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Low dose LPS does not increase TLR4 expression on monocytes in a human *in vivo* model

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ABSTRACT

Background and purpose: Toll like receptor 4 (TLR4) is the major recognition receptor for lipopolysaccharides and plays a major role in the inflammatory response. CD11b is expressed on the surface of many leukocytes including monocytes. The CD11b/CD18 complex is involved in the inflammatory response by mediating migration and adhesion of leukocytes. The aim of this human *in vivo* study was to investigate the expression of TLR4 and CD11b on the surface of human monocytes after *in vivo* low-dose LPS stimulation.

Methods: We performed a double-blind, randomized crossover study with 16 healthy males who received a bolus injection of bacterial lipopolysaccharide (LPS; 0.4 ng/kg) or normal saline. Vital parameters, blood counts, serum cytokine levels, the expression of TLR4, and CD11b on CD14 positive cells were analyzed.

Results: The experimentally induced inflammatory response was reflected by transient increases in body temperature, circulating leukocyte numbers, and plasma levels of pro- (TNF-α, IL-6) and anti-inflammatory cytokines (IL-10, IL-1ra). In contrast to a significant increase in CD11b expression, no changes in TLR4 expression on circulating monocytes were detectable.

Conclusion: Early changes in TLR4 expression on circulating monocytes are not necessarily part of the inflammatory response to low dose LPS in humans whereas the detected increase of CD11b expression might already be sufficient for optimized recognition and signalling.

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1. Introduction

Bacterial infections, sepsis, and septic multiorgan failure still remain a severe problem in clinical practice despite the development of new antibiotics and intensive investigation of the pathophysiological mechanisms [1,2]. The incidence of sepsis is rising worldwide due to the increasing amount of invasive interventions and resistance to antibiotics [3,4]. In intensive care medicine, septic multiorgan failure is one of the most common causes of death [4] .

The endotoxin lipopolysaccharide (LPS) is a component of the Gram-negative cell wall. LPS is released at growth as well as during degradation of the Gram-negative cell wall. It is capable of activating several cell types including macrophages, lymphocytes, granulocytes, endothelian, and smooth muscle cells [5]. Toll-like receptors were identified as being crucial to recognize pathogen associated molecular patterns (PAMPs) and to induce downstream effects in monocytes [6]. Toll-like-receptor 4 (TLR4) is the major recognition receptor for LPS which interacts with the extracellular coreceptors CD14 and MD2 [7]. After stimulation, two known intracellular signal pathways are activated [8]: the MyD88-dependend and – independent pathway which both end in the translocation of Nuclear Factor κB (NF-κB) into the nucleus inducing transcription of multiple genes. This transcription results in a release of cytokines in a characteristical chronology [9]. If the fine-tuned network of the immune system becomes unbalanced, cytokine secretion can result in excess. The excessive production of proinflammatory cytokines is a central element of the development of septic inflammatory response.

CD11b, also known as Mac1-Integrin, is a member of the β_2-Integrin family. CD11b merges with the common subunit CD18 forming the CD11b/CD18 complex which can be found on the surface of leukocytes and act as a receptor for several

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molecules including LPS. The most important function is the bond to Inter-Cellular Adhesion Molecule-1 (ICAM-1) which results in a strong adhesion of leukocytes to the endothelium and a subsequent transmigration into the tissue [10,11].

The interaction between LPS, CD11b, and TLR4 is still insufficiently understood. For example, it remains unclear whether upregulation of TLR4 on the surface of monocytes is a necessary step for the inflammatory response. Thus, the aim of our study was to investigate the expression of TLR4 and CD11b on the surface of human monocytes after *in vivo* low-dose LPS stimulation.

2. Methods

2.1. Subjects

Sixteen healthy, non-smoking male volunteers were included in our trial. Exclusion criteria were immunological disorders, psychiatric diseases, metabolic disorders, allergies, infections during the preceding two months, liver diseases, kidney diseases, respiratory diseases, addiction history, a regular intake of medication, intake of nonsteroidal antiinflammatory drugs (NSAID) within the last 4 weeks, and participation in other clinical trials. The study was approved by the Medical Ethical Committee of the University Hospital in Essen (References Number 07-3479), and all subjects gave written informed consent.

2.2. Protocol

We established a double blinded, randomised, placebo controlled cross over study. The volunteers received an injection of 0.4 ng/kg bodyweight LPS (*E. coli*-Lipopolysaccharid 0113:H10/LPS/United States Pharmacopeia) solved in 10 ml NaCl 0.9% or the same volume of NaCl 0.9% as placebo. Thus, each volunteer was evaluated twice with a 1–3 week interval.

