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# Neisseria meningitidis can induce pro-inflammatory cytokine production via pathways independent from CD14 and toll-like receptor 4



## Abstract

Fulminant meningococcal sepsis (FMS) is considered the prototypical Gram-negative sepsis. Lipopolysaccharide (LPS) is thought to be the main toxic element that induces pro-inflammatory cytokine production after interaction with CD14 and toll-like receptor 4 (TLR4). However, there is increasing evidence that LPS is not the sole toxic element of meningococci. The aim of the present

study was to determine the role of CD14 and TLR4 in pro-inflammatory cytokine induction by meningococci. To this end, cytokine induction by isolated meningococcal LPS, wild-type *N. meningitidis* H44/76 (LPS+-meningococci) matched for concentrations of LPS and LPS-deficient *N. meningitidis* H44/76lpxA (LPS--meningococci) was studied in human PBMCs and murine peritoneal macrophages (PMs). Pre-incubation of PBMCs with WT14, a monoclonal antibody against CD14, abolished TNF- $\alpha$  and IL-1 $\beta$  induction by *E. coli* LPS, while cytokine induction by meningococcal LPS was only partially inhibited. When LPS+- and LPS--meningococci at higher concentrations were used as stimuli, anti-CD14 had a minimal effect. In C3H/HeJ murine PMs, devoid of a functional TLR4, minimal IL-1 $\alpha$ , IL-6 and TNF- $\alpha$  production was seen after stimulation with 10 ng/mL *E. coli* or meningococcal LPS. However, at higher concentrations (1000 ng LPS/mL) the production of TNF- $\alpha$ , but not IL-1 $\alpha$  or IL-6, occurred also independently of TLR4. The expression of a functional TLR4 in murine PMs had no effect on the cytokine induction by LPS+- or LPS--meningococci. It is concluded that pro-inflammatory cytokine induction by *N. meningitidis* can occur independently of CD14 and TLR4.

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## Full text

### Introduction

Invasive infections with the Gram-negative bacterium *Neisseria meningitidis* are an important cause of morbidity and mortality worldwide [1]. Particularly fulminant meningococcal sepsis (FMS), considered to be the prototypical Gram-negative septic shock, is feared for its high fatality rate. Lipopolysaccharide (LPS, endotoxin), an important structural and functional component of the outer membrane of Gram-negative bacteria, is thought to be responsible for the major pathophysiological events during meningococcal septicaemia [2]. LPS induces the synthesis of interleukin-1 $\beta$  (IL-1 $\beta$ ) and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), pro-inflammatory cytokines pivotal in the development of septic shock [3, 4].

Recently, the principal pattern-recognition receptors for LPS have been characterised more precisely. After the interaction of LPS with LPS-binding protein (LBP), LPS is shuttled to CD14, a glycosylphosphatidylinositol (GPI) - linked cell-surface glycoprotein present on cells of the myeloid lineage [5, 6]. As CD14 lacks a transmembrane domain, association with the toll-like receptor 4 (TLR4) and its co-factor MD-2 is required for transmembrane signalling [7-9].

TLR4 imparts ligand specific recognition of LPS and binding of LPS induces physical proximity between CD14 and TLR4 prior to nuclear translocation of NF- $\kappa$ B [10, 11]. In addition to its role in the recognition of LPS, CD14 has also been shown to mediate responses after stimulation with other bacterial components [12].

In spite of the importance of LPS in the pathogenesis of FMS, there is convincing evidence that LPS is not the sole toxic element. Anti-LPS strategies have failed to ameliorate the clinical course of FMS [13-16], and it was recently shown in various laboratories that a mutant strain of *N. meningitidis* devoid of LPS is able to induce cytokine production [17-19]. Thus, bacterial components other than LPS, i.e. non-LPS components, may have an important role in the pathogenesis of FMS.

The aim of the present study was to determine the role of CD14 and TLR4 in pro-inflammatory cytokine induction by the meningococcus.

## Materials and methods

### *Neisseria meningitidis* strains

The wild-type serogroup B *N. meningitidis* H44/76 strain (designated as LPS<sup>+</sup> *N. meningitidis*) was isolated from a patient with invasive meningococcal disease [20]. The meningococcal strain H44/76/*lpxA* (designated as LPS<sup>-</sup> *N. meningitidis*) is a viable isogenic mutant, completely devoid of LPS in the outer membrane. This mutant was constructed by insertional inactivation of the *lpxA* gene, essential for the first committed step of the biosynthesis of LPS. The absence of LPS in this strain was confirmed as described previously [21]. Absence of LPS-activity in the LPS<sup>-</sup> *N. meningitidis* batch-suspension was assessed by non-reactivity in the Limulus amoebocyte lysate assay.

Bacteria were grown overnight in liquid medium, heat-inactivated (1 h, 56 °C) and washed in phosphate buffered saline (PBS). Bacteria were quantified by spectrophotometry.

### Lipopolysaccharides (LPS)

A commercially available TCA extracted *E. coli* 055:B5 LPS was purchased from Sigma-Aldrich Co (Rutherford, NY). Meningococcal LPS was isolated in our laboratory by phenol/water extraction according to Westphal and Jann [22]. After extraction, this meningococcal LPS was treated with Proteinase K (Sigma-Aldrich Co), DNA-ase and RNA-ase (Roche diagnostics) for additional purification, recovered by ultracentrifugation, freeze-dried and solved in sterile PBS.

