

# Whole-blood incubation with the *Neisseria meningitidis* *lpxL1* mutant induces less pro-inflammatory cytokines than the wild type, and IL-10 reduces the MyD88-dependent cytokines

Hans Christian D Aass<sup>1,2</sup>, Marit Hellum<sup>1,2</sup>, Anne-Marie Siebke Trøseid<sup>1</sup>, Petter Brandtzaeg<sup>1,2,3</sup>, Jens Petter Berg<sup>1,2</sup>, Reidun Øvstebø<sup>1</sup> and Carola Elisabeth Henriksson<sup>1,2</sup>

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## Abstract

Levels of bacterial LPS, pro-inflammatory cytokines and IL-10 are related to the severity of meningococcal septicaemia. Patients infected with a *Neisseria meningitidis* *lpxL1* mutant (*Nm*-mutant) with penta-acylated lipid A present with a milder meningococcal disease than those infected with hexa-acylated *Nm* wild type (*Nm*-wt). The aim was to compare the pro-inflammatory responses after *ex vivo* incubation with the heat-inactivated *Nm*-wt or the *Nm*-mutant in citrated whole blood, and the modulating effects of IL-10. Concomitantly, we measured intracellular IL-6, IL-8 and TNF- $\alpha$  to elucidate which cell types were responsible for the pro-inflammatory responses. Incubation with *Nm*-wt ( $10^6$ /ml;  $10^7$ /ml;  $10^8$ /ml) resulted in a dose-dependent increase of the MyD88-dependent pro-inflammatory cytokines (IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$ ), which were mainly derived from monocytes. In comparison, only  $10^8$ /ml of the *Nm*-mutant significantly increased the concentration of these cytokines. The MyD88-independent cytokines (IP-10, RANTES) were evidently increased after incubation with the *Nm*-wt but were unaffected by the *Nm*-mutant. Co-incubation with IL-10 significantly reduced the concentrations of the MyD88-dependent cytokines induced by both the *Nm*-wt and the *Nm*-mutant, whereas the MyD88-independent cytokines were almost unaffected. In summary, the *Nm*-mutant is a weaker inducer of the MyD88-dependent/independent cytokines than the *Nm*-wt in whole blood, and IL-10 attenuates the *Nm*-stimulated increase in MyD88-dependent pro-inflammatory cytokines.

## Keywords

IL-10, *Neisseria meningitidis*, *lpxL1*, whole blood, MyD88, pro-inflammatory cytokines

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## Introduction

*Neisseria meningitidis* (*N. meningitidis*) is a Gram-negative diplococcus and the causative agent of worldwide meningococcal meningitis and sepsis.<sup>1</sup> The bacteria are carried asymptotically in the nasopharynx in approximately 10% of the world's population, varying from 4–5% in infants to 20–24% in young adults to 7–8% in 50-yr-old adults.<sup>2,3</sup> However, in rare occasions, the bacteria pass the mucosal barrier and enter the bloodstream. A high bacterial load in the circulation is associated with an increased risk of developing

<sup>1</sup>Department of Medical Biochemistry, Oslo University Hospital, Oslo, Norway

<sup>2</sup>Institute of Clinical Medicine, University of Oslo, Oslo, Norway

<sup>3</sup>Department of Pediatrics, University of Oslo, Oslo, Norway

### Corresponding author:

Hans Christian D Aass, The Flow Cytometry Core Facility, Blood Cell Research Group, Department of Medical Biochemistry, Oslo University Hospital, Ullevål, Kirkeveien 166, N-0407 Oslo, Norway.  
Email: h.c.aass@medisin.uio.no



shock, organ dysfunction, thrombus formation, bleeding and, in many cases, death.<sup>4,5</sup>

The lipid A part of the lipopolysaccharides (LPS) in the outer membrane of the *N. meningitidis* wild type (wt) is a strong inducer of inflammation, and high LPS levels in plasma from patients with meningococcal sepsis are associated with an increased severity of the disease and decreased survival.<sup>4-6</sup> The severity of systemic meningococcal sepsis also correlates to the plasma concentrations of IL-10.<sup>7</sup> IL-10 is a major anti-inflammatory regulator of myeloid cells that are activated by bacteria or LPS,<sup>8</sup> and has been identified as the single most important plasma component suppressing the ability of monocytes to produce pro-inflammatory cytokines and to induce pro-coagulant activity.<sup>9</sup> Therefore, IL-10 is a physiologic inhibitor that may counterbalance many LPS responses.<sup>10</sup>

The cellular inflammatory response is initiated by the soluble LPS binding protein that transfers an LPS molecule to the membrane-bound CD14 receptor on monocytes and macrophages.<sup>11</sup> CD14 presents the LPS molecule to the TLR4/myeloid differentiation protein-2 (MD-2) complex,<sup>12</sup> which, in turn, activates the MyD88-dependent and the MyD88-independent pathways. The activation of the MyD88-dependent pathway results in rapid activation of NF- $\kappa$ B and MAPKs, and the subsequent release of pro-inflammatory cytokines like IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$ .<sup>13</sup> LPS activate the MyD88-dependent pathway, but other bacterial components are also able to activate this pathway through TLR2.<sup>14</sup> However, only LPS, and not any other parts of the outer membrane of *N. meningitidis*, are able to activate the MyD88-independent pathway.<sup>13</sup> Here, IFN regulatory factor 3 is activated,<sup>15</sup> which induces the production of IFN- $\beta$  and also a late-phase activation of NF- $\kappa$ B and MAPKs.<sup>12</sup> The IFN- $\beta$  binds to the type 1 IFN- $\alpha/\beta$  receptor, which activates STAT1 and leads to type 1 IFN- $\alpha/\beta$ , IFN- $\gamma$ -induced protein 10 (IP-10; CXCL10) and regulated on activation, normal T cell expressed and secreted (RANTES; CCL5).<sup>15</sup>

The lipid A part of LPS is essential for recognition by the TLR4 receptor complex, and the acylation pattern of lipid A determines, to a large part, the biological activity of LPS. In *N. meningitidis* wt, the lipid A is hexa-acylated. In the laboratory, a *N. meningitidis* mutant has been made by insertional inactivation of the *lpxLI* gene, which is required for addition of a secondary acyl chain to the lipid A molecule.<sup>16</sup> *N. meningitidis* with a mutated *lpxLI* gene has five, instead of six, acyl chains in the lipid A moiety, and this mutant activates human TLR4 less efficiently than the wt.<sup>13</sup> The *N. meningitidis lpxLI* mutant is also found naturally occurring in patients, and compared with patients infected with the wt meningococcus, these patients appear to have a milder meningococcal disease with less systemic inflammation and coagulopathy.<sup>13,17,18</sup> Fransen et al. have previously shown that the

spontaneously occurring *lpxLI* mutant induced low levels of the pro-inflammatory cytokines IL-6, TNF- $\alpha$  and IL-1 $\beta$  in peripheral blood mononuclear cells (PBMCs).<sup>13</sup> The effect of the *lpxLI* mutant on the pro-inflammatory response has also been studied using human cell lines<sup>19,20</sup> or cell lines originated from rodents.<sup>21,22</sup> However, to our knowledge, the effect of the *N. meningitidis lpxLI* mutant on the pro-inflammatory response in human whole blood has not yet been studied. *Ex vivo* incubation in whole blood is an experimental model closer to the *in vivo* environment than *in vitro* incubation of isolated cells,<sup>23</sup> and whole blood contains soluble factors that may influence cytokine release.<sup>24</sup>

