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In Vivo Lipopolysaccharide Exposure of Human Blood Leukocytes Induces Cross-Tolerance to Multiple TLR Ligands

Alex F. de Vos,1 Jennie M. Pater, Petra S. van den Pangaart, Martijn D. de Kruif, Cornelis van ‘t Veer, and Tom van der Poll

In vitro and in vivo experiments in mice have shown that exposure of cells to the TLR4 ligand LPS induces tolerance toward a second exposure to LPS and induces cross-tolerance to certain other TLR ligands. Recently, we found that LPS tolerance in experimental human endotoxemia and Gram-negative sepsis is associated with elevated levels of IL-1R-associated kinase M, an intracellular negative regulator of MyD88-dependent TLR signaling. In the present study, we investigated whether in vivo exposure of humans to LPS induces tolerance in circulating leukocytes to other TLR agonists that rely either on MyD88-dependent or on MyD88-independent signaling. Analysis of TNF, IL-1β, IL-6, and IL-10 levels in whole blood demonstrated that leukocytes were hyporesponsive to ex vivo LPS restimulation 3–8 h after i.v. LPS injection (4 ng/kg). Reduced cytokine release during the same interval was also observed in whole blood further stimulated with MyD88-dependent ligands for TLR2, TLR5, and TLR7 or with whole bacteria. Strikingly, blood leukocytes were also tolerant to a ligand for TLR3, which signals solely through a MyD88-independent (Toll IL-1R domain-containing adaptor-inducing IFN-β [TRIF]-dependent) pathway. The hyporesponsiveness of leukocytes to TLR3 ligation was associated with reduced rather than increased levels of the recently identified TRIF inhibitor SARM. Taken together, these data indicate that systemic LPS challenge of human volunteers induces cross-tolerance to multiple TLR ligands that signal in a MyD88-dependent or MyD88-independent manner and suggest that LPS exposure of human blood leukocytes may hamper the inflammatory response to various microbial components. The Journal of Immunology, 2009, 183: 533–542.

Upon i.v. injection in humans, LPS, the toxic component of the cell wall of Gram-negative bacteria, causes an immediate systemic release of a variety of inflammatory mediators including cytokines, which subsequently cause fever and leukopenia followed by leukocytosis. Within hours after systemic administration of LPS, the host becomes temporarily refractory to restimulation with LPS, a phenomenon we designate here as “early transient” LPS tolerance. Early transient LPS tolerance is characterized by a desensitization of various types of leukocytes, including monocytes, polymorphonuclear leukocytes (1, 2), and lymphocytes (3) to release TNF, IL-1β, or IL-6 in response to a second exposure to LPS (4, 5) and subsides 24 h after i.v. LPS injection. Early transient LPS tolerance differs in its kinetics from classical LPS (or endotoxin) tolerance, observed in experimental animals (6) and leukocytes of patients with sepsis (7–10), systemic inflammatory response syndrome (11), major surgery (12), and trauma (13, 14), which lasts for several days to weeks. The induction of LPS tolerance during these clinical conditions may in the short term be beneficial by preventing excessive inflammation, but in the longer term be deleterious by hampering an adequate defense response to opportunistic infections. Despite the difference in kinetics, recent data indicate that early transient and classical LPS tolerance may be regulated through similar mechanisms (10, 15).

Activation of cells by LPS is initiated by an interaction with TLR4 in complex with CD14 and MD-2 on the cell surface and the subsequent recruitment of the adaptor molecules MyD88 and TRIF to the intracellular domain of TLR4 (16, 17). These adaptors trigger the activation of downstream kinases, such as IL-1R-associated kinase (IRA)K2 1, IRAK-4, PI3K, and p38 MAPK and ultimately the liberation of cytoplasmic NF-κB. Translocation of NF-κB to the nucleus will initiate the transcription of TNF and other inflammatory cytokines. Both the MyD88 and TRIF pathways are activated by TLR4 ligands such as LPS, whereas ligands for TLR2 (like bacterial lipoteichoic acid (LTA), peptidoglycan (PGN), and Pam3Cys), TLR5 (bacterial flagellin), TLR7 (viral ssRNA or synthetic imidazoquinolines), and TLR9 (bacterial and CpG DNA) activate only the MyD88 pathway (16). In contrast, TLR3 ligands such as viral dsRNA or synthetic polyinosinic:polycytidylic acid (poly(I:C)) trigger activation of the TRIF pathway alone, which besides NF-κB-mediated gene transcription may initiate IFN regulatory factor (IRF) 3-mediated transcription of type I IFNs (16).

