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Lipopolysaccharide-induced expression of cell surface receptors and cell activation of neutrophils and monocytes in whole human blood

N.E. Gomes[1], M.K.C. Brunialti[1], M.E. Mendes[1], M. Freudenberg[2], C. Galanos[2] and R. Salomão[1]

[1]Disciplina de Infectologia, Universidade Federal de São Paulo, São Paulo, SP, Brasil
[2]Max-Planck-Institute for Immunobiology, Freiburg, Germany

Abstract

Lipopolysaccharide (LPS) activates neutrophils and monocytes, inducing a wide array of biological activities. LPS rough (R) and smooth (S) forms signal through Toll-like receptor 4 (TLR4), but differ in their requirement for CD14. Since the R-form LPS can interact with TLR4 independent of CD14 and the differential expression of CD14 on neutrophils and monocytes, we used the S-form LPS from *Salmonella abortus equi* and the R-form LPS from *Salmonella minnesota* mutants to evaluate LPS-induced activation of human neutrophils and monocytes in whole blood from healthy volunteers. Expression of cell surface receptors and reactive oxygen species (ROS) and nitric oxide (NO) generation were measured by flow cytometry in whole blood monocytes and neutrophils. The oxidative burst was quantified by measuring the oxidation of 2',7'-dichlorofluorescein diacetate and the NO production was quantified by measuring the oxidation of 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate. A small increase of TLR4 expression by monocytes was observed after 6 h of LPS stimulation. Monocyte CD14 modulation by LPS was biphasic, with an initial 30% increase followed by a 40% decrease in expression after 6 h of incubation. Expression of CD11b was rapidly up-regulated, doubling after 5 min on monocytes, while down-regulation of CXCR2 was observed on neutrophils, reaching a 50% reduction after 6 h. LPS induced low production of ROS and NO. This study shows a complex LPS-induced cell surface receptor modulation on human monocytes and neutrophils, with up- and down-regulation depending on the receptor. R- and S-form LPS activate human neutrophils similarly, despite the low CD14 expression, if the stimulation occurs in whole blood.

Key words: Lipopolysaccharide; Neutrophils; Monocytes; Cell surface receptors; Reactive oxygen species; Nitric oxid

Introduction

Interaction between the innate immune system and microbial constituents is the basis of pathogen recognition and induced cellular activation (1). Lipopolysaccharide (LPS), present in Gram-negative bacteria, is a primary target for the recognition of bacteria, and elicits many of their pathogenic effects as well as a protective immune response (2). The interaction between LPS and cells of the innate immune system, such as neutrophils and monocytes, triggers an inflammatory response, driven to control the infection process, but that also may trigger the pathophysiological response leading to severe sepsis and septic shock (3).

LPS-induced cell activation is mediated by the Toll-like receptor 4 (TLR4) (4), and is dependent on LPS-binding protein and the CD14 receptor [as membrane-bound (mCD14) or soluble (sCD14) protein] (5). Signal transduction by TLRs requires association with myeloid differentiation protein 2 (6) and occurs by two pathways, one depending on the myeloid differentiation primary response gene (88) (MyD88) and the other MyD88-independent. The former induces activation of mitogen-activated protein kinases and translocation of transcriptional factor κB (NF-κB). The MyD88-independent signal activates interferon regulatory factor 3 and also NF-κB, although on a late basis (7). Both signaling pathways are involved in oxidative burst and

Correspondence: R. Salomão, Rua Pedro de Toledo, 781, 15º andar, 04039-032 São Paulo, SP, Brasil.
Fax: +55-11-5081-5394/5579-8226. E-mail: rsalomao@unifesp.br

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induction of nitric oxide (NO) (8,9).

LPS-induced cell activation in whole blood has the advantage of preserving the microenvironment of LPS and cell interaction as it occurs *in vivo*; however, studies of the effects of LPS on different cell populations in whole blood are scarce. Flow cytometry permits the study of the effects of LPS in whole blood at the cellular level (10,11). Macrophages are considered to be the primary targets of LPS, and neutrophils have also been reported as highly LPS-responsive cells. In the present study, we evaluated the modulation of cell surface receptors, oxidative burst and NO production by neutrophils and monocytes in whole blood of human volunteers.