In this study, the aim was to compare the pro-inflammatory responses, and the modulating effects of recombinant human IL-10 (rhIL-10), in citrated whole blood after *ex vivo* incubation with heat-inactivated *N. meningitidis* wt or heat-inactivated *N. meningitidis lpxLI* mutant. In addition to plasma cytokine levels, intracellular cytokine levels were measured to elucidate which cell types were responsible for the inflammatory responses.

## Material and methods

### Ethics statement

The project was approved by the Regional Committee for Medical Research Ethics, South-Eastern Norway Regional Health Authority (2011/1413).

### Materials

Stock solution (10  $\mu$ g/ml) of rhIL-10 (R&D Systems, Abingdon, UK) was reconstituted in Tris-NaCl buffer (TBS; Sigma, St Louis, MO, USA). Brefeldin A (10 mg/ml) was purchased from Sigma Aldrich (Oslo, Norway). The monoclonal antibodies (abs) anti-CD45 fluorescein isothiocyanate (FITC) (clone J33), anti-CD3-phycoerythrin-cyanine 7 (PE-Cy7) (clone UCHT1), anti-CD14-allophycocyanin (APC) (clone RMO52), anti-TNF- $\alpha$  phycoerythrin (PE) (clone IPM2) and the PerFix-nc kit used for intracellular staining were purchased from Beckman Coulter (Indianapolis, IN, USA). Anti-IL-6 PE abs (clone MQ2-13A5) and anti-IL-8 PE abs (clone B-K8) were purchased from Nordic Biosite (Oslo, Norway).

### The *N. meningitidis* wt and the *N. meningitidis lpxLI* mutant

*N. meningitidis* strain H44/76 is a serogroup B (B:15:P1.7,16 with immunotype L,3,7,9) reference strain initially isolated from a Norwegian patient with lethal meningococcal septicaemia.<sup>25</sup> The *N. meningitidis* wt has six acyl chains in the lipid A portion of the LPS

molecule (hexa-acylated), whereas the *lpxLI* mutant has five acyl chains (penta-acylated). The *N. meningitidis lpxLI* mutant was obtained by insertional inactivation of the *lpxLI* gene in the *N. meningitidis* strain (H44/76), and kindly provided by Arie van der Ende (Academic Medical Centre, Amsterdam, the Netherlands) and Peter van der Ley (National Institute of Public Health and Environment, Bilthoven, the Netherlands) to the National Institute of Public Health, Oslo, Norway, for research purposes. It has previously been reported that the *N. meningitidis lpxLI* mutant makes normal amounts of LPS and, compared with the wt, it is only the lipid A part that is altered.<sup>16,26</sup> It has also been shown that the laboratory-engineered *lpxLI* mutant has an identical ability to induce pro-inflammatory cytokines compared with similar strains isolated from patients.<sup>13</sup> The meningococci were grown and heat-inactivated (56°C, 30 min) as previously described.<sup>13,27</sup> To quantify the number of *N. meningitidis* (either wt or *lpxLI*), bacterial DNA was isolated with the MagNA Pure LC DNA isolation Kit I (Roche Diagnostics, Mannheim, Germany) and the DNA copy number, equivalent to the number of bacteria, was estimated by spectroscopic measurements at 260 nm (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE, USA) combined with a genome calculator (<http://cels.uri.edu/gsc/cndna.html>) using a *N. meningitidis* genome size of 2,272,351 base pairs.<sup>28</sup>

#### **Incubation of the *N. meningitidis* wt and the *N. meningitidis lpxLI* mutant in citrated whole blood**

After signed consent was obtained, whole blood from healthy volunteers was drawn into 3.5 ml citrate tubes (Vacuette 3.5 ml; Greiner Bio One GmbH, Kremsmünster, Austria), and the first tube was discarded. Whole blood was immediately incubated with heat-inactivated *N. meningitidis* wt, *N. meningitidis lpxLI* mutant (both 10<sup>7</sup>/ml) or vehicle (TBS) [37°C, 12 rpm Hulamixer (Invitrogen Dynal AS, Oslo Norway)].

To determine the optimal time point to measure pro-inflammatory cytokines, whole blood from four donors was centrifuged [2500 g, 15 min at room temperature (RT)] after 0, 4, 6 and 12 h, and plasma was collected for measurements of cytokines.

After these initial experiments, an incubation period of 4 h was chosen, and whole blood from six donors was incubated with increasing concentrations of *N. meningitidis* wt or *N. meningitidis lpxLI* mutant (10<sup>6</sup>/ml, 10<sup>7</sup>/ml or 10<sup>8</sup>/ml). In some cases, rhIL-10 (25 ng/ml) or vehicle (TBS) was added to whole blood 15 min prior to the addition of the bacteria. Similar IL-10 concentrations have previously been used in *in vitro* studies,<sup>29</sup> and have also been observed in patients with severe fulminant meningococcal sepsis.<sup>30</sup>

#### **Quantification of cytokines in plasma**

Citrated whole blood was prepared by centrifugation at 2500 g for 15 min at RT. Then, 1 ml plasma was collected in a 1.5-ml Eppendorf tube and centrifuged at 10,000 g for 10 min at 4°C. The top supernatant (800 µl) was collected prior to freezing, and stored at -80°C. Measurements of cytokines were performed using a microsphere-based multiplexing bioassay system with Xmap technology (Austin, TX, USA) with a Luminex IS 100 instrument (Bio-Rad, Hercules, CA, USA), powered with the Bio-Plex manager Software version 6.0 (build 617). The anti-inflammatory cytokine IL-10, the MyD88-dependent cytokines IL-1β, IL-6, IL-8 and TNF-α,<sup>13</sup> and the MyD88-independent cytokines IP-10 and RANTES,<sup>15</sup> were measured (Bio-Rad, Oslo, Norway) in duplicates.

