtures were loaded onto a 5 mm × 300 µm i.d. C18 PEPMAP100 trapping column with water with 0.1% formic acid at 20 µL/min. After loading and washing for 3 min, peptides were eluted onto a 15 cm × 75 µm i.d. C18 PEPMAP100 nanocolumn (Thermo Fisher Scientific). A mobile phase gradient at a flow rate of 300 nL/min and with a total run time 120 min was used: 2% – 40% of solvent B in 87 min; 40% – 80% B in 1 min; 80% B during 1 min, and back to 2% B. Solvent A was 100:0 water/acetonitrile (v/v) with 0.1% formic acid, and solvent B was 0:100 water/acetonitrile (v/v) with 0.1% formic acid. In the nanospray source a stainless-steel emitter (Thermo Fisher Scientific) was used at a spray voltage of 2 kV with no sheath or auxiliary gas flow. The ion transfer tube temperature was 250 °C. Spectra were acquired in data-dependent mode with a survey scan at

m/z 300 – 1650 at a resolution of 70 000 followed by MS/MS fragmentation of the top 10 precursor ions at a resolution of 17 500. Singly charged ions were excluded from MS/MS experiments and fragmented precursor ions were dynamically excluded for 20 s.

PEAKS Studio version Xpro (Bioinformatics Solutions, Inc., Waterloo, Canada) software was used to search the MS data against the from UniProt human reference proteome (downloaded 13 July 2021). Search parameters: trypsin digestion with up to 2 missed cleavages; fixed modification carbamidomethylation of cysteine; variable modification oxidation of methionine; precursor mass tolerance of 20 ppm; fragment mass tolerance of 0.02 Da. The false discovery rate was set at 0.1% on the peptide level. Label free quantitation of the different groups was performed with the PEAKS Q module.

STATISTICAL ANALYSIS

For *in vitro* data, the means of all groups were compared to each other, and pairwise differences were calculated using one-way ANOVA (with Dunnett's multiple testing adjustment). P values ≤0.05 obtained from above test were considered statistically significant. Data are expressed as arithmetic mean ± standard deviation. Clinical data were tested using the 1-way repeated-measures analysis of variance (with Dunnett's multiple testing adjustment), where post-dose time points were compared with baseline). P values ≤0.05 obtained from above test were considered statistically significant. Data are expressed as arithmetic mean ± standard deviation. For proteomics data, volcano plots were made in which 3 hour post-dose samples were compared to the baseline samples. All analysis and visualization were done using Graphpad Prism version 9.2.0 (Graphpad Sofware, San Diego, CA, USA).

Results

IN VIVO LPS ADMINISTRATION INCREASES CIRCULATING LEUKOCYTE AND NEUTROPHIL NUMBERS, WHILE MONOCYTES AND LYMPHOCYTES DECREASE

Twelve subjects were dosed with 1 ng/kg LPS. Blood samples were taken 15 minutes before dosing, and at 0.5, 1.75, 3, 6, 9, 24 and 48 hours after LPS administration for assessment of leukocyte differentiation. After LPS administration, total circulating leukocyte numbers significantly increased to $10.48*10^9$/L compared to $5.10*10^9$/L at baseline (Figure 1A). This is mainly caused by a 3.6-fold increase in circulating neutrophils, which peaked at 8 hours after LPS dosing ($9.03*10^9$/L compared to $2.51*10^9$/L at baseline, Figure 1B). In contrast to neutrophils, monocytes almost completely disappeared 1.75h after LPS administration ($0.07*10^9$/L compared to $0.46*10^9$/L at baseline) which normalized again at 6h after LPS administration ($0.68*10^9$/L, Figure 1C). The number of circulating lymphocytes also significantly dropped upon LPS administration, with the lowest point reached at 3 hours ($0.56*10^9$/L, compared to $1.90*10^9$/L at baseline, Figure 1D). All these effects on circulating leukocyte numbers normalized at 24 hours after LPS administration, although the lymphocyte numbers remained slightly reduced compared to baseline. As is known from literature,[14,21,22] circulating neutrophils mainly display a phenotype with decreased expression of CD16 or CD62L after LPS administration. Indeed, the percentage of $CD62L^{dim}$ and $CD16^{dim}$ neutrophils was increased after LPS administration compared to baseline (Figure 1E). This increase in $CD62L^{dim}$ and $CD16^{dim}$ neutrophils was significant at 3h and 6h post LPS administration, while the increase of $CD16^{dim}$ neutrophils was also significant at 9 hours (Figure 1F).