At noon, a peripheral intravenous catheter was placed and the first blood samples for baseline levels were drawn. Afterwards, either LPS solution or NaCl 0.9% were injected. Further blood samples were taken 1, 1.75, 3, 4, 6, and 24 h after injection. Simultaneously, heart rate, blood pressure, and body temperature were measured. Blood samples were composed of one 10 ml lithium-heparin blood container and two EDTA blood containers. Additional blood samples were taken one week and 24 h before the first trial day and 1 week after the last trial day.

2.3. Blood sampling

Plasma samples were prepared by immediate centrifugation (10 min, 4 °C, 2000 g) of peripheral blood with Eppendorf blood containers (Eppendorf AG, Germany) containing Lithium–heparin for anticoagulation. The supernatant plasma was aliquoted and frozen at −80 °C. EDTA blood was taken with Eppendorf EDTA blood containers (Eppendorf AG, Germany).

2.4. Blood count analysis

EDTA blood was used for an automatic blood count analysis (KX 21 N, Sysmex, Norderstedt, Germany). Numbers and relative distribution of leukocytes, lymphocytes, monocytes, and neutrophil granulocytes were analyzed.

2.5. Cytokine assay

Cytokine assessments for IL-1β, IL-1ra, IL-6, IL-10 and TNFα were performed using multiplexed bead-based assays (Bioplex Cytokine assays, BioRad laboratory industries, Hercules, California, USA). Plasma samples were diluted 1:4 with High performance ELISA buffer (HPE) and afterwards the samples were prepared according to the manufacturer information and were analyzed by a triple-laser FACSCanto II flow cytometer using FACSDiva software (BD Biosciences, NY, USA). The absolute cytokine levels were calculated based on the mean fluorescence intensity of cytokine standard dilutions with a 4 Parameter Logistics (4PL) curve model using GraphPad Prism 5 (GraphPad Software Inc, CA, USA). The detection limits of the assays were 0.2 pg/ml for IL-6, 0.4 pg/ml for IL-1β and IL-10, 70 pg/ml for IL-1ra, and 3.0 pg/ml for TNF-α.

2.6. Isolation of peripheral blood mononuclear cells (PBMCs)

Mononuclear cells were divided by centrifugation with a Ficoll gradient. Blood samples were taken before and 3 h after LPS administration with heparin-coated Ficoll blood containers (Vacutainer CPT 8 ml, BD, Heidelberg, Germany). Centrifugation was performed at 1650*g* for 20 min. The layer with lymphocytes and monocytes was transferred into 14 ml of PBS and centrifugated again 10 min with 450*g*. The cell pellet was dissolved again in PBS to a cell concentration of 4 to $9 \times 10^6/\mu l$.

2.7. CD11b and TLR4 assay

After washing them twice in FACS-buffer (Dulbecco's PBS supplemented with 2% fetal calf serum and 0.1% NaN_3), PBMCs were resuspended and incubated for 10 min at room temperature with FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) to avoid non-specific binding to Fc receptors. After a second wash, the cells were then incubated for 45 min at 4 °C in a respective volume of 70 μl with fluorochrome-coupled antibodies (anti-CD14-PE, anti-TLR-4-Alexa647 or respective isotype control, and anti-CD11b-FITC or respective isotype control, AbD Serotec, Düsseldorf, Germany). After a final wash, cells were resuspended in 300 μl of FACS-buffer respectively, and analyzed with a 3-laser FACS Canto II flow cytometer (Becton Dickinson, Heidelberg, Germany) using the BD FACS Diva Software. After compensation, the cells were gated by anti-CD14-PE and median flourescence signals for anti-CD11b-FITC and anti-TLR-4-Alexa647 were assessed. To avoid false positive results, the fluorescence median of control samples containing the respective isotype control instead of TLR-4 or CD11b antibody were subtracted from the antibody-signals.

2.8. Statistics

All analyses were calculated using PASW statistics 18 for Windows (IBM, USA). Repeated measurements of vital signs and blood count were analyzed by ANOVA followed by a Fisher-PLSD ANOVA post hoc-analysis. Cytokine values were converted in relative values whereas each corresponding control condition was set at 0. Relative values were analyzed by Friedman test followed by Wilcoxon signed-rank test comparing the different time points to 0 h. Significance was defined as $p < 0.05$.

3. Results

3.1. Vital signs

Heart rate was significantly increased between 1.75 and 4 h after LPS injection and returned to normal values after 6 h ($p < 0.001$; Fig. 1a). In parallel, body temperature significantly increased on average by 0.9 °C between 2 h and 3 h after injection and normalized 24 h after injection ($p < 0.001$; Fig. 1b).

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