#### **The intracellular cytokine expression of different cell types**

To determine which cell type expresses IL-6, IL-8 and TNF-α, whole blood from eight healthy volunteers was pre-incubated with Brefeldin A (10 µg/ml) for 15 min. Brefeldin A inhibits the release of cytokines by blocking the transport of proteins from the endoplasmic reticulum to the Golgi apparatus. Whole-blood samples were stimulated with either *N. meningitidis* wt (10<sup>8</sup>/ml), *N. meningitidis lpxLI* mutant (10<sup>8</sup>/ml) or vehicle for 4 h. In order to perform intra- and extracellular staining of the white blood cells (WBCs), the low fixation procedure of the PerFix-nc kit was performed to fix and permeabilize cells, as well as to lyse the red blood cells. The cells were stained with the following final concentrations: 8.3 ng/ml anti-CD45 abs, 40 ng/ml anti-CD3 abs and 5.0 ng/ml anti-CD14 abs. The intracellular staining was performed with either 8.3 ng/ml of anti-IL-6 abs, 4.2 ng/ml of anti-IL-8 abs or 5 ng/ml of anti-TNF-α abs. All abs were titrated prior to analysis. Cells were stained for 30 min sheltered from light, and then washed with 3 ml of the final 1 × reagent (Perfix-nc kit buffer 3). After washing, the cells were pelleted by centrifugation at 500 g (5 min), the supernatant removed, and the cells re-suspended in 300 µl final 1 × reagent. Samples were analysed on a Accuri C6 (Becton Dickinson, San Jose, CA, USA). The intracellular expression of IL-6, IL-8 and TNF-α was presented as PE median fluorescence intensity (MFI). For the three intracellular cytokines, the delta MFI (ΔMFI) was calculated as: MFI of cells (incubated with *N. meningitidis* wt or *N. meningitidis lpxLI* mutant) – MFI of cells (incubated with vehicle only).

#### **Statistical analysis**

Data are presented as median (range) throughout. The non-parametric Friedman test without correction for multiple comparisons (GraphPad Prism

version 7.02; GraphPad, La Jolla, CA, USA) was used to statistically test the differences in cytokine expression and release between vehicle and *N. meningitidis* wt and between vehicle and *N. meningitidis lpxLI* mutant. Comparisons of medians were also performed between the *N. meningitidis* wt and the *N. meningitidis lpxLI* mutant and between the presence and absence of rhIL-10. For these comparisons we used the non-parametric Wilcoxon rank sum test. To compare the levels of intracellular cytokines between different types of cells (monocytes, granulocytes and lymphocytes), we calculated the  $\Delta$ MFI (both after incubation with the *N. meningitidis* wt and the *N. meningitidis lpxLI* mutant) and performed a non-parametric Kruskal–Wallis test.  $P < 0.05$  was considered statistically significant.

## Results

### *The time course of cytokine plasma levels after incubation with the N. meningitidis wt or the N. meningitidis lpxLI mutant*

After incubation with the *N. meningitidis* wt, compared with vehicle, the plasma levels of the MyD88-dependent cytokines IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$  were significantly increased at 2 h, peaked at 6 h and remained at a high level also at 12 h (Figure 1a–d, red and black lines;  $P < 0.05$ ). After incubation with the *N. meningitidis lpxLI* mutant, only IL-1 $\beta$  was significantly increased compared with vehicle, and only at 12 h (Figure 1a–d, blue and black lines). All cytokines peaked/almost peaked at 4–6 h (Figure 1a–d),

After incubation with the *N. meningitidis* wt, compared with vehicle, the MyD88-independent cytokines IP-10 and RANTES significantly increased at 2 h and peaked at 4 h (Figure 1e and f, red and black lines;  $P < 0.05$ ). IP-10 remained at the same level at 6 and 12 h, whereas the level of RANTES decreased and was similar to vehicle only, at these time points. After incubation with the *N. meningitidis lpxLI* mutant, the plasma levels of IP-10 and RANTES remained low between 0 to 12 h, similar to the levels observed for vehicle only (Figure 1e and f, blue and black lines).

After incubation with the *N. meningitidis* wt, IL-10 was significantly increased at 12 h compared with vehicle only, but not at the other time-points (Figure 1g, red and black lines). Compared with incubation with vehicle only, IL-10 was not increased after incubation with the *N. meningitidis lpxLI* mutant at any time-point (Figure 1g, blue and black lines).

### *The cytokine plasma levels after incubation with the N. meningitidis wt or the N. meningitidis lpxLI mutant in increasing concentrations*

After incubation with  $10^6$ /ml,  $10^7$ /ml and  $10^8$ /ml of the *N. meningitidis* wt, the MyD88-dependent cytokines

IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$  were significantly increased compared with vehicle only (Figure 2a–d;  $P < 0.05$ ).

With the *N. meningitidis lpxLI* mutant, only the  $10^8$ /ml concentration induced significant differences in the plasma levels of the MyD88-dependent cytokines, compared with vehicle only, and IL-8 was the only cytokine that reached the same level as after incubation with the *N. meningitidis* wt, with concentrations of [1015 (853–3054)] and [1545 (1128–2350)] pg/ml, respectively (Figure 2a–d;  $P < 0.05$ ).

After incubation with the *N. meningitidis* wt ( $10^6$ /ml,  $10^7$ /ml and  $10^8$ /ml), the MyD88-independent cytokines IP-10 and RANTES, were significantly increased compared with vehicle only (Figure 2e and f;  $P < 0.05$ ).

After incubation with the *N. meningitidis lpxLI* mutant ( $10^6$ /ml,  $10^7$ /ml and  $10^8$ /ml), the levels of IP-10 and RANTES were not significantly increased compared with vehicle only and IP-10 and RANTES never reached the same levels as after incubation with the *N. meningitidis* wt (Figure 2e and f;  $P > 0.05$ ).

After incubation with the *N. meningitidis* wt ( $10^7$ /ml and  $10^8$ /ml), IL-10 was significantly increased compared with vehicle only (Figure 2g;  $P < 0.05$ ).