LPS STIMULATION ACTIVATES NEUTROPHILS

For the *in vitro* studies, whole blood of 6 healthy volunteers was stimulated with 2 ng/mL, 10 ng/mL or 100 µg/mL LPS or left unstimulated for 1 hour. Afterwards, flow cytometry was performed to measure expression of CD11b, CD62L, CD63 and CD64. Representative histograms of *in vitro* stimulated neutrophils are shown in Figure 2A. Expression of CD11b on neutrophils dose-dependently increased after LPS stimulation (Figure 2B). Unstimulated neutrophils had an CD11b MFI of 32.0 ± 5.5, increasing to 63.0 ± 13.0 after stimulation with 2 ng/mL LPS. This increase was even stronger after stimulation with 10 ng/mL and 100 µg/mL (80.0 ± 10.2 and 116.4 ± 14.3, respectively). In contrast to CD11b, CD62L expression decreased after LPS stimulation. The MFI of CD62L on unstimulated neutrophils was 29.1 ± 5.3, which significantly decreased to 3.8 ± 2.1, 4.7 ± 3.5, and 4.0 ± 2.0 after stimulation with LPS at 2 ng/mL, 10 ng/mL, and 100 µg/mL LPS, respectively. Expression of CD63 only significantly changed after LPS stimulation at 100 µg/mL (0.73 ± 0.44 for 100 µg/mL vs. 0.17 ± 0.06 for unstimulated), although the MFI values remained relatively low compared to the other markers.

CD64 expression increased significantly after LPS stimulation at 10 ng/mL and 100 µg/mL (4.2 ± 1.2 for 10 ng/mL and 5.2 ± 1.3 for 100 µg/mL vs 2.5 ± 0.4 for unstimulated).

The same flow cytometric analysis was performed on whole blood collected before (-15 min) and after (0.5, 3, 6, 9, 24h) i.v. administration of LPS (1 ng/kg) to 12 healthy volunteers. Representative histograms are shown in Figure 2C. Similarly to the *in vitro* stimulation experiment, CD11b expression significantly increased after LPS administration, peaking at 3 hours (MFI of 22.3 ± 11.6 at 3h vs. 10.9 ± 6.2 at baseline). The decrease in CD62L expression was also found *in vivo*, with the lowest expression found at 6h (27.61 ± 6.38) and 9h (27.60 ± 3.59) after LPS administration (baseline: 40.21 ± 12.18). Although only minor LPS effects were found on expression of CD63 and CD64 *in vivo*, these differences were significant at 3 hours post LPS administration (Figure 2D).

LPS STIMULATION INDUCES THE RELEASE OF NEUTROPHIL GRANULE PROTEINS

In addition to surface activation markers on neutrophils, neutrophil activation was measured by determining the protein excretion of MPO, elastase and LL37 via degranulation in plasma both after *in vitro* as well as *in vivo* LPS stimulation. Elastase levels were significantly increased after *in vitro* LPS activation at 2 ng/mL for one hour (2292 ± 827 ng/mL for 2 ng/mL vs. 892 ± 663 ng/mL for unstimulated whole blood) (Figure 3A). Elastase levels were further increased with increasing levels of LPS, 2558 ± 863 ng/mL for 10 ng/mL and 5833 ± 687 ng/mL for 100 µg/mL. Interestingly, levels of myeloperoxidase (MPO) and LL37 were only increased after stimulation with 100 µg/mL LPS (2737 ± 761 ng/mL for 100 µg/mL LPS vs. 439 ± 254 ng/mL for unstimulated and 65.6 ± 14.2 for 100 µg/mL LPS vs. 42.3 ± 8.1 ng/mL for unstimulated respectively). These effects are calcium-dependent, since LPS stimulation at 100 µg/mL in K_2EDTA anti-coagulated blood did not result in a strong increase in elastase, MPO or LL37 (Figure S2).