Recently, it has been shown that the rough (R)-form LPS can interact with TLR4 without CD14 (12,13). Re-form LPS (lipid A + two 3-deoxy-D-manno-2-octurosonic acid residues), but not the smooth (S)-form LPS, can induce tumor necrosis factor-alpha (TNF-α) responses also in the absence of CD14 (12). R-form LPS was a potent activator of mast cells (that lack CD14), while S-form LPS was practically devoid of stimulatory activity (13). Since human neutrophils either lack or express low amounts of mCD14, it became important to study if LPS-induced neutrophil activation is different when stimulated by S- or R-form LPS. For this reason, we used both LPS types in the present study.

Material and Methods

Reagents

S-form LPS from *Salmonella abortus equi* and R-form LPS from *Salmonella minnesota* mutants (R595 (Re)) were extracted from parent bacteria and purified as previously described (14).

The following monoclonal antibodies were used: CD66b-fluorescein isothiocyanate (FITC), clone G1OF5; CD14-peridinin-chlorophyll-protein (PerCP), clone M8P9; CD11b-allophycocyanin (APC), clone D12; CD11c-APC, clone S-HCL-3; CD15-APC, clone HI98, and CXCR2-APC, clone 6C6 were obtained from BD Biosciences (USA). TLR2-phycoerythrin (PE), clone TL2.1 and TLR4-PE, clone HTA125 were obtained from eBioscience (USA). p50-PE, clone sc-8414 and p65-PE, clone sc-8008 were obtained from Santa Cruz Biotechnology (USA).

Healthy volunteers

The study was approved by the Universidade Federal de São Paulo Ethics Committee (process No. 1304/05) and written informed consent was obtained from all volunteers. Blood samples from 21 volunteers (mean age: 28 ± 7 years, 38% males) were used.

Cell surface receptor expression in whole blood in response to R- or S-form LPS

Expression of cell surface receptors on monocytes and neutrophils was determined in whole blood as previously described (15). Cells were stained with 6 µL CD14-PerCP and 5 µL CD66b-FITC in order to identify monocytes and neutrophils, respectively. For kinetics, the cells were also stained with 20 µL TLR4-PE and 2 µL CD11b-APC (tube 1), 20 µL TLR2-PE and 2 µL CD11c-APC (tube 2), or 2 µL CXCR2-APC (tube 3). A dose-response assay was performed with CD11b-APC.

Event acquisition and analyses were performed using the CellQuest software (BD Biosciences) and an FACSCalibur 4-color flow cytometer (BD Biosciences). Monocyte analyses were carried out using forward and side scatter parameters combined with CD14-positive- and CD66b-negative-stained cells, and neutrophil analyses were performed using forward and side scatter parameters combined with CD66b-positive- and CD14-negative-stained cells. Surface receptor expression was measured as the geometric mean fluorescence intensity (GMFI).

Analysis of oxidative burst in whole blood after stimulation with R- or S-form LPS

The oxidative burst was quantified by measuring the oxidation of 2',7'-dichlorofluorescein diacetate (DCFH-DA, Sigma, USA) in whole blood as previously described (16). Neutrophils in whole blood were characterized by side scatter and forward scatter parameters and negativity for CD14 and monocytes was characterized by side scatter and forward scatter parameters and positive staining for CD14. Reactive oxygen species (ROS) generation is reported in histograms and expressed as the GMFI.

Analysis of NO production in whole blood after stimulation with R- or S-form LPS

NO production was quantified by measuring the oxidation of 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (Invitrogen, USA) in whole blood by the method of Zelnickova et al. (17), modified. Cells were stained with 5 µL CD14-PE and 5 µL CD15-FITC for monocyte and neutrophil identification, respectively. NO generation is reported in histograms and expressed as the GMFI.

Statistical analysis

Data are reported as means ± SEM and were compared by analysis of variance (ANOVA) and the *post hoc* Tukey honestly significant difference (HSD) test. The software used was SPSS 13.0 for Windows (USA).

Results

LPS-induced modulation of the expression of cell surface receptors in whole blood

Induction of CD11b in monocytes and neutrophils by LPS: dose-response. Whole blood samples were stimulated for 30 min with different amounts of R- or S-form LPS (1-100 ng/mL) and the expression of CD11b on monocytes and neutrophils was determined by fluorescence-activating

cell sorting (FACS). The 100-ng LPS/mL dose was chosen and used for stimulation throughout the study (data not shown).