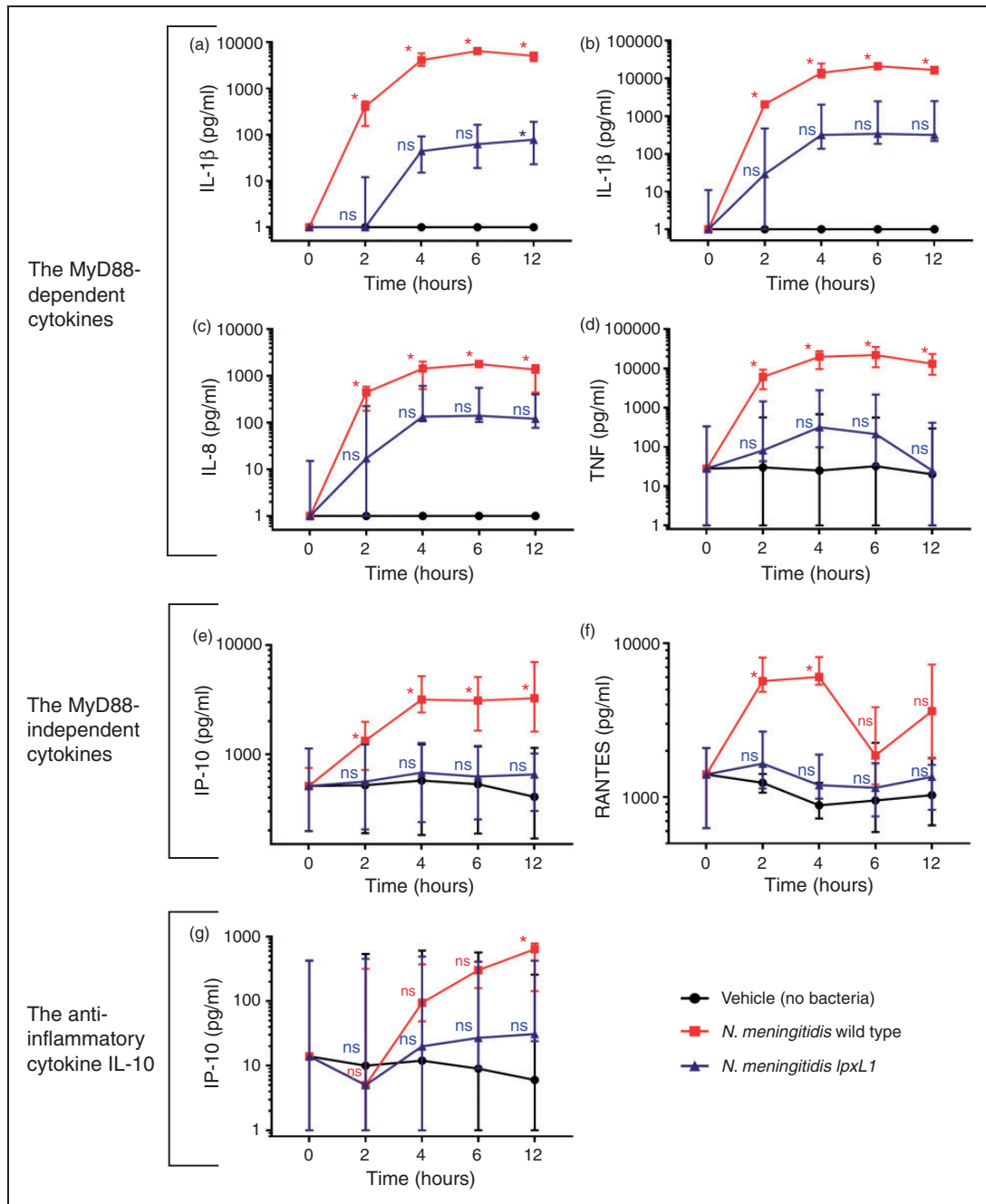
After incubation with the *N. meningitidis lpxLI* mutant, IL-10 was only significantly increased at  $10^8$ /ml compared with vehicle only (Figure 2g;  $P < 0.05$ ).

After incubation with the *N. meningitidis lpxLI* mutant at  $10^7$ /ml and  $10^8$ /ml, IL-10 reached the same level as after incubation with *N. meningitidis* wt.

### *The intracellular cytokine expression of different cell types after whole blood incubation with the N. meningitidis wt or the N. meningitidis lpxLI mutant*

To determine which cell type expresses IL-6, IL-8 and TNF- $\alpha$ , whole blood was pre-incubated with Brefeldin A prior to the addition of *N. meningitidis* wt or *lpxLI* mutant (both  $10^8$ /ml). Subsequently, whole blood was subjected to flow cytometry analysis (Figure 3a). The WBCs (gate I) were separated from erythrocytes in a CD45/side scatter (SSC) dot plot and the WBCs (gate I) were further displayed in a CD3/SSC dot plot with the CD3<sup>+</sup> lymphocytes in gate III. The CD3<sup>+</sup> cells in the CD3/SSC dot plot (gate II) were then further analysed in a CD14/SSC dot plot. Monocytes were selected as CD14<sup>+</sup> (gate V), and granulocytes as CD14<sup>−</sup> cells with high SSC (gate IV). Representative overlay histograms of IL-6, IL-8 and TNF- $\alpha$  for monocytes, lymphocytes and granulocytes are shown (Figure 3a). The intracellular expression of IL-6, IL-8 and TNF- $\alpha$  was measured as PE MFI. CD14<sup>+</sup> monocytes showed significantly increased intracellular concentrations of IL-6, IL-8 and TNF- $\alpha$  after incubation