In vitro and in vivo studies in mice have revealed that LPS tolerance is partially regulated by a number of intracellular inhibitors of the TLR4/MyD88 signal transduction pathway (18). These intracellular inhibitors include an inactive splice variant of MyD88 (MyD88short), IRAK-M, and suppressor of cytokine signaling 1.

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(SOCS-1) (which all three cause inhibition of IRAK phosphorylation), SHIP (a PI3K inhibitor), and A20 (a negative regulator of NF-κB). Mice deficient in either IRAK-M, SOCS-1, or SHIP are defective in the induction of LPS tolerance. Recently, we found that various inhibitors of the MyD88 pathway are expressed in leukocytes during human endotoxemia and that IRAK-M in particular was associated with early LPS hyporesponsiveness (15). Similarly, our laboratory demonstrated that patients with severe Gram-negative sepsis displayed classical LPS tolerance in association with elevated IRAK-M mRNA levels in circulating leukocytes, which correlated with an adverse outcome (10). Besides intracellular inhibitors of the MyD88 pathway, a recent study identified SARM in LPS-stimulated PBMC as a specific negative regulator of TRIF-dependent TLR signaling (19).

In analogy to LPS tolerance, it has been found that exposure to ligands for TLR2 (20–25), TLR5 (26), or TLR9 (27, 28) also induced hyporesponsiveness to subsequent stimulation with the same ligand (self-tolerance) and that TLR ligands can substitute for each other inducing cross-tolerance. Cells treated first with bacterial lipoprotein or MALP-2 (both TLR2 ligands) did not respond to subsequent LPS stimulation (20, 23, 29) and cells pretreated with LPS did not respond to LTA (30) or flagellin (26). However, despite LPS-, LTA-, Pam3Cys-, and CpG DNA-induced self-tolerance, LTA-, Pam3Cys-, or CpG DNA-pretreated cells still (partially) responded to LPS (25, 27, 28, 30, 31) and LPS-pretreated cells still responded to MALP-2 (24) or Pam3Cys (31). In this respect, Dalpke et al. (28) showed that in vivo pretreatment of mice with LPS, LTA, or CpG DNA induced self-tolerance and that LPS and LTA, but not CpG DNA induced cross-tolerance to the other TLR ligands. These findings indicate that tolerance induced by different TLR ligands occurs via distinct mechanisms.

So far, our understanding of cross-tolerance is based on in vitro experiments and in vivo studies in mice, but it is unknown to what extent in vivo TLR ligand exposure of humans induces cross-tolerance in blood leukocytes. In the present study, we elaborated on our recent findings in human endotoxemia, showing that the hyporesponsiveness of circulating leukocytes to LPS after in vivo exposure to LPS was associated with elevated IRAK-M mRNA and protein levels (15). We argued that if IRAK-M indeed mediates early transient LPS tolerance in this human model, circulating leukocytes should also be hyporesponsive to other MyD88-dependent TLR agonists. Therefore, in the present study, we investigated whether i.v. LPS administration to human volunteers hampered the cytokine response of blood leukocytes when stimulated ex vivo with ligands specific for TLR2 (LTA, PGN, zymosan), TLR5 (flagellin), and TLR7 (S-27609). In addition, we determined whether cellular hyporesponsiveness also occurred upon stimulation of circulating leukocytes with a MyD88-independent TLR
agonist (polyI:C), a TLR3 agonist. Finally, we examined the capacity of blood leukocytes to respond to whole bacteria after in vivo exposure to LPS. The data of our study reveal that human blood leukocytes become refractory to multiple TLR ligands after systemic LPS exposure.

Materials and Methods
Subjects and endotoxemia model
The study was approved by the institutional scientific and ethics committees. Written informed consent was obtained from all subjects, in total six healthy male volunteers (age 25–35 years). Medical histories, physical examinations, hematomatological and biochemical screenings, and electrocardiograms were all normal. The subjects fasted overnight before LPS administration. The volunteers were challenged (at t = 0) with LPS (Escherichia coli LPS, lot G; U.S. Pharmacopoeia) as a bolus i.v. injection at a dose of 4 ng/kg body weight as previously described (32–34). Blood was collected from a cannulated forearm vein directly before LPS administration (t = 0) and at 1, 2, 3, 4, 6, 8, and 24 h thereafter. Blood was drawn in EDTA-K3 containing tubes (Vacutainer Systems; BD Biosciences) for leukocyte and differential counts and assessment of plasma TNF levels and in sodium heparin tubes for ex vivo stimulations.