Kinetics of LPS-induced modulation of surface receptor expression on monocytes. The modulation of receptor expression on monocytes was monitored between 5 min and 24 h after the addition of LPS to whole blood cultures. Although no changes in TLR4 expression were observed by 180 min, a significant up-regulation by both R- and S-form LPS was seen after 6 h of culture (P = 0.039; Figure 1A), with a return to normal after 24 h (data not shown). No changes in TLR2 expression were detectable during the observation period (data not shown). CD14 expression was increased after 15-30 min (P = 0.034 and P = 0.050) of stimulation, returned to normal levels at 180 min and decreased after 6 h (P < 0.001; Figure 1B), a condition that persisted up to the end of the 24-h observation period. Increased CD11b expression was observed as early as 5 min after the addition of LPS and was highest after 6 h of incubation (Figure 1C), with similar effects for both LPS forms. R- and S-form LPS-induced CD11c expression on monocytes reached no statistical significance compared to control (Figure 1D).

Kinetics of LPS-induced modulation of surface receptor expression on neutrophils. The modulation of receptor expression on neutrophils was monitored between 5 min and 24 h after the addition of LPS to whole blood cultures. The expression of TLR2 and TLR4 was not modulated by R- or S-form LPS (data not shown). CD14 surface expression on neutrophils was only marginal compared to that on monocytes. Thus, human neutrophils have little or no mCD14 on their surface, and its expression was not induced by either R- or S-form LPS (data not shown).

Both LPS forms induced a decrease of CXCR2 expression on neutrophils, already present after 15 min and observed throughout the 6 h of the experiment (Figure 2A).

CD66b expression was significantly up-regulated by both R- and S-form LPS (P = 0.028 and P = 0.006, after 3