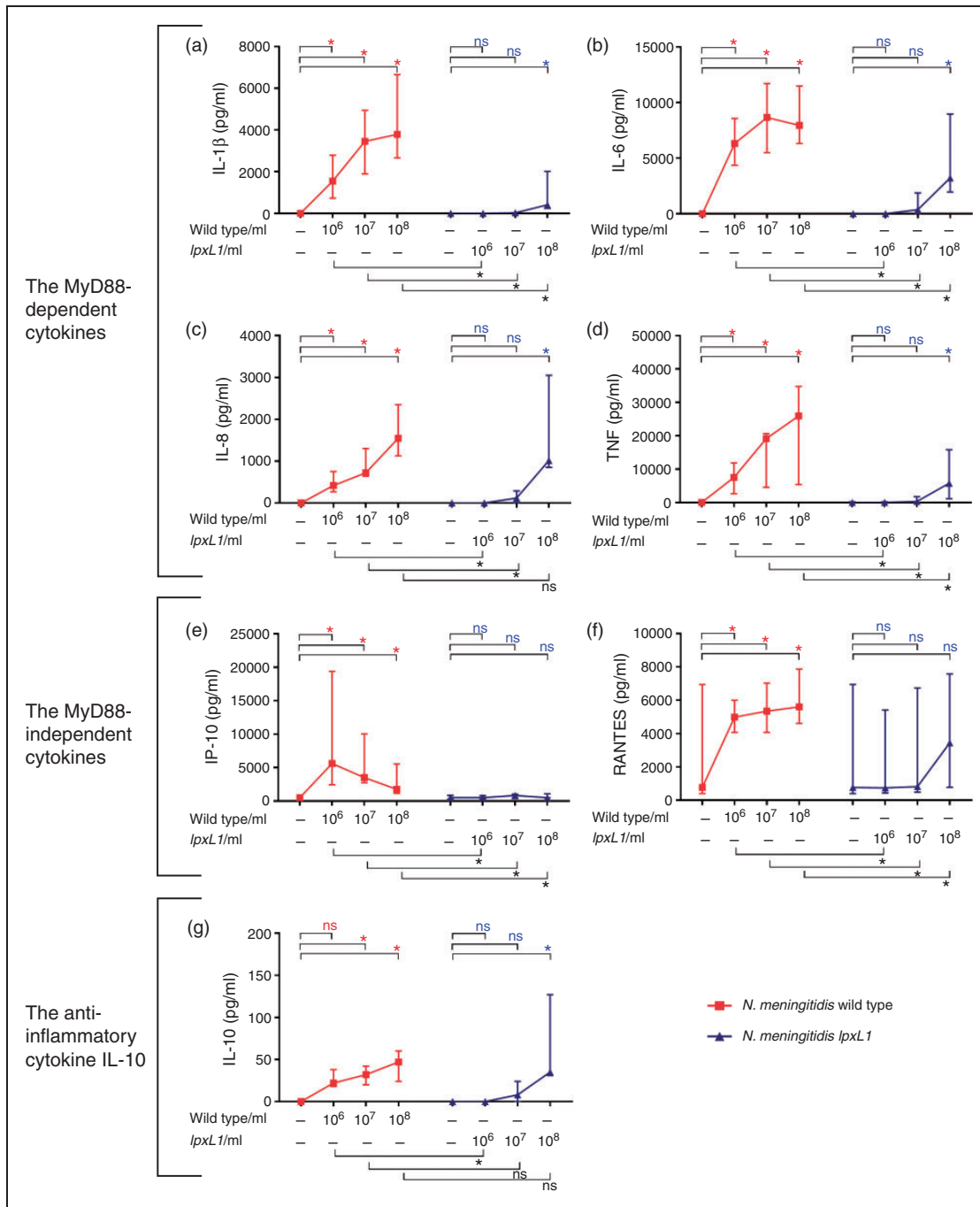




**Figure 1.** Time course of cytokine plasma levels. Whole blood from four donors was incubated (0, 2, 4, 6 and 12 h) with *N. meningitidis* wt ( $10^7$ /ml) (red line), *N. meningitidis* *lpxL1* mutant ( $10^7$ /ml) (blue line) or vehicle (no bacteria) (black line), plasma collected and measurements of cytokines performed. The concentrations [median (range)] (pg/ml) of the (a–d) MyD88-dependent cytokines IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$  (e, f), the MyD88-independent cytokines IP-10 and RANTES, and (g) the anti-inflammatory cytokine IL-10 are presented. The y-axis is presented in a log scale. \*Significant differences in cytokine levels between the *N. meningitidis* wt and vehicle or between the *N. meningitidis* *lpxL1* mutant and vehicle ( $P < 0.05$ ). ns: non-significant differences.

with either the *N. meningitidis* wt or the *N. meningitidis* *lpxL1* mutant compared with vehicle only (Figure 3b;  $P < 0.05$ ). Granulocytes showed significantly increased intracellular concentrations of IL-6, IL-8 and TNF- $\alpha$  after incubation with the *N. meningitidis* wt, and increased IL-8 after incubation

with the *N. meningitidis* *lpxL1* mutant, compared with vehicle only (Figure 3b;  $P < 0.05$ ). In contrast, CD3<sup>+</sup> lymphocytes did not show any significant increase in intracellular IL-6, IL-8 or TNF- $\alpha$ , neither after incubation with the *N. meningitidis* wt nor with the *N. meningitidis* *lpxL1* mutant, compared with vehicle