The commercial *E. coli* LPS preparation was found to be contaminated with protein (8.5%) (by Lowry-assay) and nucleic acids (6.1%) (by spectrophotometry). The *N. meningitidis* LPS preparation was free of protein (< 1.0%), nucleic acid content was (4.4%).

Quantification of LPS in *N. meningitidis* H44/76 bacteria was performed by TSDS-PAGE followed by silverstaining of LPS and assay for 2-keto-3-deoxyoctanate as described previously [17]; 1 ng of LPS correlated with  $0.7 \times 10^6$  LPS<sup>+</sup> *N. meningitidis* H44/76 bacteria.

### Anti-CD14 antibody

The monoclonal anti-CD14 antibody WT14 was produced as described previously [23]. Binding of LPS to human macrophages was determined by flow cytometry using fluorescein isothiocyanate (FITC) labelled *E. coli* 055:B5 LPS. FITC labeling of LPS was performed as described by Pollack *et al.* [24]. Purified human monocytes were preincubated with control antibody (which did not bind monocytes), WT-14 antibody or an excess of unlabelled LPS. The cells were washed and FITC-labelled LPS was allowed to bind the monocytes in the presence of 10% pooled human serum (as a source of LPS-binding protein). Monocytes were identified based on size and granularity. In stimulation experiments with PBMCs, WT14 alone did not induce cytokines.

### Mice

Murine C57Bl/6J peritoneal macrophages (PMs) were used to study cytokine induction by meningococcal LPS and LPS<sup>+</sup>- or LPS<sup>-</sup>-meningococci. The role of TLR4 in cytokine induction by meningococcal LPS and non-LPS components was assessed in PMs isolated from C3H/HeJ or C3H/HeN mice. The mice were obtained from the Jackson Laboratories (Bar Harbour, ME, USA) and were bred in

our local facility; 6 to 8 weeks old mice, weighing 20 to 25 grams were used. The animals were fed standard laboratory food (Hope Farms, Woerden, the Netherlands) and housed under specific pathogen-free conditions. C3H/HeJ mice have a non-functional TLR4, owing to a base-transversion in the gene coding for TLR4. The parent strain C3H/HeN possesses the normal functional TLR4.

## Human and murine cells; culture conditions

Blood for the isolation of human peripheral blood mononuclear cells (PBMCs) was drawn in 10-mL EDTA anti-coagulated tubes (Vacutainer System, Beckton Dickinson, Rutherford, NJ) from healthy volunteers. PBMCs were isolated after density gradient centrifugation on Ficoll-Hypaque (Pharmacia Biotech AB). The cells from the interphase were aspirated and washed 3 times in sterile PBS.  $5 \times 10^5$  cells per well were incubated in 200  $\mu$ L 96 wells plates (Greiner BV, Alphen a/d Rijn, the Netherlands) with the various stimuli for 24 hours at 37 °C and 5% CO<sub>2</sub>. The anti-CD14 antibody WT14 was preincubated (20 mg/mL) with the PBMCs for 2 hours at 37 °C and 5% CO<sub>2</sub>.

Resident peritoneal macrophages (mPMs) of C3H/HeN or C3H/HeJ mice were harvested by rinsing the peritoneal cavity aseptically with sterile PBS (4 °C).  $1 \times 10^5$  cells per well were incubated with the various stimuli. Culture conditions were similar to those for human PBMCs.

RPML 1640 (Dutch modification, Flows Lab, Irvine, Scotland) supplemented with L-glutamine (2 mMol), pyruvate (1 mMol) and gentamycin (50 mg/mL) was used as culture medium. For experiments with human PBMCs, 5% fresh pooled human serum obtained from 5 healthy volunteers was added.

## Cytokine assays

Human IL-1 $\beta$  and TNF- $\alpha$  were determined by radioimmunoassay (RIA) as described previously [25]. The lower limit of detection was 0.08 ng/mL. Murine IL-1 $\alpha$  (mIL-1 $\alpha$ ) and mTNF- $\alpha$  were determined by RIA as described [26]. mIL-6 was determined by ELISA (Pelikine Compact murine IL-6 ELISA kit, Central laboratory of the Netherlands red cross). The lower limit of detection was 0.04 ng/mL.

## Calculations and statistics

For calculations and graphs, the net cytokine production, i.e. the cytokine concentration in the stimulated sample minus the concentration in the unstimulated sample, was used. Median cytokine production was used to calculate the % decrease in cytokine production. Data were analysed using the Mann-Whitney test for unpaired, non-parametrical data (GraphPad Prism, GraphPad software Inc.); a  $p$ -value  $< .05$  was considered significant.

## Results

### Cytokine production after stimulation with meningococcal LPS, LPS<sup>+</sup>- and LPS<sup>-</sup> - Meningococci

Cytokine production by human PBMCs and murine C57Bl/6J PMs was studied using a low or a high concentration of meningococcal LPS (6 or 600 ng/mL for human PBMCs and 10 or 1 000 ng/mL for murine PMs) and matched concentrations of LPS-containing (LPS<sup>+</sup>) or LPS-deficient (LPS<sup>-</sup>) meningococci. Figure 1 shows the results for IL-1b in human PBMCs. At these concentrations, LPS<sup>+</sup>-meningococci induced 3 - 4 fold more IL-1b than equivalent amounts of purified meningococcal LPS. The LPS<sup>-</sup>-meningococci induced sizeable amounts of pro-inflammatory cytokines, however less than the wild-type strain. TNF- $\alpha$  production followed a similar pattern (data not shown). In murine PMs, the pattern of the production of IL-1a, TNF- $\alpha$  and IL-6 after stimulation with the three different stimuli was similar to that observed in human PBMCs for IL-1b and TNF- $\alpha$ , although a higher concentration of stimulus was needed to obtain measurable cytokine production.