After *in vivo* LPS stimulation (2 ng/kg, 10 subjects), elastase levels were significantly increased compared to baseline, peaking at 6 hours (841 ± 1234 ng/mL at 6h vs. 98 ± 23 ng/mL at baseline, Figure 3B), despite a high variation between subjects. MPO levels were also highly variable between subjects, but again a significant increase was observed compared to baseline, peaking at 3h (109 ± 55 ng/mL at 3h vs. 22 ± 11 ng/mL at baseline). LL37 levels were less variable between subjects and peaked later than the other granule proteins, at 10 hours after LPS administration (108 ± 16 ng/mL at 10h vs. 57 ± 12 ng/mL at baseline).

DIFFERENTIAL EXPRESSED PROTEINS AFTER IN VIVO LPS ADMINISTRATION

To assess the impact of LPS on the neutrophil proteome, neutrophils were isolated and analyzed using shotgun proteomics before and 3 hours after *in vivo* LPS administration (2 ng/kg, n=4, Figure 4A). After LPS administration, only 4 proteins showed increased expression compared to baseline, while 22 proteins were decreased (Figure 4B). A table of all differentially expressed proteins is shown in Figure 4C, with the most significantly differentially expressed proteins listed first in the table. The proteins that are increased (14-3-3 protein gamma, protein transport protein SEC31A, beta-actin like protein 2 and 40S ribosomal protein SA) all play a role in regulation of the immune response[23,24] and intracellular protein synthesis and transportation. Of the decreased proteins (22 in total), 5 are normally stored in neutrophil granules. This decrease can therefore be attributed to degranulation of the cells upon activation. The other proteins are part of the cytoskeleton (keratins), play a part in the cell metabolism, are involved in signaling pathway modulation or are cytoplasmic proteins. It should be noted that keratins are notorious contaminants in proteomics and we cannot rule out that these differences stem from contamination.

Discussion

First, we aimed to provide a time course of neutrophil activation after *in vivo* LPS administration. A decline in lymphocytes and monocytes was seen in blood after i.v. LPS administration, while the number of neutrophils, and thereby the number of total leukocytes strongly increased. This is in line with literature.[12,25-27] As shown before,[14,21,22] the neutrophils mainly display a $CD16^{dim}$ (banded) and $CD62L^{dim}$ (hypersegmented) phenotype, in contrast to homeostasis where neutrophils are mainly of a $CD16^{hi}CD62L^{hi}$ phenotype. For the cause of this decrease in CD62L expression, it is impossible to distinguish between activation and subsequent shedding of CD62L, or influx of $CD62L^{dim}$ neutrophils. The peak of neutrophil counts and the lowest expression of CD62L *in vivo* coincide at 6 hours after LPS administration (Figure

S4), indicating that this decrease in CD62L expression is likely due to influx of neutrophils from the bone marrow. Besides CD62L, CD11b was investigated as a marker of activation on neutrophils.[18,19] A rapid increase in CD11b expression was observed peaking at 4 hours after LPS administration. Also, the expression of CD63 and CD64 were significantly increased after *in vivo* LPS exposure, peaking at 4 hours, although this increase was only moderate. CD63, also known as lysosome-associated membrane protein (LAMP3), is part of the tetraspanin family. In neutrophils CD63 is expressed on membranes of primary granules, controlling granule sorting of proteins such as neutrophil elastase.[28,29] Therefore, increased cell surface expression can be used as a marker of neutrophil degranulation.[30] It has been shown *in vitro* that aging neutrophils increase the surface expression of CD63, and surface CD63 expression was restricted to the apoptotic neutrophil population.[31] CD64 (Fcγ receptor I) is not constitutively expressed on neutrophils, but can be induced upon stimulation. CD64 expression on neutrophils is a marker for sepsis,[32,33] and a trial in which healthy volunteers received 2 ng/kg LPS showed an increase in CD64 neutrophil expression upon endotoxemia.[34] Coinciding with the expression of CD63 and CD64, azurophilic granule proteins MPO, elastase and LL37[35] were rapidly increased after LPS administration. Interestingly, at the level of the neutrophil proteome, little but significant differences were observed in protein expression at 3 hours after LPS administration compared to baseline. These differences point to neutrophil degranulation. However, changes in protein expression were not as prominent as expected for the massive neutrophil activation and degranulation indicated by the experiments discussed above. In this context, it is important to note that activation markers such as receptors are already contained in the granules. Upon cell activation, they are transported to the cell membrane, and the exact cellular location does not impact the total neutrophil proteome. Additionally, activation of neutrophil receptor relocalization leads to enhanced adhesion of neutrophils on the blood vessel and subsequent degranulation. These neutrophils are not present and analyzable in blood samples. As shown previously by de Kleijn et al., significant changes in the transcriptome of neutrophils were found in human experimental endotoxemia, mostly due to neutrophil activation by inflammatory cytokines.[27]