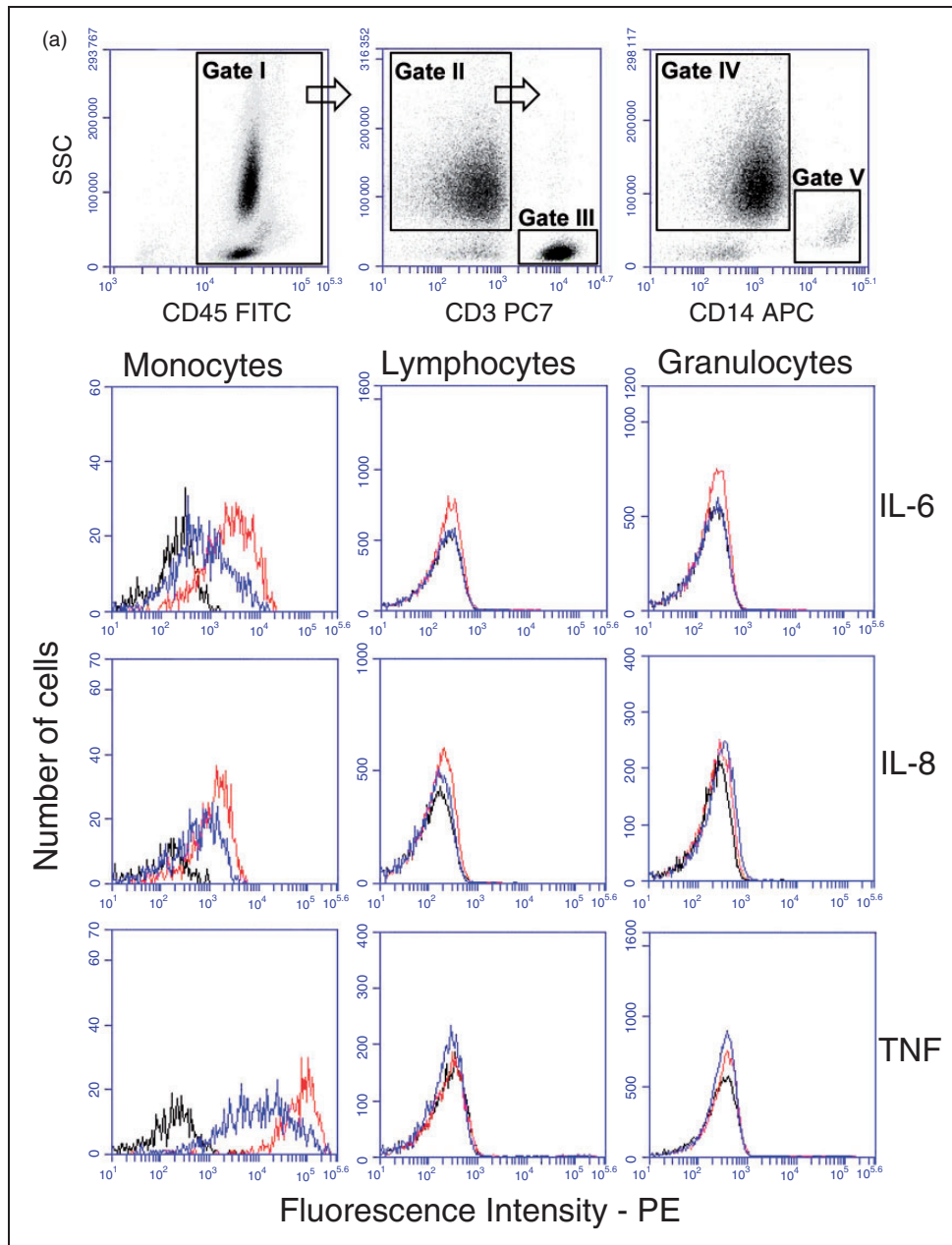


**Figure 2.** Plasma cytokine levels released upon increasing concentrations of bacteria. Whole blood from six donors was incubated for 4 h with increasing concentrations of *N. meningitidis* wt (wild type), *N. meningitidis* *lpxL1* mutant (*lpxL1*) or vehicle (no bacteria), plasma collected and measurements of cytokines performed. The concentrations [median (range)] (pg/ml) of (a–d) the MyD88-dependent cytokines IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$  (e, f), the MyD88-independent cytokines IP-10 and RANTES, and (g) the anti-inflammatory IL-10 are presented. \*(Top of charts) indicates significant differences in cytokine levels between the wt and vehicle or between the mutant *lpxL1* and vehicle ( $P < 0.05$ ). \*(Below charts) indicates significant differences in cytokine levels between the wt and the mutant *lpxL1*,  $P < 0.05$ .

ns: non-significant differences.

only. The remaining cells, i.e. all cells that did not appear within the gates of the three selected populations, were not positive for IL-6, IL-8 or TNF- $\alpha$  (data not shown).

The intracellular levels of IL-6 and TNF- $\alpha$  were also significantly higher in monocytes incubated with *N. meningitidis* wt compared with the *N. meningitidis* *lpxL1* mutant, but, in contrast, the cytokine



**Figure 3.** Intracellular cytokine levels in different cell types. Whole blood from eight donors was incubated for 4 h with *N. meningitidis* wt ( $10^8$ /ml), *N. meningitidis* *lpxLI* mutant (*lpxLI*) ( $10^8$ /ml) or vehicle (no bacteria), and flow cytometry measurements of intracellular cytokines performed. In (a) the gating strategy used to identify the different types of WBCs is shown. WBCs are shown in gate I,  $CD3^+$  lymphocytes in gate III, granulocytes in gate IV and  $CD14^+$  monocytes in gate V. The cytokine fluorescence intensity (FI) levels of IL-6, IL-8 and TNF- $\alpha$  for monocytes, lymphocytes and granulocytes are displayed in overlay histograms of one representative donor, with lines in black (vehicle), red ( $10^8$ /ml *N. meningitidis* wt) and blue ( $10^8$ /ml *N. meningitidis* *lpxLI* mutant). In (b) intracellular levels of IL-6, IL-8 and TNF- $\alpha$  in monocytes, lymphocytes and granulocytes are presented as median (range) of MFI. \*(Top of charts) indicates significant differences in intracellular cytokine MFI between the wt and vehicle or between the *lpxLI* mutant and vehicle ( $P < 0.05$ ). \*(Below charts) indicates significant differences between the wt and the *lpxLI* mutant ( $P < 0.05$ ). ns: non-significant differences.

IL-8 had the same level with both bacteria.  $\Delta$ MFI was calculated and the  $\Delta$ MFI of IL-6, IL-8 and TNF- $\alpha$  were all significantly higher in monocytes compared with granulocytes and compared with

lymphocytes in whole blood incubated with either *N. meningitidis* wt or the *N. meningitidis* *lpxLI* mutant (data not shown in figure) (Kruskal-Wallis test,  $P < 0.05$ ).

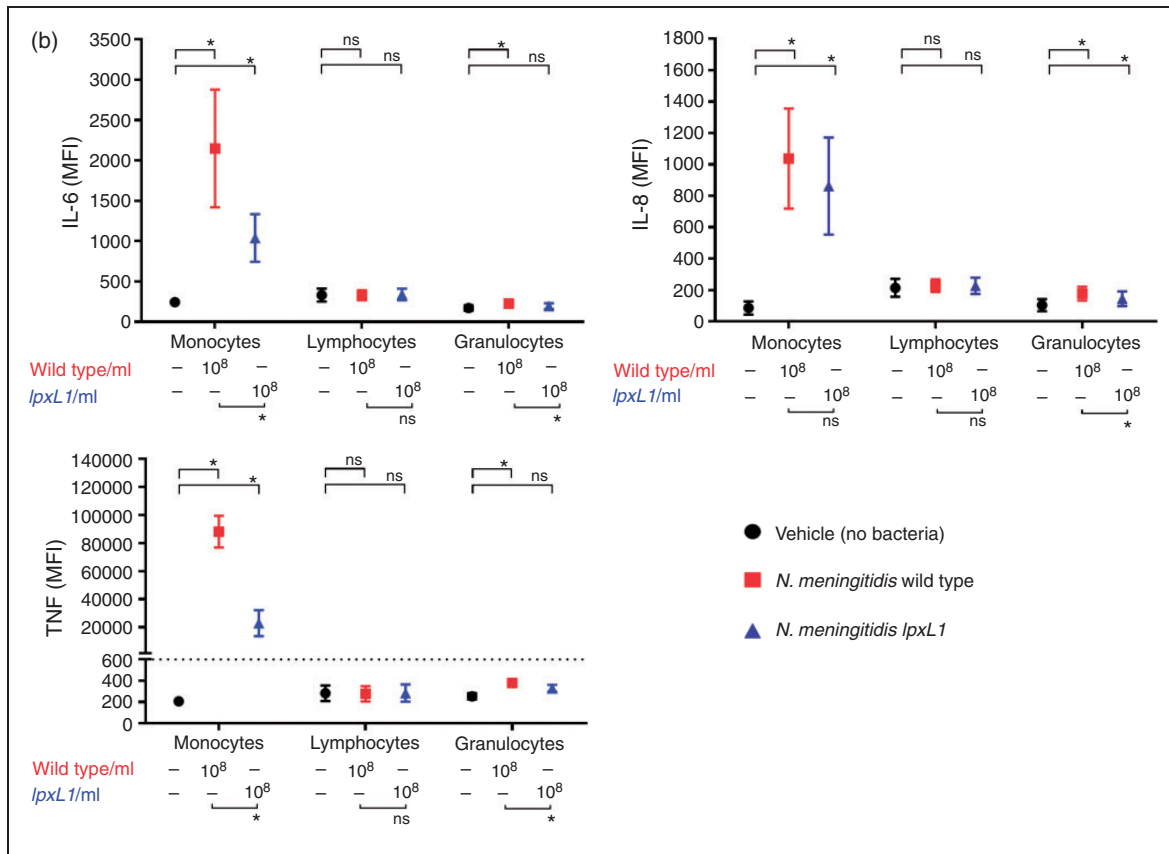


Figure 3. Continued.