### Effect of anti-CD14 on the binding of *E. coli* LPS to monocytes and cytokine induction by *E. coli* LPS

Flow-cytometric (FACS) analysis of the binding of FITC-labeled *E. coli* LPS to human monocytes showed that the anti-CD14 antibody WT14 completely abrogated the binding of *E. coli* LPS to monocytes (Figure 2). As expected, anti-CD14 inhibited the *E. coli* LPS (10 ng/mL) induced IL-1b and TNF $\alpha$  production with  $> 95\%$  (Figure 3).

## Effect of anti-CD14 on cytokine induction by meningococcal LPS, LPS<sup>+</sup>- and LPS<sup>-</sup>-meningococci

Figure 4 shows the effect of anti-CD14 on the IL-1b and TNF-a production by human PBMCs after stimulation with isolated meningococcal LPS, LPS<sup>+</sup>-meningococci and LPS<sup>-</sup>-meningococci. Meningococcal LPS and both bacterial strains were used at matched concentrations as described above. Anti-CD14 showed a trend towards inhibition of IL-1b and TNFa production at both low and high concentrations of meningococcal LPS. However, in contrast to *E. coli* LPS (Figure 3), inhibition was incomplete (64% for IL-1b and 45% for TNFa) and did not reach statistical significance. After stimulation with the high concentration of meningococcal LPS (600 ng/mL), the production of IL-1b and TNF-a was even less inhibited (53% and 24%, respectively).

IL-1b and TNF-a production after stimulation with the low concentration ( $6 \times 10^6$ /mL) of LPS<sup>+</sup>-meningococci was inhibited by anti-CD14 (78% for IL-1b and 61% for TNF-a,  $p < .05$ ). After stimulation with LPS<sup>-</sup>-meningococci this reduction was 55% for both cytokines ( $p = .08$  and  $.03$  for IL-1b and TNF-a, respectively). At the high concentration of both bacterial strains ( $6 \times 10^8$  meningococci/mL), no significant inhibition of cytokine production occurred, however, an inhibiting trend on TNF-a production was present.

## Cytokine induction by meningococcal LPS, LPS<sup>+</sup>-meningococci and LPS<sup>-</sup>-meningococci in C3H/HeJ and C3H/HeN mice

As a reference, PMs of C3H/HeN and C3H/HeJ mice were stimulated with *E. coli* LPS (10 ng/mL). Figure 5 shows the results for IL-1a and TNF-a. In accordance with previous studies, production of IL-1a and TNF-a by C3H/HeJ PMs was reduced to values approximately equivalent to the blank. The IL-6 production was similarly affected (data not shown).

Figure 6 shows the IL-1a and TNF-a production by mPMs of C3H/HeN and C3H/HeJ mice after stimulation with meningococcal LPS, or matched concentrations of LPS<sup>+</sup>- or LPS<sup>-</sup>-meningococci. Similar to the pattern for *E. coli* LPS, minimal IL-1a production occurred in C3H/HeJ mPMs after stimulation with the low or high dose of meningococcal LPS. The IL-6 production was similarly affected (data not shown). However, TNF-a production was only affected after stimulation with the low dose (10 ng/mL) of meningococcal LPS,

while after stimulation with the high dose (1 000 ng/mL) only 18% reduction occurred as compared with the production in C3H/HeN PMs.

LPS<sup>+</sup>-meningococci in low concentrations induced 58% less IL-1a in C3H/HeJ mice as compared to C3H/HeN mice ( $p = .08$ ). IL-6 showed a similar pattern (data not shown). At high concentrations however, virtually equal amounts of IL-1a and IL-6 were produced by C3H/HeJ and C3H/HeN mPMs. Remarkably, TNF- $\alpha$  production was not affected in C3H/HeJ mice.

LPS<sup>-</sup>-meningococci induced equal amounts of IL-1a, IL-6 and TNF- $\alpha$  in murine PMs of C3H/HeJ and C3H/HeN mice.

## Discussion

In the present study we demonstrated that *N. meningitidis* can induce cytokines in human PBMCs or murine peritoneal macrophages by pathways independent from CD14 and TLR4.

The finding that anti-CD14 did not completely inhibit the production of pro-inflammatory cytokines after stimulation with meningococcal LPS is in contrast with the findings for *E. coli* LPS. In accordance with previous studies we found that IL-1b and TNF- $\alpha$  induction by *E. coli* LPS is completely CD14-dependent [6]. Because our meningococcal LPS preparation appeared to be less contaminated than the commercially purchased *E. coli* LPS, the observed CD-14 independent cytokine production in experiments with meningococcal LPS is rather explained by the specific molecular characteristics of meningococcal LPS than contaminants. Indeed, recently Gangloff *et al.* showed that LPS molecules with a short polysaccharide tail that lacks the typical O antigen, such as meningococcal LPS, may engage CD14-independent pathways for the induction of TNF- $\alpha$  [27].