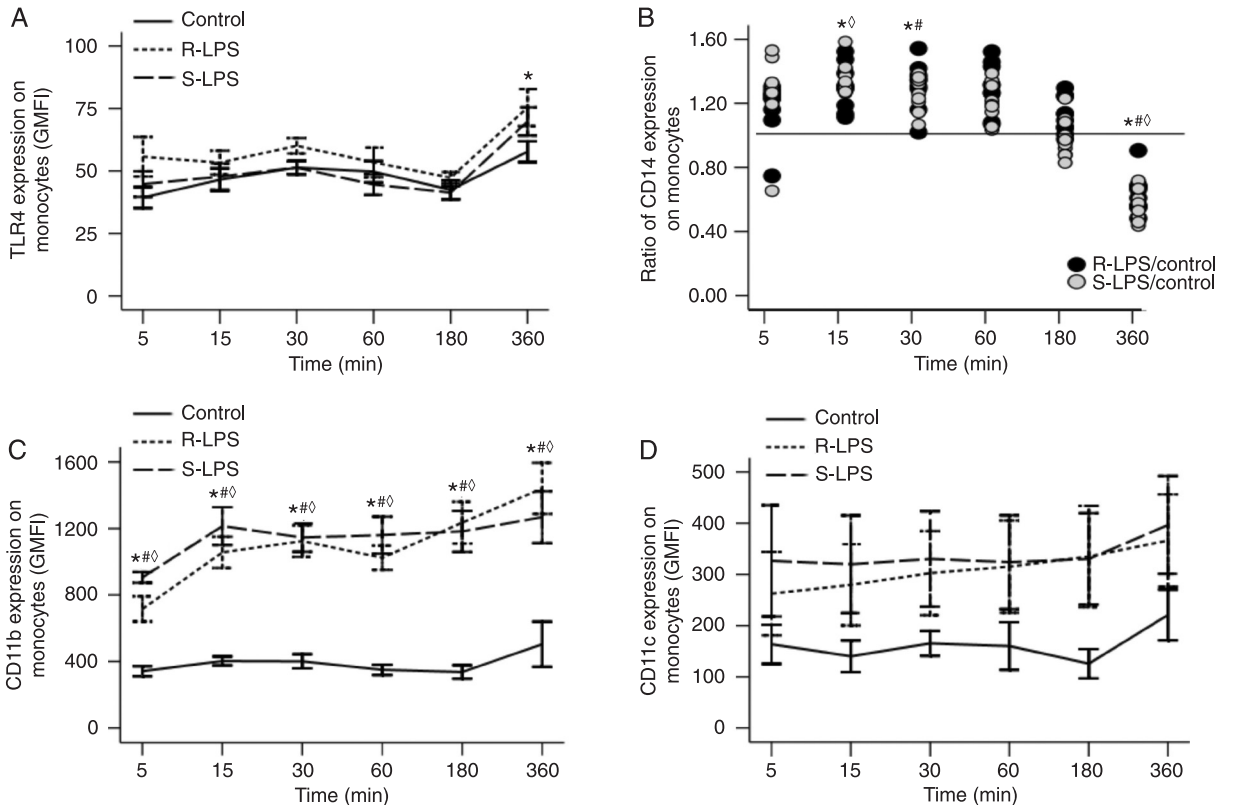

Figure 1. Expression of cell surface receptors on monocytes in whole blood after incubation with medium (control) or with 100 ng/mL rough (R)- or smooth (S)-form lipopolysaccharide (LPS). *A*, *C*, and *D*, Monocytes were gated based on forward and side scatter parameters and positive staining for CD14. The cell surface receptor expression was measured as geometric mean fluorescence intensity (GMFI) and is reported as means ± SEM for each time. *B*, Monocytes were gated based on forward and side scatter parameters and CD14 expression is represented as the ratio of CD14 expression between stimulated cells and controls. *A*, Results of TLR4 expression obtained from 4 individuals. *B*, Results of CD14 expression obtained from 10 healthy volunteers. *C*, Results of CD11b expression obtained from 5 individuals. *D*, Results of CD11c expression obtained from 4 individuals. *P ≤ 0.05 for group comparison (ANOVA), $^{\lozenge}$P ≤ 0.05 for S-form LPS versus control (Tukey *post hoc* test) and $^{\#}$P ≤ 0.05 for R-form LPS versus control (Tukey *post hoc* test).