### The cytokine plasma levels after incubation with the *N. meningitidis* wt or the *N. meningitidis lpxL1* mutant in the absence and presence of rhIL-10

The inhibitory effect of rhIL-10 on cytokine production was measured in plasma obtained from whole blood after 4 h incubation with increasing concentrations of *N. meningitidis* wt, *N. meningitidis lpxL1* mutant or vehicle, and only data from  $10^8$ /ml are presented in Table 1.

After incubation with the *N. meningitidis* wt, the plasma levels of the MyD88-dependent cytokines IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$  were significantly reduced by the presence of rhIL-10 with the following fold reductions: 62.1 (IL-1 $\beta$ ), 7.9 (IL-6), 6.0 (IL-8) and 24.5 (TNF- $\alpha$ ) (Table 1;  $P < 0.05$ ).

Also, after incubation with the *N. meningitidis lpxL1* mutant, the release of the MyD88-dependent cytokines in whole blood was significantly reduced in the presence of rhIL-10, with fold reductions of 67.8 (IL-1 $\beta$ ), 36.1 (IL-6), 8.5 (IL-8) and 84.8 (TNF- $\alpha$ ) (Table 1;  $P < 0.05$ ).

After incubation with the *N. meningitidis* wt, the levels of the MyD88-independent cytokines IP-10 and RANTES only showed a modest fold reduction of 1.7 and 1.1, respectively (only IP-10 was significantly decreased), in the presence of rh-IL10.

After incubation with the *N. meningitidis lpxL1* mutant in the presence of rhIL-10, IP-10 and RANTES were not significantly reduced with fold reductions of only 0.9 (IP-10) and 2.9 (RANTES).

### Discussion

*Ex vivo* incubation of *N. meningitidis* wt in citrated whole blood resulted in a dose-dependent increase of the MyD88-dependent cytokines IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$ . In contrast, only the highest concentration of the *N. meningitidis lpxL1* mutant ( $10^8$ /ml) was able to significantly increase the plasma levels of the MyD88-dependent cytokines. At this concentration, the *lpxL1* mutant induced the same IL-8 level as found for the wt, but for IL-1 $\beta$ , IL-6 and TNF- $\alpha$  the levels were significantly lower. These data are in accordance with previous publications where the *N. meningitidis lpxL1* mutant bacterium has been shown to have a reduced capacity to induce TLR-4 mediated NF- $\kappa$ B activation and cytokine production in human PBMCs and cell lines as compared with the *N. meningitidis* wt.<sup>13,21</sup> Fransen et al. concluded that the discrepancy in the concentrations of MyD88-dependent cytokines induced by the wt and the *lpxL1* mutant was only present at a low concentration of bacteria.<sup>13</sup> However, we found



**Table 1.** The effect of rhIL-10 on the MyD88-dependent and -independent cytokines.

	IL-1 $\beta$	IL-6	IL-8	TNF- $\alpha$	IP-10	RANTES
Vehicle (no bacteria)	<b>0</b> (0–0)	<b>0</b> (0–0)	<b>0</b> (0–0)	<b>0</b> (0–179)	<b>515</b> (274–855)	<b>773</b> (405–6943)
<i>N. meningitidis</i> wt	<b>3790</b> (2666–6654)	<b>7945</b> (6317–11,481)	<b>1545</b> (1128–2350)	<b>25,929</b> (5377–34,772)	<b>1769</b> (1152–5555)	<b>5601</b> (4615–7871)
<i>N. meningitidis</i> wt + rhIL-10 (25 ng/ml)	<b>61</b> (36–172)	<b>1011</b> (862–2029)	<b>256</b> (173–355)	<b>1060</b> (332–2773)	<b>1031</b> (617–2040)	<b>5062</b> (1388–6900)
Fold reduction	<b>62.1*</b>	<b>7.9*</b>	<b>6.0*</b>	<b>24.5*</b>	<b>1.7*</b>	<b>1.1<sup>ns</sup></b>
<i>N. meningitidis lpxL1</i> mutant	<b>407</b> (245–2015)	<b>3214</b> (1947–8956)	<b>1015</b> (853–3053)	<b>5766</b> (1155–15,816)	<b>583</b> (482–1169)	<b>3443</b> (776–7576)
<i>N. meningitidis lpxL1</i> mutant + rhIL-10 (25 ng/ml)	<b>6</b> (0–30)	<b>89</b> (37–513)	<b>119</b> (63–246)	<b>68</b> (23–567)	<b>663</b> (388–1425)	<b>1171</b> (1057–6718)
Fold reduction	<b>67.8*</b>	<b>36.1*</b>	<b>8.5*</b>	<b>84.8*</b>	<b>0.9<sup>ns</sup></b>	<b>2.9<sup>ns</sup></b>

Whole blood from six donors was incubated for 4 h with *N. meningitidis* wt ( $10^8$ /ml), *N. meningitidis lpxL1* mutant ( $10^8$ /ml) or vehicle, in the absence or presence of recombinant IL-10 (rhIL-10), plasma collected, and measurements of cytokines performed. The concentrations [median (range)] (pg/ml) of the cytokines, and fold reduction between  $\pm$  rhIL-10 are shown. \*Indicates significant differences in cytokine levels with and without rhIL-10 in whole blood incubated with either the *N. meningitidis* wt, or the *N. meningitidis lpxL1* mutant ( $P < 0.05$ ).

ns: non-significant differences.

that in whole blood, the *lpxL1* mutant was a weaker inducer of all cytokines even at high concentrations (except for IL-8). Whole blood contains soluble factors that may influence cytokine release,<sup>24</sup> and we do not know if the observed discrepancy in the levels of MyD88-dependent cytokines between the wt and the *lpxL1* mutant would have disappeared at higher bacteria concentrations than  $10^8$ /ml. However, concentrations of  $10^6$ /ml– $10^8$ /ml for the *N. meningitidis* wt or the *lpxL1* mutant are clinically relevant concentrations found in patients with meningococcal sepsis.<sup>27,31</sup>

We observed that both the extracellular and intracellular level of IL-8 was similar after addition of  $10^8$ /ml *N. meningitidis* wt and  $10^8$ /ml *N. meningitidis lpxL1* mutant (but not for  $10^7$ /ml bacteria). It is interesting that the monocytes were able to express IL-8, an important signalling molecule for recruitment of other immune cells, in a less LPS-dependent manner. In a lepirudin-anticoagulated human whole-blood model, Hellerud et al. compared the levels of IL-8 after incubation (for 2 h) with a *N. meningitidis* wt or a *lpxA*-mutant (LPS deficient),<sup>32</sup> and in agreement with our study they also found similar IL-8 levels at high bacterial concentrations ( $10^8$ /ml). The similar levels of IL-8 in plasma after stimulation with either the *N. meningitidis* wt or the *N. meningitidis lpxL1* mutant may suggest that other components than LPS may favour the expression of IL-8, and less for IL-1 $\beta$ , IL-6 and TNF- $\alpha$ . Hellerud et al. suggested that the production of IL-8 is more dependent on complement than IL-6 and TNF- $\alpha$ .<sup>32,33</sup> To study complement activation, lepirudin is the preferred anticoagulant in the tube. The use of citrated whole blood in our study is not optimal for activation of complement, but we cannot exclude that also in our

study the IL-8 expression was more dependent on the activation of complement than of LPS.