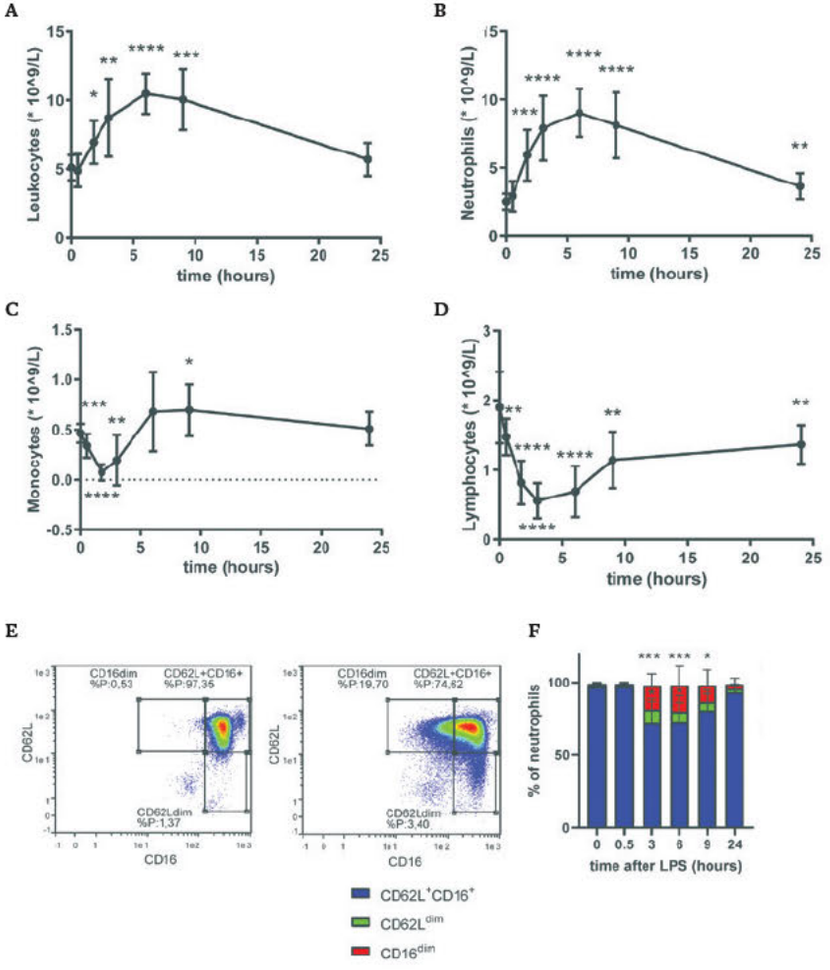
When comparing the *in vivo* and *in vitro* LPS-driven neutrophil responses, some differences can be observed. First of all, the dose of LPS required for neutrophil activation is higher in *in vitro* experiments. While *in vivo* a dose of 1 or 2 ng/kg of LPS drove significant neutrophil responses, corresponding to <0.05 ng of LPS per mL of blood depending on the size/weight of the volunteer, *in vitro* higher concentrations (ranging from 2 ng/mL to 100 µg/mL) were necessary to measure effects. This discrepancy in required trigger concentration can be explained by the fact that in whole blood cultures only direct effects of LPS on leukocytes are studied, while *in vivo* other factors play a role, such as general inflammation of the endothelium of the vessel wall.[36,37] Another factor complicating comparison of *in vitro* and *in vivo* results is the above discussed effect that as soon as neutrophils get activated, they will adhere to the vessel wall and thereby will not be analyzed after blood draw. This could also explain the relatively limited increase in neutrophil activation markers, for instance CD11b, upon *in vivo* LPS exposure. While *in vitro* more LPS was needed to activate neutrophils, the LPS-driven expression of activation markers CD62L and CD11b was much more pronounced compared to *in vivo.* In contrast to activation markers, granule protein plasma levels are clearly increased upon *in vivo* LPS stimulation in line with the *in vitro* data. Interestingly, the baseline levels of elastase and MPO in the *in vivo* study were lower than the unstimulated control in the *in vitro* experiments. This would mean that an hour of whole blood incubation already triggers a low level of degranulation although this effect was not seen for LL37. Furthermore, MPO and LL37 levels were only significantly increased at the highest LPS concentrations, indicating that low levels of LPS alone is not sufficient to induce high level degranulation, and that additional activation triggers are needed to activate neutrophil degranulation *in vivo,* such as interaction with the endothelium.