CD14 has been shown to bind a range of microbial components other than LPS such as peptidoglycan, lipoproteins, lipoteichoic acid, lipoarabinomannan and various other components [12, 28]. Gram-negative bacteria contain some of these non-LPS components (peptidoglycan, lipoproteins) which are proficient to engage CD14. Recently, Ingalls *et al.* identified CD14 as a principal ligand for cytokine induction by LPS-deficient meningococci [19]. Our finding that, after stimulation with the low dose ( $6 \times 10^6$  bacteria/mL) LPS-free meningococci, anti-

CD14 considerably (55%) inhibited IL-1 $\beta$  and TNF $\alpha$  production is in accordance with this role for CD14. However, as inhibition was incomplete, the non-LPS components of *N. meningitidis* may engage also CD14-independent pathways. At higher concentrations ( $6 \times 10^8$  bacteria/mL), we found that pro-inflammatory cytokine production was completely CD14-independent. We presume that at these concentrations, non-specific receptors like the  $\beta_2$ -integrins or scavenger receptors are engaged [29, 30].

TLR4 has been shown to confer ligand-specific recognition of LPS. However, there is recent evidence that TLR2, and not TLR4, is involved in recognition of LPS isolated from *Porphyromonas gingivalis* or *Leptospira spp.* Thus, probably various types of LPS may interact differentially with TLRs [31, 32]. In the present study we showed that in mice, TLR4 is the principal signal transferring receptor for meningococcal LPS at lower concentrations (10 ng/mL). At higher concentrations (1 000 ng/mL) however, TNF $\alpha$  - but not IL-1 $\alpha$  or IL-6 - was also produced independently of TLR4. Hirschfeld *et al.* [31] reported that engagement of TLR4 and TLR2 will result in the differential expression of cytokine genes. Combined with our recently published suggestion that the molecular conformation of the lipid-A moiety in meningococcal LPS, with six symmetrically distributed fatty-acids, favors the engagement of TLR2 [33], we speculate that the TLR4 independent induction of TNF $\alpha$  by meningococcal LPS, is mediated by TLR2.

Our experiments with LPS-free and LPS-containing meningococci indicate that TLR4 is not exclusively engaged in cytokine induction by the Gram-negative bacterium *N. meningitidis*. Using TLR4-nonfunctional C3H/HeJ mPMs, IL-1 $\alpha$  production after stimulation with the wild-type LPS<sup>+</sup>-meningococci was only moderately decreased compared to the parent C3H/HeN murine PMs, and TNF $\alpha$  production was not decreased at all after stimulation with low dosages of LPS<sup>+</sup>-meningococci. As cytokine production after stimulation with LPS<sup>-</sup>-meningococci in C3H/HeJ mPMs was not affected, it is likely that the signaling *via* non-TLR4 pathways by LPS<sup>+</sup> meningococci occurs by non-LPS components of the meningococcus. This is underscored by the finding of Massari *et al.*, who found that isolated outer membrane porins of the meningococcus can signal *via* TLR2 [34].

It should be noted that our data on the role of TLR4 in proinflammatory cytokine induction by *N. meningitidis* are derived from experiments with murine peritoneal

macrophages. However, recent studies with cells transfected with human receptors, *i.e.* chinese hamster ovary fibroblasts transfected with human TLR4 and TLR2 [19] or with HeLa cells transfected with human TLR2 [35], suggest that for the response to meningococci non-TLR4 pathways are also of importance in the human situation.

Recently, we and others provided evidence for a role of non-LPS components of *Neisseria meningitidis* in cytokine induction and therefore in the pathogenesis of FMS [17-19]. In the present study we show that pro-inflammatory cytokine induction by *N. meningitidis* can occur independently of CD14 and TLR4.

Therefore, it can be speculated that therapeutic strategies designed to block either LPS, CD14 or TLR4 are likely to be insufficient for the complete abrogation of the harmful pro-inflammatory cytokine response during FMS.