Ex vivo blood stimulations
Whole blood was mixed with an equal volume of plain RPMI 1640 medium (Life Technologies) or with RPMI 1640 containing TLR ligands or heat-killed bacteria as described previously (5). TLR ligands consisted of LPS (E. coli 0111:B4, ultrapure; 100 ng/ml end concentration; InvivoGen), LTA (Staphylococcus aureus; 10 μg/ml end concentration; provided by R. Miller, 3M Pharmaceuticals, St. Paul, MN). For bac-

Cytokine assays
TNF, IL-1β, IL-6, and IL-10 were measured by cytometric bead array (CBA; BD Biosciences). ELISA was performed to determine the levels of IFN-α (Bender MedSystems), IFN-β (BioSource International), and IFN-ω (Bender MedSystems), all according to the instructions of the manufacturers. TNF concentrations after in vivo LPS injection are given as pg/ml plasma, whereas cytokine levels after ex vivo stimulation of whole blood are presented as pg/ml in the 1:2 mixture of plasma and culture medium. The limit of detection for TNF was 10 pg/ml, for IL-1β was 156 pg/ml, for IL-6 was 100 pg/ml, for IL-10 was 40 pg/ml, for IFN-α was 15 pg/ml, for IFN-ω was 6 pg/ml, and for IFN-ω was 18 pg/ml.

RT-PCR and Western blot analysis of SARM
SARM mRNA analysis was performed in essence as described elsewhere (15). Briefly, total RNA obtained with the PAXgene System (PreAnalytiX; Qiagen) was treated with RQ1 RNase-Free DNase (Promega) and reverse transcribed using oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase (Invitrogen). RT-PCR were performed on 4-fold diluted cDNA samples using LightCycler 480 SYBR Green I Master (Roche Diagnostics) with 0.2 μM SARM sense and antisense oligonucleotide primers in a LightCycler (Roche Diagnostics) apparatus. PCR conditions were: 6 min 95°C hot start, followed by 40 cycles of amplification (95°C for 10 s, 60°C for 5 s, 72°C for 1 s). For quantification, standard curves were constructed by PCR on serial dilutions of a concentrated cDNA and data were analyzed using LightCycler software. DNA sequences of the SARM primers were: sense, 5’-ACTGCAAGGATTGGGTGCAT-3’ and antisense, 5’-TCAATGGTGCGCTCTGTTGAT-3’. Gene expression is presented as a ratio of the expression of the housekeeping gene β2-microglobulin, since we previously found that the expression of β2-microglobulin in vivo LPS-exposed blood leukocytes remained constant to the expression of ferritin L chain, a known LPS-unresponsive gene (36). All PCR generated the DNA products of the expected length. SARM protein analysis was performed in essence as described previously (15). Briefly, eryth-

FIGURE 2. Intravenous LPS induces cross-tolerance in blood leukocytes to the TLR2 ligand LTA. Volunteers (n = 6) were injected i.v. with LPS (4 ng/kg) at t = 0 and blood collected at the indicated time points was mixed with an equal volume of RPMI 160 medium containing LTA (10 μg/ml end concentration) and stimulated for 24 h. Secretion of TNF (A), IL-1β (B), IL-6 (C), and IL-10 (D) in diluted plasma was determined by CBA. Asterisks indicate significant differences compared with t = 0 (*, p ≤ 0.05 and **, p ≤ 0.01).
in TBS, and incubated overnight with rabbit anti-SARM Abs (Abcam) in 1% BSA in PBS at 4°C. After washing, membranes were probed with peroxidase-labeled secondary Ab (DakoCytomation) for 1 h at room temperature in 1% BSA in PBS. After washing, the membranes were incubated with Lumi-LightPlus Western Blotting Substrate (Roche) and positive bands were detected using a GeneGnome Syngene Bio Imaging System. Intensity of the bands was quantified using the GeneTools software supplied by Syngene.

Statistics

All data are expressed as means ± SEM. Changes in time were calculated by one-way ANOVA followed by Dunnett’s post hoc test where appropriate. Values of p < 0.05 were considered to represent statistical significant differences.