In general, *in vitro* and *in vivo* activation of neutrophils by LPS were quite comparable when looking at activation markers and granule proteins. Although the minimal LPS concentration required to drive neutrophil responses, and the neutrophil response sizes, differed upon *in vivo* and *ex vivo* LPS exposure, the nature of the observed responses were well comparable between the *in vivo* and *in vitro* challenge models. Based on these data, both the *in vitro* whole blood LPS stimulation assay and the human endotoxemia model could be valuable tools for evaluation of the effects of future drugs modulating neutrophil responses, both during preclinical and clinical development.

Table 1 Demographics of the different studies.

Study	Age, years Median (Q25-Q75)	Gender (% male)	Ethnicity (% white)	BMI kg/m^2 median (Q25-Q75)
In vivo, 1 ng/kg (n=12)	25.0 (23.0-30.75)	91.7	91.7	25.05 (21.15-26.23)
In vivo, 2 ng/kg (n=10)	25.0 (23.0-32.5)	100	50	24.75 (21.43-25.65)
In vitro, flow cytometry exp (n=6)	37.5 (30.5-40.75)	16.7	83.3	N/A
In vitro, granule protein exp (n=6)	25.0 (22.75-42.50)	50	100	N/A

Figure 1 Circulating leukocyte levels after in vivo LPS administration.



*Blood was collected before (-15 min) and after (0.5, 1.75, 3, 6, 9, 24, 48h) LPS administration (1 ng/kg, n=12). The number of leukocytes (A), neutrophils (B), monocytes (C) and lymphocytes (D) was determined by hematocytometry. Total neutrophils were analyzed for CD16 and CD62L expression (E), where an increase in CD62L^{dim} and CD16^{dim} neutrophils was found (F). Mean and SD are shown. Statistics was performed using 1-way repeated-measures analysis of variance with Dunnett's post hoc test, where post-dose time points were compared to t=0h. * P≤0.05, ** P≤0.005, *** P≤0.001, **** P≤ 0.0001.*

Figure 2 Neutrophil activation markers CD11b, CD62L, CD63 and CD64. Whole blood of 6 subjects (1 male, 5 female, median age 37.5 years, 2 separate experiments) was stimulated with 2 ng/mL, 10 ng/mL, 100 µg/mL or no LPS for 1 hour. CD11b, CD62L, CD63, CD64 expression on neutrophils was measured by flow cytometry. Representative histograms of 1 subject are shown (A). MFI is shown in panel (B). CD11b, CD62L, CD63, CD64 expression on neutrophils of 12 subjects receiving 1 ng/kg LPS was measured by flow cytometry at -1h prior to dosing up until 24 hours LPS administration. Representative histograms of 1 subject are shown (C), mean with SD is shown in (D). Statistics for panel B was performed using one-way ANOVA with Dunnett's post hoc test, statistics for panel D was performed using 1-way repeated-measures analysis of variance with Dunnett's post hoc test, where post dose time points were compared to t=-1h. * P≤0.05, ** P≤0.005, *** P≤0.001, **** P≤ 0.0001.