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## Figures

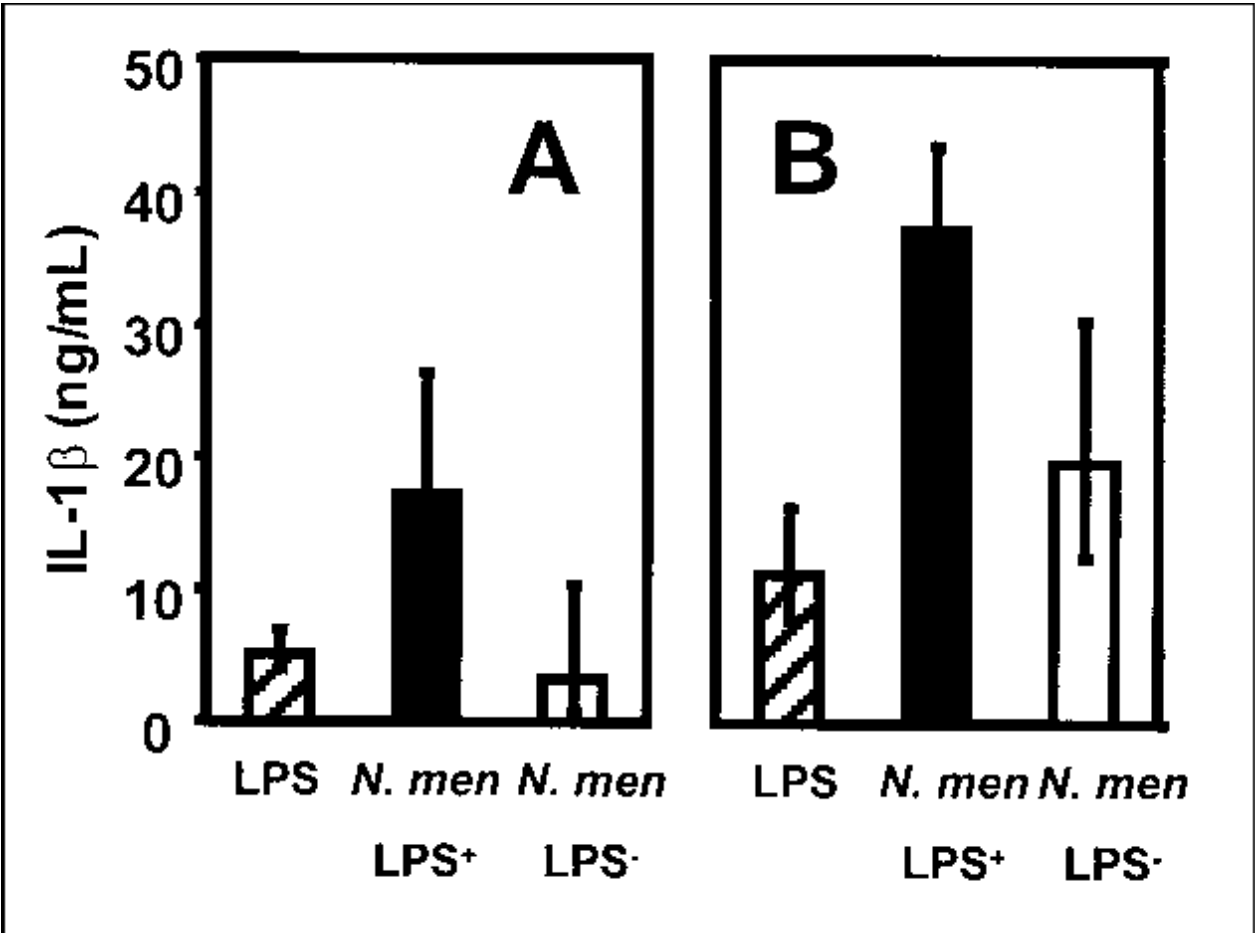


Figure 1 : IL-1 $\beta$  production by human PBMCs after stimulation with low (panel A, 6 ng/mL meningococcal LPS or 6  $\times$  10<sup>6</sup> meningococci/mL) and high dose (panel B, 600 ng/mL meningococcal LPS or 6  $\times$  10<sup>8</sup> meningococci/mL) purified meningococcal LPS (hatched bars), LPS<sup>+</sup> *N. meningitidis* H44/76 (black bars) or LPS<sup>-</sup> *N. meningitidis lpxA* (white bars). Median values are presented, error bars indicate inter-quartile range (IQR). n = 15 for the LPS experiments, n = 25 or 30 for the experiments with the low or high bacterial concentration, respectively.

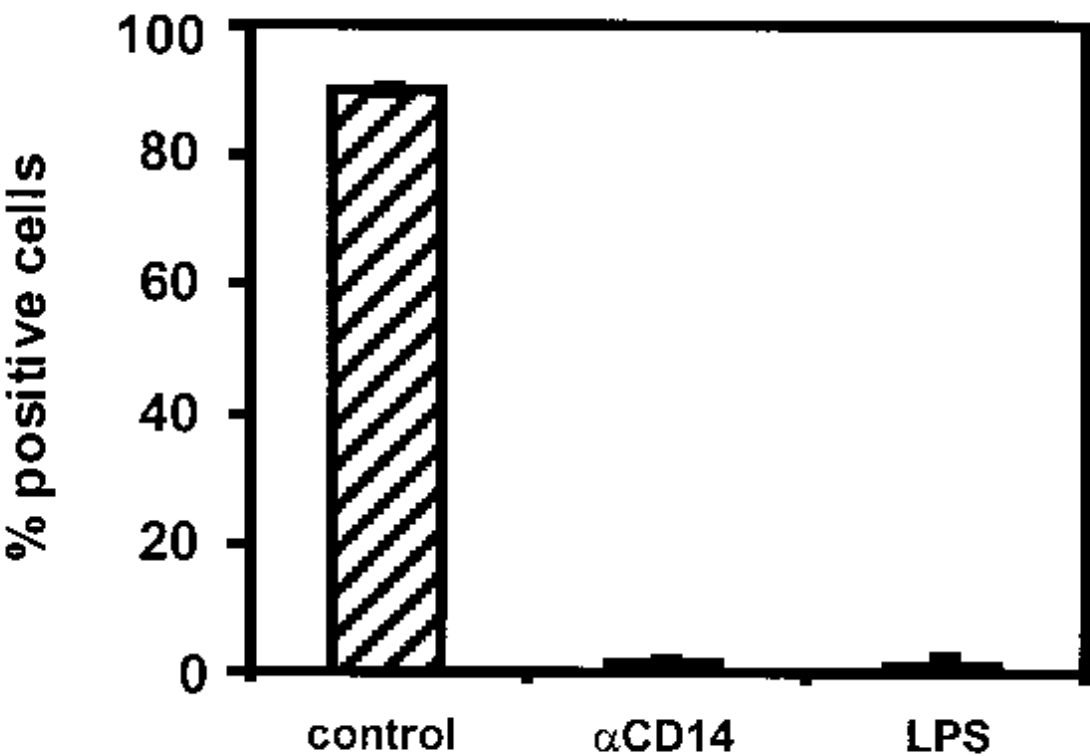


Figure 2 : Binding of FITC-labelled *E. coli*/LPS to human monocytes after preincubation with a control antibody, the anti-CD14 antibody WT14 or an excess of unlabelled LPS. Results are given as the mean percentage of positive cells + / - SD, n = 4, performed on the same batch of frozen monocytes.