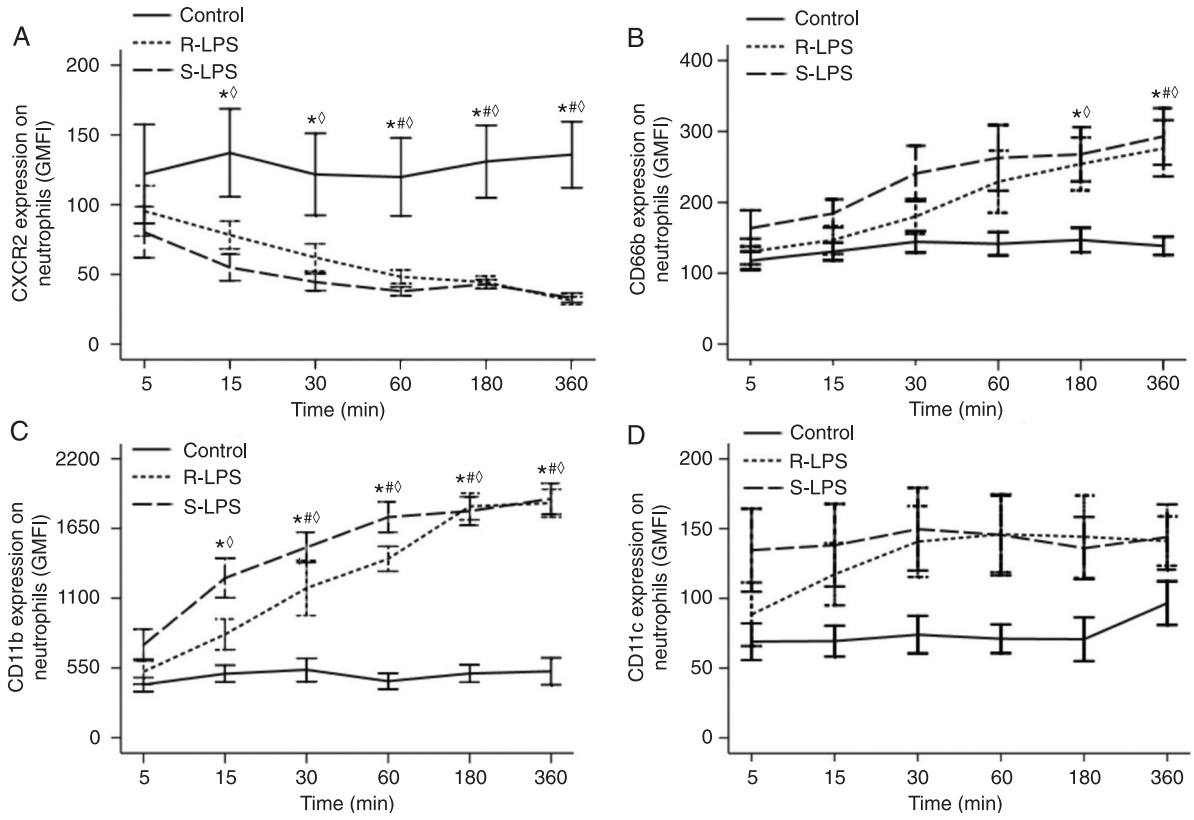

Figure 2. Expression of cell surface receptors on neutrophils in whole blood after incubation with medium (control) or with 100 ng/mL rough (R)- or smooth (S)-form lipopolysaccharide (LPS). *A*, *C*, and *D*, Neutrophils were gated based on forward and side scatter parameters and positive staining for CD66b. The cell surface receptor expression was measured as geometric mean fluorescence intensity (GMFI) and is reported as means ± SEM for each time. *B*, Neutrophils were gated based on forward and side scatter parameters and CD66b expression was measured as GMFI and is reported as means ± SEM for each time. *A*, Results of CXCR2 expression obtained from 6 individuals. *B*, Results of CD66b expression obtained from 9 individuals. *C*, Results of CD11b expression obtained from 4 individuals. *D*, Results of CD11c expression obtained from 4 individuals. *P ≤ 0.05 for group comparison (ANOVA), $^{\lozenge}$P ≤ 0.05 for S-form LPS versus control (Tukey *post hoc* test) and $^{\#}$P ≤ 0.05 for R-form LPS versus control (Tukey *post hoc* test).

h and 6 h of stimulation, respectively, Figure 2B). S-form LPS seems to have an earlier effect in inducing CD66b expression (Figure 2B). CD66b expression on stimulated cells remained elevated up to 24 h of incubation (data not shown).

CD11b expression on neutrophils, similar to that on monocytes, was rapidly up-regulated by 15 min of stimulation, increased gradually with time (Figure 2C), persisting at an elevated level up to 24 h of stimulation (data not shown). When the Tukey test was performed, both LPS forms showed activity. In contrast, the expression of CD11c on neutrophils was low and did not significantly increase after blood was cultured with R- or S-form LPS (Figure 2D).

LPS-induced production of ROS and NO by monocytes and neutrophils in whole blood

A dose-dependent production of ROS was generated in monocytes and neutrophils within 30 min when whole blood samples were stimulated with LPS. No significant differences in ROS generation activity were observed between the two LPS forms (Figure 3A and B). A significant induction of NO was observed in monocytes (Figure 4A), but not in neutrophils (Figure 4B) after 30 min of stimulation with 100 ng/mL R- and S-form LPS (P = 0.019).

Discussion

We investigated the modulation of a wide range of cell surface receptors on human monocytes and neutrophils involved in bacterial recognition, chemotaxis, and cell-to-cell interactions, in whole blood of human volunteers using the S- and R-forms of LPS.

In agreement with numerous other studies we show that not only monocytes, but also neutrophils, express the signaling receptor for LPS, TLR4 (11). However, in contrast to some reports (18,19), we observed no down-regulation of

TLR4 on monocytes during the initial period of stimulation (up to 3 h after LPS), but rather a weak, transient increase at 6 h after LPS. In addition, we found no changes in TLR4 expression in LPS-stimulated neutrophils. We also investigated the impact of LPS activation on the expression of TLR2 on both cell types. No major changes in TLR2 levels on the surface of monocytes and neutrophils were observed when whole blood samples were stimulated with LPS up to 24 h.