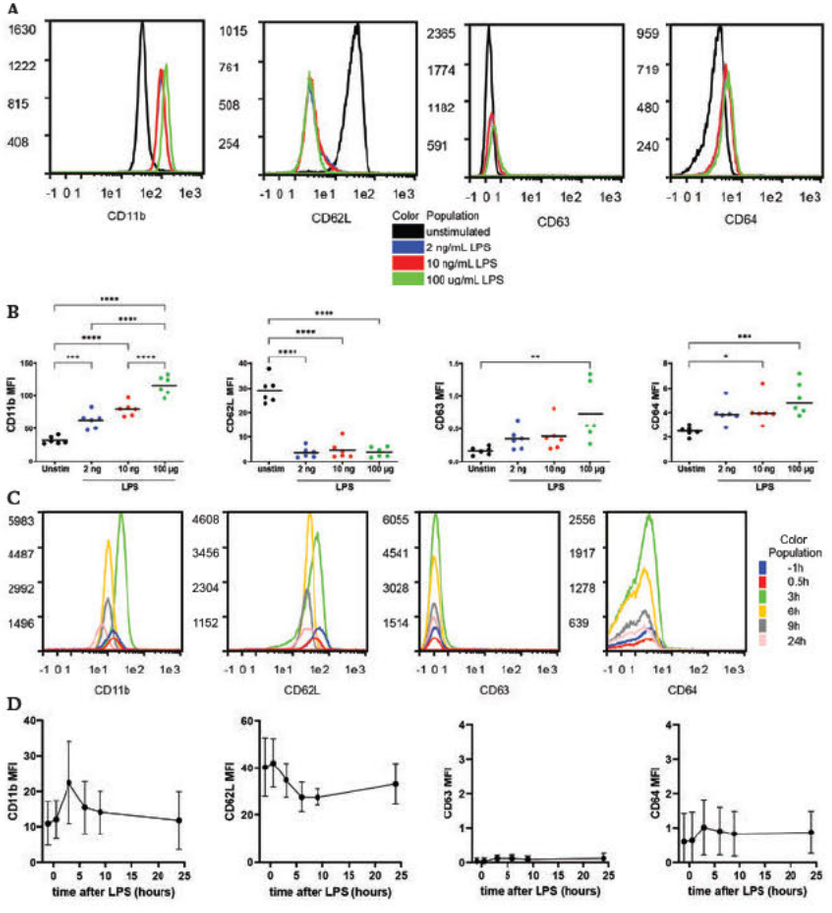


Figure 3 Granule protein measurements. Whole blood of 6 subjects (3 male, 3 female, median age 25 years) was stimulated with 2 ng/mL, 10 ng/mL, 100 µg/mL or PBS control (Unstim) for 1 hour in 2 separate experiments. Supernatants were collected and elastase, MPO and LL37 levels were determined by ELISA (A). Samples before (-1h) and after (1h, 2h, 3h, 4h, 6h, 10h and 24h) LPS administration (2 ng/kg, n=10) were assayed for elastase, MPO and LL37 levels by ELISA (B). Statistics for panel A was performed using one-way ANOVA with Dunnett's post hoc test, statistics for panel B was performed using 1-way repeated-measures analysis of variance with Dunnett's post hoc test, where post dose time points were compared to t=-1h. * P≤0.05, ** P≤0.005, *** P≤0.001, **** P≤ 0.0001.

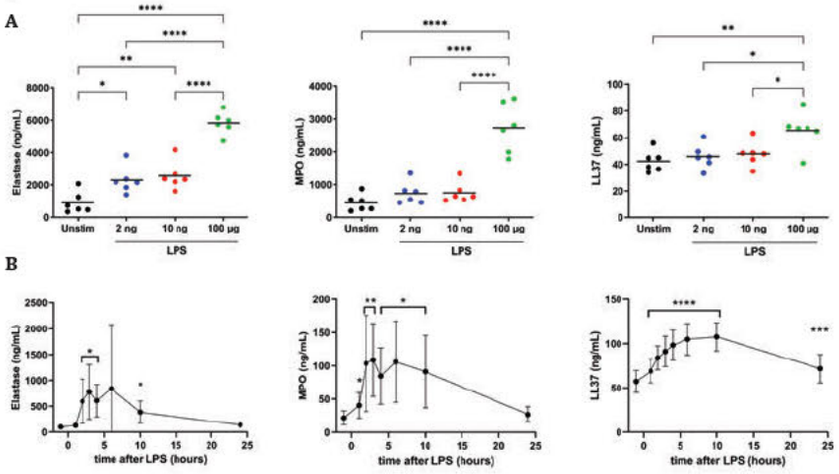
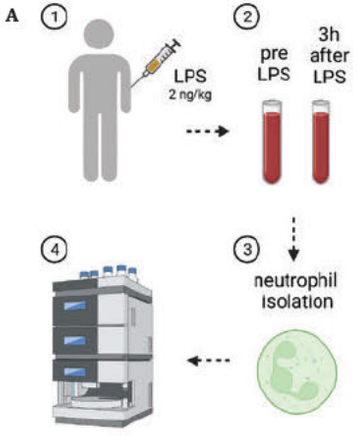