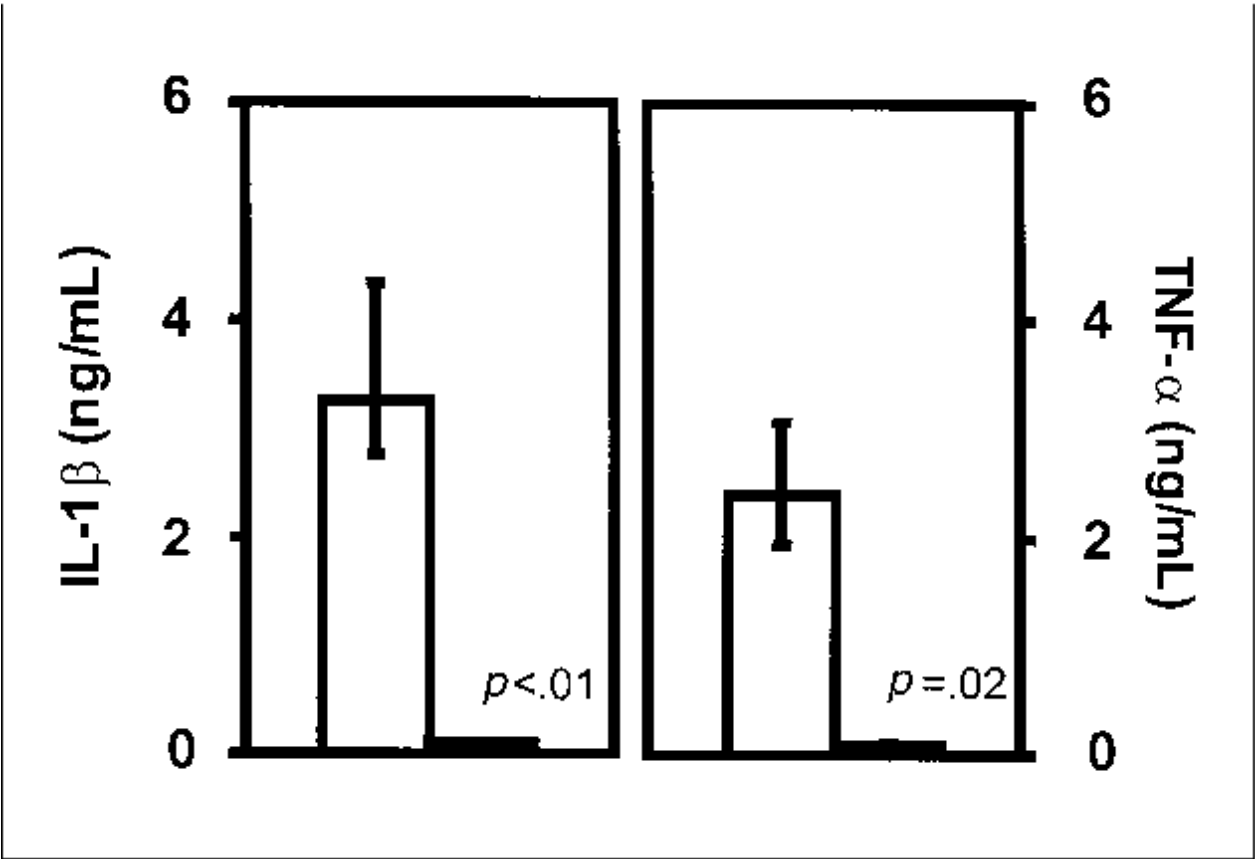


Figure 3 : IL-1b and TNF-a production by human PBMCs preincubated for 2 hours with RPMI (white bars) or 20 mg/mL anti-CD14 (hatched bars) prior to stimulation for 24 hours with 10 ng/mL *E. coli* LPS. Median values are presented + / - IQR, n = 5. *p*-values apply to the comparison between preincubation with or without anti-CD14.