While the expression of the LPS-binding co-receptor CD14 on monocytes has been well documented, the presence of CD14 on neutrophils is not so clear. Some investigators claim that CD14 is expressed in polymorphonuclear neutrophils, although to a lesser extent in monocytes (18), and a positive modulation of CD14 expression on neutrophils by LPS has been reported (20). FACS analysis of the present data showed that the levels of CD14 on unstimulated neutrophils were marginal or absent and, in agreement with Wright et al. (21), not affected by LPS stimulation. This absence of significant amounts of CD14 is also indirectly supported by results of an earlier study (22), in which only R-form LPS preparations elicited oxidative responses in isolated neutrophils, while the S-form LPS was virtually inactive. Since it is known today that only the R-form LPS can activate TLR4-positive cells independent of CD14 (12), retrospectively the inability of the S-form LPS to activate neutrophils under serum-free conditions (22) can be explained by the absence of CD14 on human neutrophils.

As previously shown (23), LPS modulated the expression of mCD14 on monocytes. The rapid increase of mCD14 expression (15 and 30 min after the addition of LPS) was followed by a strong decrease of expression between 6 and 24 h later compared to control. This kinetics is not fully consistent with finding reported by others. Some investigators (24) did not detect a change in response of peripheral blood mononuclear cells (PBMC) after the addition of the S-form LPS after 120 min, others (25) observed an initial decrease followed by a sharp increase after 24 h of PBMC incubation, while still others (23) described a positive modulation of CD14 on whole blood monocytes during 4 h of incubation. The finding of LPS-induced down-regulation of CD14 raises the question of whether this effect might be involved in the down-regulation of the pro-inflammatory response during re-exposure of target cells to LPS. Here, however, the concomitant unsuppressed or even enhanced production of anti-inflammatory proteins (26) had to be explained.

We show here that both LPS forms substantially up-regulated the expression of CD11b on monocytes and neutrophils. CD11b up-regulation persisted during the 6 h of LPS stimulation on monocytes and neutrophils, in contrast to Haugen et al. (27), who found both up- and down-regulation of its expression. These discrepancies may be due to different experimental conditions. CD11 molecules (a, b, c, d)/CD18 β2 integrins are important for cell adhesion to the endothelium (28) and CD11b/CD18 has

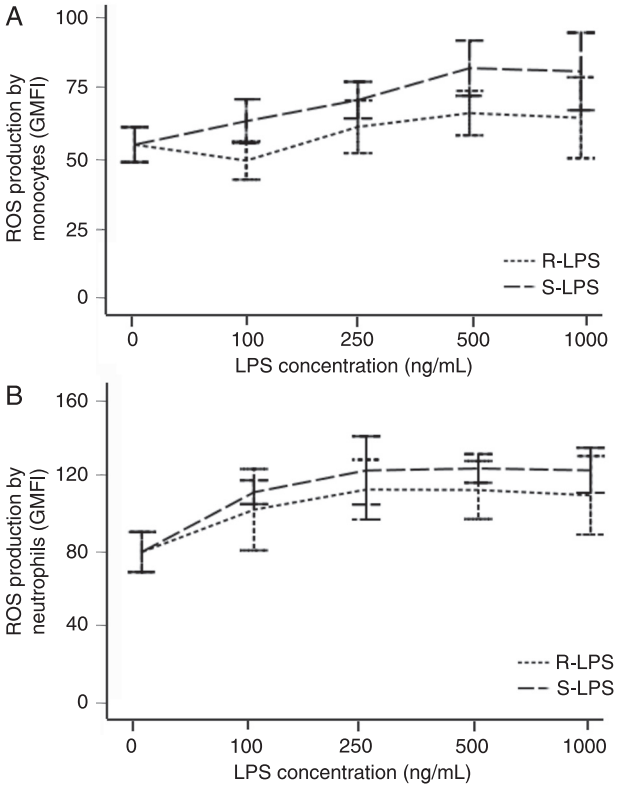

Figure 3. Production of reactive oxygen species (ROS) by monocytes (A) and neutrophils (B) in response to different concentrations of rough (R)- or smooth (S)-form lipopolysaccharide (LPS, 0, 100, 250, 500, and 1000 ng/mL) applied for 30 min. ROS production is reported as geometric mean fluorescence intensity (GMFI) and is reported as means ± SEM for each concentration. Results obtained from 3 individuals.