Mollnes et al. have systematically examined to what extent time and temperature influence the degree of *in vitro* complement activation in plasma anticoagulated with heparin, citrate and EDTA.<sup>34</sup> The authors showed that EDTA inhibited C3 and terminal complement activation in plasma more efficiently than citrate and heparin, but increasing the temperature to 37°C *in vitro* overcame inhibitory effects present in EDTA plasma but even more in citrated plasma. We incubated citrated whole blood for 4 h at 37°C, which therefore are conditions that probably will allow activation of the complement system to some degree.

To our knowledge, measurements of the MyD88-independent cytokines (IP-10 and RANTES) have not previously been performed in human PBMCs, cell lines or whole blood after incubation with the *N. meningitidis lpxL1* mutant. However, after incubation with purified penta-acylated lipid A, extracted from the *N. meningitidis lpxL1* mutant, in concentrations ranging from 0.1 pg/ml to 1 ng/ml, Pupo et al. did not find an increase in IP-10 in the culture supernatant obtained from a human monocyte macrophage cell line (MM6).<sup>20</sup> In accordance with these results, we found that the *N. meningitidis lpxL1* mutant with penta-acylated lipid A did not significantly induce the MyD88-independent cytokines IP-10 and RANTES, not even after incubation with the highest concentration ( $10^8$ /ml).

In the present study we found that the monocytes were the main producers of IL-6, IL-8 and TNF- $\alpha$  in whole blood, and incubation with the *N. meningitidis* wt gave significantly higher intracellular levels of IL-6 and TNF- $\alpha$  (but not IL-8) than incubation with the *N. meningitidis lpxL1* mutant. Our findings that the

monocyte is a key orchestrator of LPS responsiveness are in accordance with a study by Sabroe et al.<sup>35</sup> The authors examined the expression of TLR2, TLR4 and CD14, and found that the monocytes expressed relatively high levels of cell surface TLR2, TLR4 and CD14, whereas neutrophils expressed all three molecules at low levels.<sup>35</sup> A quantitative cell-surface CD14 antigen study by Antal-Szalmast et al. showed that the neutrophils expressed 30 times fewer CD14 copies on the surface compared with the monocytes.<sup>36</sup> CD14 plays a crucial part in introducing the LPS molecules to the TLR4/MD-2 complex and it is possible that a different expression of CD14 on the surface of monocytes and neutrophils may explain the different concentrations of intracellular IL-6 and TNF- $\alpha$ , and possibly for IL-8.

In our study, we observed a great donor to donor variation in the levels of all the pro-inflammatory cytokines released in whole blood stimulated with either the *N. meningitidis* wt or the *N. meningitidis lpxL1* mutant. This phenomenon has been described by others,<sup>37</sup> and the variation may reflect the individual biological diversity in response to bacterial invasion. Netea et al. have proposed that patients may develop systemic meningococcal septicaemia due to innate moderate production of pro-inflammatory cytokines (low responder) that allows uncontrolled growth of bacteria.<sup>38</sup> This is supported by Westendorp et al. who showed that individuals with a low production of TNF- $\alpha$  and a high production of IL-10 were at risk of developing fulminant meningococcal septicaemia because they were more susceptible to the development of a large bacterial burden.<sup>39</sup>

In our study, the presence of rhIL-10 significantly reduced the concentrations of the MyD88-dependent cytokines (IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$ ) when whole blood was either stimulated with the *N. meningitidis* wt or the *lpxL1* mutant. However, the presence of rh-IL10 did almost not affect the cytokine levels of the MyD88-independent pathway (IP-10 and RANTES). Our results in whole blood are in accordance with Chang et al.,<sup>40</sup> who demonstrated that IL-10 inhibited the MyD88-dependent signalling mechanisms and cytokine release in dendritic cells. They also showed that IL-10 did not exert any inhibition on the MyD88-independent signalling pathway proteins.<sup>40</sup> IL-10 is identified as the single most important plasma component suppressing the ability of monocytes to produce pro-inflammatory cytokines and to induce monocyte-associated pro-coagulant activity.<sup>9</sup> However, it is clear that despite elevated levels of IL-10, sepsis often progress rapidly leading to multiple organ failure and death within 24 h. Evidently, the high anti-inflammatory level in sepsis patients is not sufficient to prevent uncontrolled pro-inflammatory activation. In our study, the median plasma levels of IL-10 after incubation with *N. meningitidis* wt ( $10^7$ /ml) for 12 h was moderate, with a median (range) concentration of 0.642 (0.144–0.784) ng/ml.

This is in strong contrast to Lehmann et al.,<sup>30</sup> who observed 15 patients with meningococcal shock where the mean (range) level of IL-10 was much higher; 21.2 (0.25–64.5) ng/ml. The modest levels of IL-10 reached in our whole-blood model, may suggest that circulating IL-10 in meningococcal patients may derive from sources other than WBCs, such as lung, liver and spleen tissues.<sup>41,42</sup>

Taken together, in this study we evaluated the pro-inflammatory response of the *N. meningitidis* wt and the *lpxL1* mutant, with and without the presence of rhIL-10, in an *ex vivo* citrated whole blood model, i.e. an experimental model closer to the *in vivo* environment than the *in vitro* incubation of isolated cells.<sup>23,24</sup>

We demonstrated that after incubation of whole blood with low doses of the *N. meningitidis lpxL1* mutant, the induction of both the MyD88-dependent and -independent pro-inflammatory cytokines were reduced or absent. Higher concentrations of the *N. meningitidis lpxL1* mutant were required to obtain significant levels of the MyD88-dependent cytokines; IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$ , but the MyD88-independent cytokines IP-10 and RANTES were not significantly increased.

We also showed that co-incubation of rhIL-10 with *N. meningitidis* with hexa- or penta-acylated LPS lipid A reduced the MyD88-dependent cytokines, but the MyD88-independent cytokine release was rather unaffected. In conclusion, the *ex vivo* whole-blood cytokine release assay contributes to a better understanding of the pro-inflammatory responses exerted by hexa- and penta-acylated LPS components of the *N. meningitidis* bacteria, and also the major regulatory role of IL-10.

### Declaration of Conflicting Interests

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