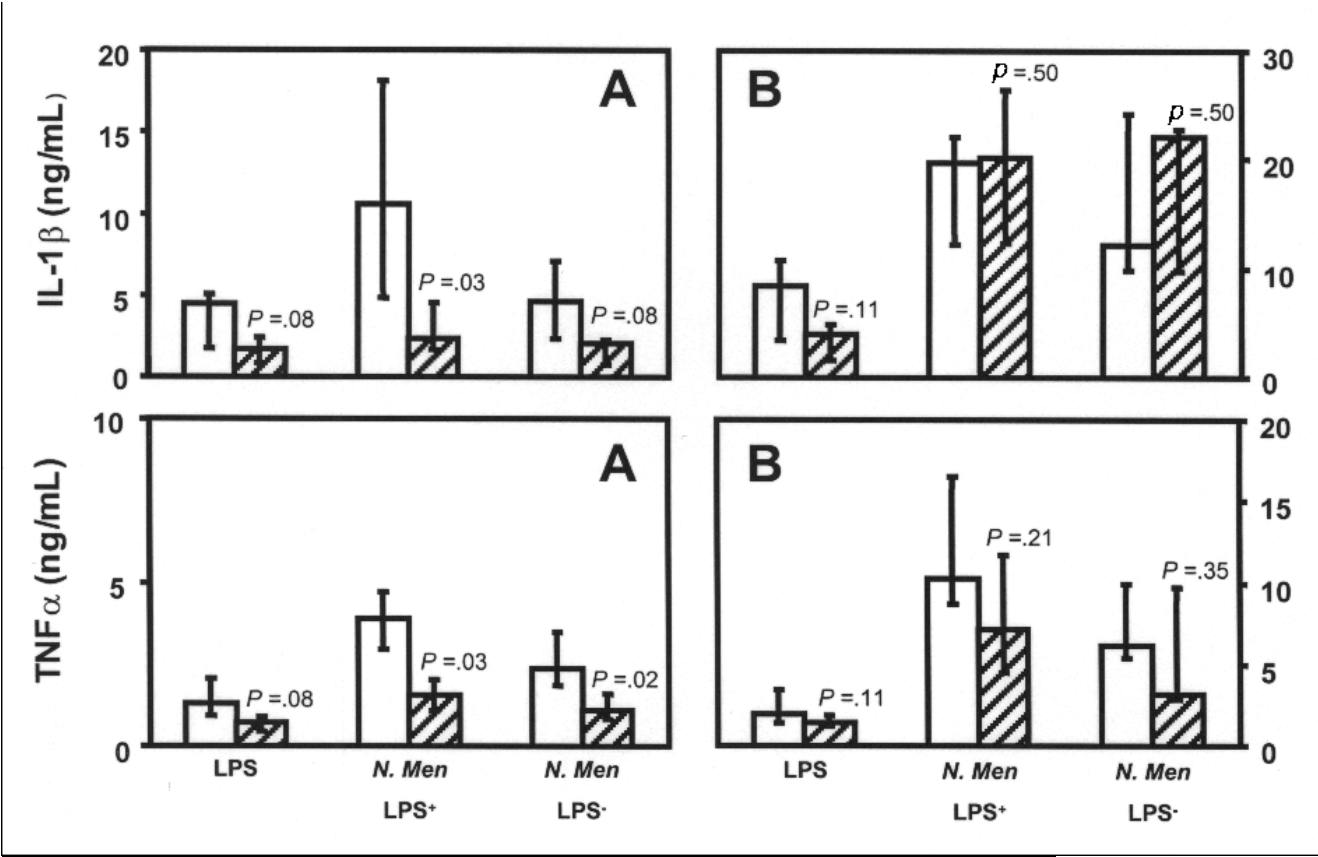
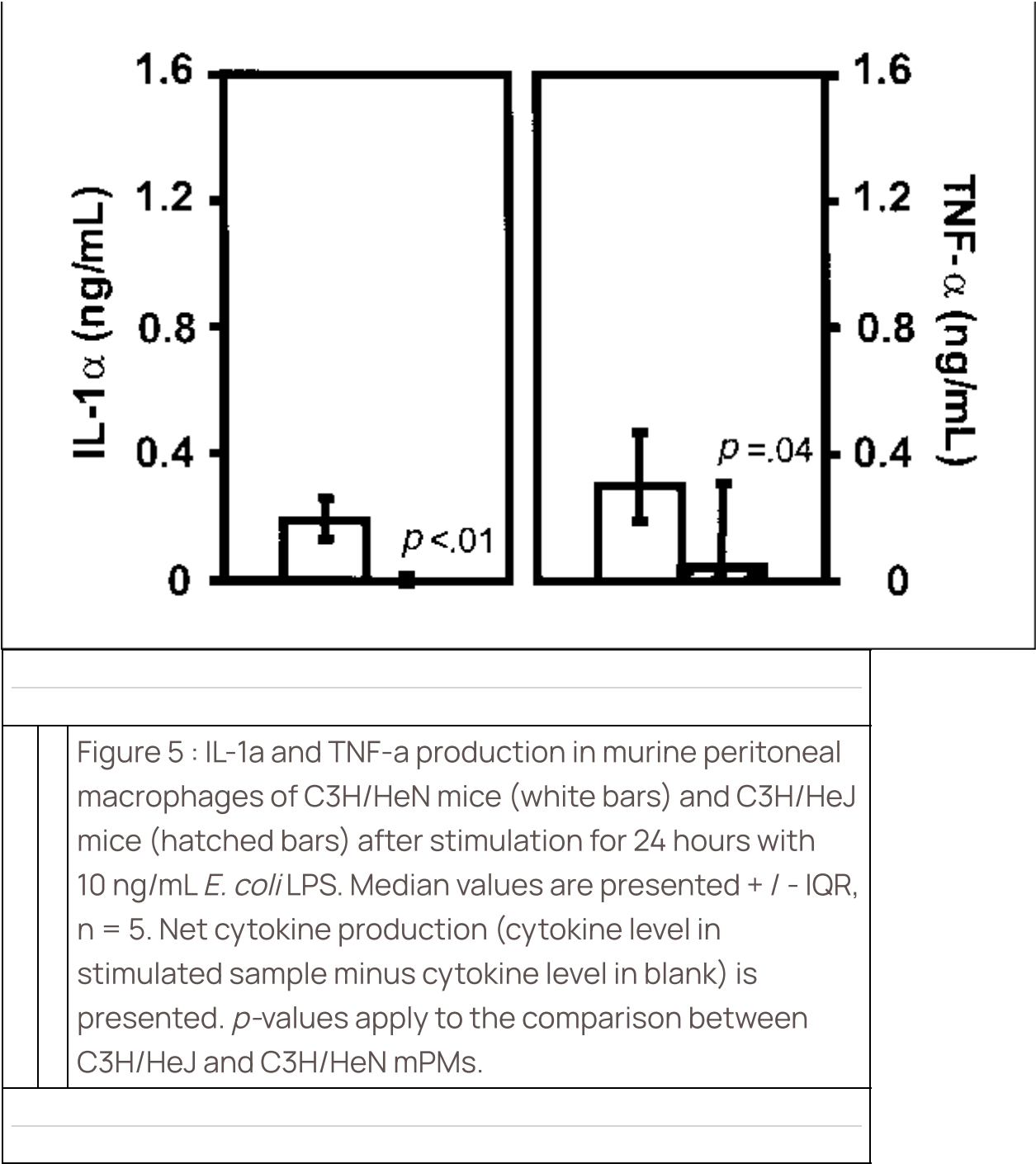