Figure 4 Proteomics of neutrophils before and after in vivo LPS stimulation.
Blood was drawn from subjects receiving 2 ng/kg LPS (n=4), before and 3 hours after LPS administration, and neutrophils were isolated. Shotgun proteomics was performed with these isolated neutrophils (A). Differentially expressed proteins are visualized in a volcano plot (B). A table with all significantly increased and decreased proteins in included in (C). Panel A was made using biorender.com.



C

Increased
14-3-3 protein gamma
Protein transport protein SEC31A
Beta-actin-like protein 2
40S ribosomal protein SA
Decreased
Hornerin
Keratin type I cytoskeletal 9
Keratin type I cytoskeletal 10
Phosphatidylethanolamine-binding protein 1
Peptidoglycan recognition protein 1
Complement C3
Protein S100-P
Adenylyl cyclase-associated protein 1
Keratin type II cytoskeletal 1
Transaldolase
Keratin type II cytoskeletal 2 epidermal
Lysozyme C
Neutrophil elastase
Malate dehydrogenase cytoplasmic
Vascular non-inflammatory molecule 2
Fructose-bisphosphate aldolase C
Fatty acid-binding protein 5
Cysteine-rich secretory protein 3
Ubiquitin-conjugating enzyme E2 N
Chitotriosidase-1
Cytochrome P450 4F3
Succinate--CoA ligase [GDP-forming] subunit beta

Supplemental material

Table S1 Flow cytometry antibody list.

Marker	Fluorochrome	Clone	Supplier
CD11b	VioBlue	REA713	Miltenyi
CD14	FITC	REA599	Miltenyi
CD16	BV605	3G8	Biolegend
CD45	VioGreen	REA747	Miltenyi
CD62L	PE	REA615	Miltenyi
CD63	APC	REA1055	Miltenyi
CD64	PERCP-VIO700	REA978	Miltenyi
CD66b	PE-VIO770	REA306	Miltenyi

PE=Phycoerythrin, BV=Brilliant Violet, FITC=Fluorescein isothiocyanate, APC=allophycocyanin, PERCP=Peridinin-Chlorophyll-Protein

Figure S1 Gating strategy neutrophil activation markers. First all cells are gated in the FSC-A/SSC-A plot, excluding debris. Then singlets are gated in the FSC-A/FSC-H, followed by CD45$^+$ PI- live cells. Then granulocytes are gated in the FSC-A/SSC-A plot, followed by neutrophils, gated as CD16$^+$CD66b$^+$. From the neutrophil gate expression of CD62L, CD63, CD64 and CD11b is measured.