Results

Intravenous LPS induces ex vivo LPS tolerance in leukocytes

As expected (5, 15, 32–34, 37), i.v. LPS administration induced a transient release of TNF, IL-6, and IL-10, but not IL-1β in plasma, changes in circulating leukocyte numbers and LPS tolerance in whole blood. Plasma TNF concentrations became elevated and peaked at 60 and 90 min, respectively, after i.v. LPS administration and returned to baseline levels at 4 h after i.v. LPS (Fig. 1A), whereas IL-10 and IL-6 peaked at 3 and 4 h, respectively, after i.v. LPS (data not shown). LPS also induced a transient reduction of circulating monocyte and lymphocyte numbers, whereas neutrophil numbers increased (Fig. 1B). At 3 h after i.v. LPS, monocyte and lymphocyte numbers had declined to ~10 and 20%, respectively, of the numbers before LPS challenge. After this time point, monocyte numbers gradually increased, reaching baseline numbers at 8 h after i.v. LPS. After the decline, lymphocyte numbers increased 8 h after i.v. LPS and returned to baseline levels at t = 24 h. Neutrophil numbers increased >2-fold at 6, 8, and 24 h after i.v. LPS.

Similar to previous studies, in vivo LPS exposure induced immediate ex vivo hyporesponsiveness of leukocytes to LPS (4, 5, 15). Stimulation of whole blood obtained before i.v. LPS administration with 100 ng/ml LPS for 24 h induced release of TNF, IL-1β, IL-6, and IL-10 (Fig. 1, C–F). Plasma levels of these cytokines were undetectable or low in LPS-stimulated blood obtained at 3, 6, and 8 h after i.v. LPS challenge and returned to baseline levels at 24 h after i.v. LPS (Fig. 2). Cytokine concentrations (all in pg/ml) were measured in supernatants; levels below the detection limit of the assay (see Materials and Methods) were expressed as equivalent to the detection limit. Data are means ± SE.

Table 1. Intravenous LPS injection induces hyporesponsiveness to the TLR2 ligands PGN and zymosan upon ex vivo stimulation of whole blood leukocytes*p

<table>
<thead>
<tr>
<th>TLR2 Ligand</th>
<th>Time</th>
<th>TNF</th>
<th>IL-1β</th>
<th>IL-6</th>
<th>IL-10</th>
</tr>
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<tbody>
<tr>
<td>PGN (10 μg/ml)</td>
<td>0</td>
<td>75.4 ± 33.0</td>
<td>298.8 ± 10.0</td>
<td>6833.0 ± 1746.0</td>
<td>47.2 ± 7.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>21.2 ± 10.4</td>
<td>156.0 ± 0</td>
<td>1333.0 ± 568.2*</td>
<td>41.6 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>10.0 ± 0*</td>
<td>156.0 ± 0</td>
<td>163.3 ± 54.0*</td>
<td>40.0 ± 0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>11.9 ± 1.9*</td>
<td>159.7 ± 3.7</td>
<td>183.9 ± 42.0*</td>
<td>40.0 ± 0</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>33.8 ± 8.6</td>
<td>156.0 ± 0</td>
<td>1482.0 ± 314.5*</td>
<td>40.0 ± 0</td>
</tr>
<tr>
<td>Zymosan (20 μg/ml)</td>
<td>0</td>
<td>243.2 ± 56.0</td>
<td>426.0 ± 82.1</td>
<td>13176.0 ± 2416.0</td>
<td>88.2 ± 21.7</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>14.5 ± 4.2*</td>
<td>160.0 ± 3.3*</td>
<td>954.4 ± 292.7*</td>
<td>40.0 ± 0*</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>10.0 ± 0*</td>
<td>156.0 ± 0*</td>
<td>168.6 ± 64.7*</td>
<td>40.0 ± 0*</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>12.6 ± 2.1*</td>
<td>165.6 ± 9.6*</td>
<td>223.1 ± 42.2*</td>
<td>40.0 ± 0*</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>77.2 ± 25.9*</td>
<td>167.4 ± 11.4*</td>
<td>2310.0 ± 599.0*</td>
<td>40.0 ± 0*</td>
</tr>
</tbody>
</table>

*p Six healthy humans were i.v. injected with LPS (4 ng/kg) at t = 0. Directly before LPS injection and at 3, 6, 8, and 24 h thereafter whole blood was collected and stimulated with the indicated TLR2 ligands for 24 h. Cytokine concentrations (all in pg/ml) were measured in supernatants; levels below the detection limit of the assay (see Materials and Methods) were expressed as equivalent to the detection limit. Data are means ± SE. *p < 0.05 vs t = 0.