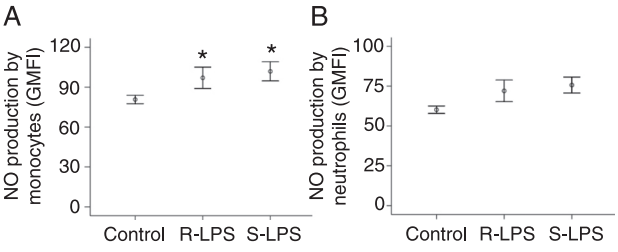

Figure 4. Nitric oxide (NO) production by monocytes and neutrophils after incubation with medium (control) or 100 ng/mL rough (R)- or smooth (S)-form lipopolysaccharide (LPS) for 30 min. Monocytes (A) were gated based on forward and side scatter parameters and positive CD14 staining, and neutrophils (B) based on forward and side scatter parameters and positive staining for CD15. NO production was measured as geometric mean fluorescence intensity (GMFI) of benzotriazolic derivate and is reported as means ± SEM for each stimulus. Results obtained from 7 individuals. *P < 0.05 compared to control (ANOVA and Tukey *post hoc* test).

already been suggested to be an LPS receptor in human macrophages (29). Zhou et al. (30) suggested that LPS can amplify its response because of CD11b. Recently, CD11b/CD18 was found to play a significant role in the optimal LPS response (31). We found a transient association between CD14 and CD11b/CD18 expression after LPS stimulation on monocytes, as previously reported for neutrophils (32), that may indicate an initial regulation driven to optimize LPS recognition and signaling.

CD66b, another protein involved in cell migration, was strongly induced by both LPS forms on the neutrophil surface. No studies have evaluated the modulation of this receptor by LPS on the neutrophil surface.

In agreement with previous studies (33), we found that LPS induced down-regulation of the chemokine receptor CXCR2 on neutrophils. The decrease of CXCR2 expression may be part of the negative regulatory effects by the host to limit inflammation.

Altogether, the enhanced co-expression of CD11b and CD14 on monocytes might indicate an up-regulation of recognizing and signaling receptors in the initial response to LPS, while the later decrease in CD14 expression and the persistently increased expression of CD11b might favor adhesion properties. The enhanced expression of CD66b and CD11b on neutrophils also seems to indicate an enhanced capacity of migration and adhesion, which somehow contrasts with the decreased expression of CXCR2.

Despite the good results obtained with monocytes and neutrophils in dose-response assays, ROS production by LPS was not consistent in all experiments and it is not a consensus in the literature (with some studies reporting little production, and others strong production). Interestingly, Kapp et al. (22), using lucigenin-dependent chemiluminescence for the detection of oxygen radicals, showed that only the R-form LPS was able to induce strong activation of isolated neutrophils. Myhre et al. (34) suggested that DCFH cannot detect all ROS production, and that various methods should be used for ROS identification. We may have missed this activity by the detection method used, and found a weak and similar ROS generation with R- and S-form LPS by DCFH oxidation.

R- and S-form LPS induced NO production by monocytes but not by neutrophils after 30 min of stimulation in whole blood. This finding agrees with a previous report that showed no stimulation of neutrophil production by S-, Ra- or Re-form LPS at any dose or time used (35). The low LPS-induced production of NO in these experiments may reflect the difficulties in detecting NO activity in human cells since LPS is one of the main inducers of NO synthase in inflammatory cells (36).

In the present study we showed that, despite their differential requirement for CD14 help, S- and R-form LPS activate cells with differential CD14 expression - monocytes and neutrophils - with similar strength and kinetics, if the stimulation is applied to whole blood. Thus, S- and R-form LPS were highly comparable in their property to modulate different surface receptors and to induce an oxidative burst and NO production in monocytes and neutrophils. Since larger amounts of soluble CD14 (sCD14) are known to substitute membrane-bound CD14 (mCD14) during activation of cells with LPS, our data show that the levels of sCD14 in plasma of healthy humans fully compensate for the shortage of mCD14 in circulating neutrophils.

Acknowledgments

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