Figure 4 : IL-1b and TNF-a production in human PBMCs preincubated for 2 hours with RPMI (white bars) or 20 mg/mL anti-CD14 (hatched bars) prior to stimulation for 24 hours with meningococcal LPS, LPS<sup>+</sup> *N. meningitidis* H44/76 and LPS<sup>-</sup> *N. meningitidis* lp. Panels A: 6 ng/mL meningococcal LPS or 6 × 10<sup>6</sup> meningococci/mL, panels B: 600 ng/mL meningococcal LPS or 6 × 10<sup>8</sup> meningococci/mL. Median values are presented, + / - IQR, n = 5 for all stimuli. *p*-values apply to the comparison between preincubation with or without anti-CD14.



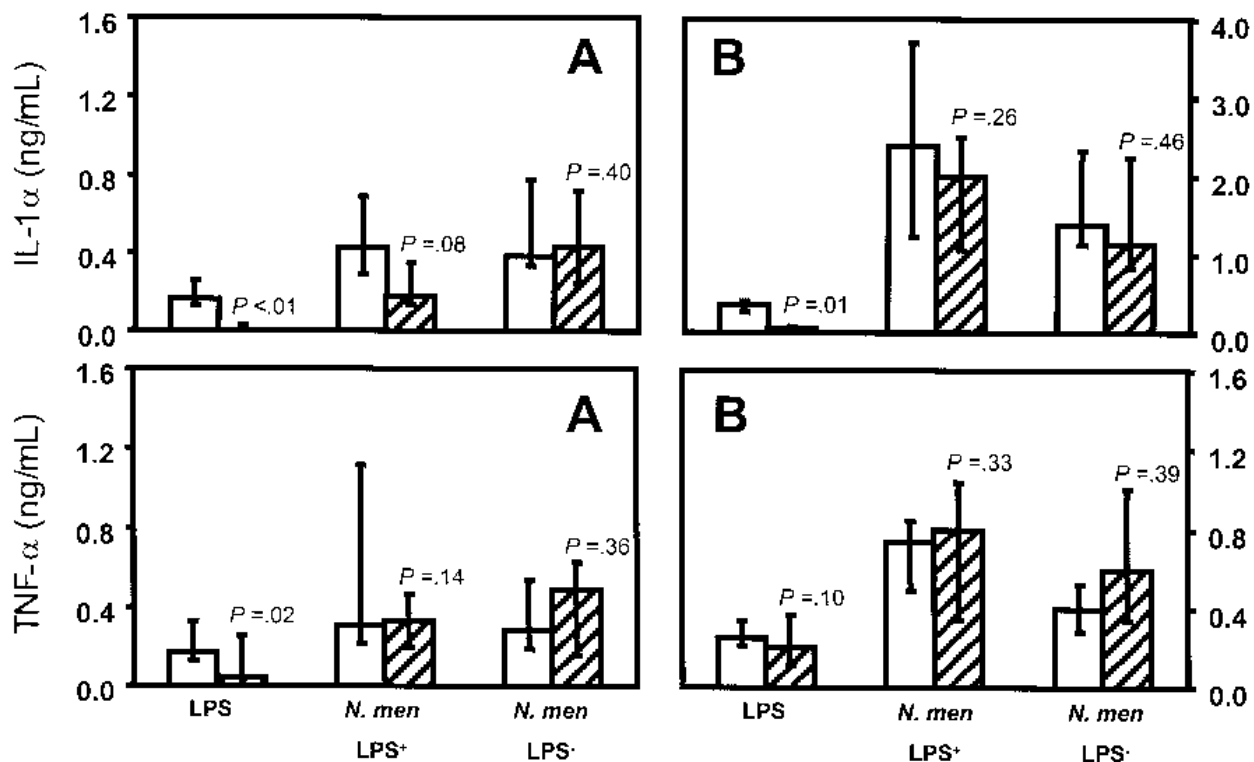


Figure 6 : IL-1a and TNF-a production in murine peritoneal macrophages of C3H/HeN mice (white bars) and C3H/HeJ mice (hatched bars) after stimulation with meningococcal LPS, LPS<sup>+</sup> *N. meningitidis* H44/76 or LPS<sup>-</sup> *N. meningitidis* lpxA. Panels A: 10 ng/mL meningococcal LPS or 10<sup>7</sup> meningococci/mL, panels B: 1 000 ng/mL meningococcal LPS or 10<sup>9</sup> meningococci/mL. Median values + / - IQR, n = 10, of net cytokine production (cytokine level in stimulated sample minus cytokine level in blank) are presented. *p*-values apply to the comparison between C3H/HeJ and C3H/HeN mPMs.

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