FIGURE 3. Intravenous LPS induces cross-tolerance in blood leukocytes to the TLR5 ligand flagellin. Volunteers (n = 6) were injected i.v. with LPS (4 ng/kg) at t = 0 and blood collected at the indicated time points was stimulated with flagellin (1 μg/ml end concentration) for 24 h. Secretion of TNF (A), IL-1β (B), and IL-6 (C) in 1/2 diluted plasma was determined by CBA. Asterisks indicate significant differences compared with t = 0 (*, p ≤ 0.05).
Intravenous LPS induces tolerance in blood leukocytes to the TLR7 ligand S-27609. Volunteers (n = 6) were injected i.v. with LPS (4 ng/kg) at t = 0 and blood collected at the indicated time points was stimulated with S-27609 (5 μg/ml end concentration) for 24 h. Secretion of TNF (A), IL-1β (B), IL-6 (C), and IL-10 (D) in 1/2 diluted plasma was determined by CBA. Asterisks indicate significant differences compared with t = 0 (*, p ≤ 0.05 and **, p ≤ 0.01).

Intravenous LPS induces hyporesponsiveness of leukocytes to TLR2 ligands

After having confirmed that i.v. LPS induces early transient tolerance to restimulation with LPS, we determined the capacity of whole blood leukocytes to release cytokines upon stimulation with various TLR2 ligands, i.e., LTA, PGN, and zymosan. All TLR2 ligands induced the release of TNF, IL-1β, and IL-6, as well as IL-10 in whole blood obtained before i.v. LPS, with the highest plasma cytokine levels after stimulation with 10 μg/ml LTA (Fig. 2 and Table I). Similar to the period of LPS tolerance, undetectable or low cytokine levels were found in LTA-stimulated whole blood obtained at 3, 6, and 8 h after i.v. LPS, with the highest plasma cytokine levels after stimulation with 10 μg/ml LTA (Fig. 2 and Table I). Similar to the period of LPS tolerance, undetectable or low cytokine levels were found in LTA-stimulated whole blood obtained at 3, 6, and 8 h after i.v. LPS, with the highest plasma cytokine levels after stimulation with 10 μg/ml LTA (Fig. 2 and Table I).

Intravenous LPS induces hyporesponsiveness of leukocytes to TLR3 and TLR7 agonists

Having established that i.v. LPS induced (cross-) tolerance to the TLR2 agonists LTA, PGN, and zymosan (which all rely on MyD88 for signaling and therefore are sensitive to inhibition by IRAK-M), we next investigated whether circulating leukocytes also displayed tolerance to other MyD88-dependent TLR agonists. Therefore, we stimulated whole blood leukocytes with the bacterial TLR5 ligand flagellin and the synthetic TLR7 ligand S-27609. Flagellin (at 1 μg/ml) induced the release of low levels of TNF, IL-1β, and IL-6 in blood obtained before i.v. LPS challenge (Fig. 3). A reduction of plasma TNF (although not statistically significant) and IL-6, but not IL-1β, was found in flagellin-stimulated blood obtained at 3, 6, and 8 h after i.v. LPS. S-27609 (at 5 μg/ml) induced high levels of cytokines in whole blood obtained before i.v. LPS, but not in whole blood obtained at 3, 6, 8, and 24 h after i.v. LPS (Fig. 4). In contrast to any of the other TLR ligands or whole bacteria (see further), S-27609 induced the release of the type I IFNs IFN-α, IFN-β, and IFN-ω in plasma (Fig. 5). IFN-α and IFN-ω levels were reduced in S-27609-stimulated blood obtained 3 h after i.v. LPS challenge (although statistically significant only for IFN-α) and returned to baseline level at later time points (Fig. 5). Together, these data indicate that LPS exposure of leukocytes triggers tolerance to various MyD88-dependent TLR ligands.