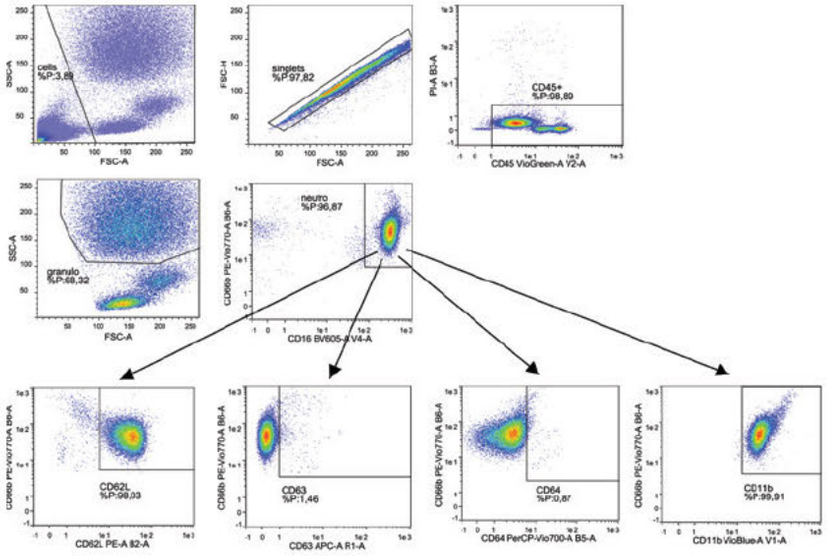


Figure S2 LPS stimulation in K_2EDTA whole blood. K_2EDTA anticoagulated whole blood was stimulated with 100 µg/ml LPS or PBS control for 1 hour. Supernatant was assayed for elastase, MPO and LL37 (n=3 for elastase and LL37, n=2 for MPO).

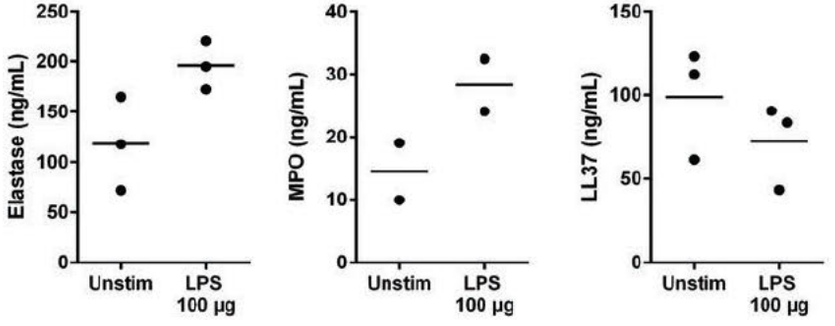


Figure S3 CD62L and CD16 expression on neutrophils, *in vitro*. CD16 and CD62L expression on unstimulated (left) and 100µg/mL LPS stimulated neutrophils (right).

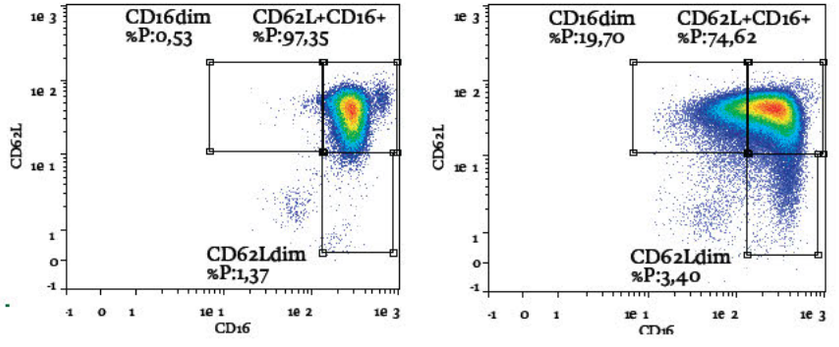
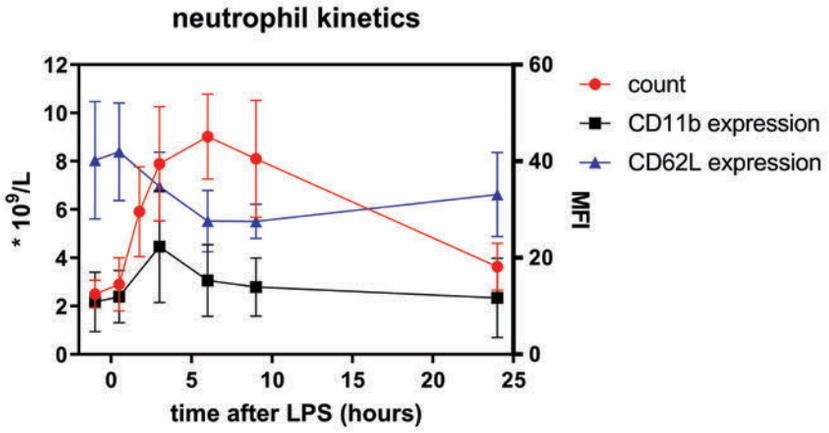


Figure S4 Neutrophil kinetics *in vivo*. The neutrophil count (red), CD11b MFI (black) and CD62L MFI (blue) over time after LPS administration.



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