Intravenous LPS induces hyporesponsiveness of leukocytes to the TLR3 agonist poly(I:C)

To determine whether i.v. LPS also induces cross- tolerance to a MyD88-independent TLR agonist, we studied cytokine release by LP-exposed leukocytes after ex vivo stimulation with the synthetic TLR3 ligand poly(I:C), which in contrast to the other TLR ligands tested in this study signals solely in a TRIF-dependent manner. Poly(I:C) (at 50 μg/ml) induced high levels of cytokines in whole blood obtained before i.v. LPS (Fig. 6). Poly(I:C)-induced cytokine release was not influenced by polymyxin B, excluding a role for possible contaminating LPS (data not shown). Of interest, blood leukocytes obtained at 3, 6, and 8 h after i.v. LPS were tolerant for poly(I:C). Indeed, the capacity of blood leukocytes to release TNF, IL-1β, IL-6, and IL-10 was strongly reduced up to 6–8 h after LPS injection. Except for IL-10, cytokines returned
to baseline levels in poly(I:C)-stimulated whole blood obtained at 24 h after i.v. LPS (Fig. 6).

**Intravenous LPS causes a decrease in the intracellular TRIF inhibitor SARM**

Several studies have revealed that LPS tolerance is mediated by intracellular inhibitors of the MyD88 pathway and previously we found that IRAK-M in particular was associated with LPS hyporesponsiveness during human endotoxemia (15) and Gram-negative sepsis (10). Since poly(I:C)-induced cytokine secretion was strongly reduced after i.v. LPS challenge (Fig. 6) and considering that SARM was recently identified as a negative regulator of TRIF-dependent TLR3 and TLR4 signaling in human PBMC (19), we analyzed the expression of SARM in leukocytes during endotoxemia. Expression of SARM (both at mRNA and protein level) was detected in leukocytes obtained before i.v. LPS administration (Fig. 7), but strikingly SARM mRNA and protein declined at the time points at which LPS tolerance was observed. This suggests that SARM is not an essential regulator of the hyporesponsiveness of leukocytes to poly(I:C) in our model.

**Intravenous LPS induces hyporesponsiveness of leukocytes to whole bacteria**

Finally, to verify whether cross-tolerance of LPS-exposed leukocytes to various purified TLR ligands also resulted in hyporesponsiveness to whole bacteria, we analyzed cytokine release in whole blood in response to *E. coli* (Fig. 8), *S. aureus*, and *S. pneumoniae* (Table II). Stimulation with 1 × 10^8 CFU/ml *E. coli* induced the release of TNF, IL-1β, IL-6, and IL-10 in whole blood obtained before i.v. LPS. The release of all of these cytokines was significantly declined in *E. coli*-stimulated whole blood obtained at 3 and 6 h after i.v. LPS (Fig. 4). Although plasma IL-1β and IL-6 concentrations returned to baseline levels in blood obtained at 8 and 24 h after i.v. LPS challenge, IL-10 levels remained low in blood from these time points. Stimulation with 1 × 10^8 CFU/ml *S. aureus* or *S. pneumoniae* resulted in a similar release of cytokines in whole blood as observed with *E. coli* and comparable decline in

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**FIGURE 5.** Secretion of IFN-α (A) and IFN-ω (B) in response to the TLR7 ligand S-27609 is inhibited in blood leukocytes from LPS-treated volunteers. Volunteers (n = 6) were injected i.v. with LPS (4 ng/kg) at t = 0 and blood collected at the indicated time points was mixed with an equal volume of RPMI 1640 medium containing S-27609 (5 μg/ml end concentration) and stimulated for 24 h. IFN-α and IFN-ω levels in 1/2 diluted plasma were determined by ELISA. Asterisks indicate significant differences compared with t = 0 (*, p ≤ 0.05).

**FIGURE 6.** Intravenous LPS induces tolerance in blood leukocytes to the TLR3 ligand poly(I:C). Volunteers (n = 6) were injected i.v. with LPS (4 ng/kg) at t = 0 and blood collected at the indicated time points was stimulated with poly(I:C) (50 μg/ml end concentration) for 24 h. Secretion of TNF (A), IL-1β (B), IL-6 (C), and IL-10 (D) in 1/2 diluted plasma was determined by CBA. Asterisks indicate significant differences compared with t = 0 (**, p ≤ 0.01).
cytokine release in blood obtained at 3, 6 and 8 h after i.v. LPS administration (Table II). Thus, i.v. LPS induces tolerance of leukocytes to whole bacteria.

Discussion

Previously, we and others showed that i.v. injection of LPS in humans induces early transient tolerance in blood leukocytes characterized by diminished capacity to secrete cytokines upon re-stimulation with LPS (4, 5, 15). Recent investigations performed by our laboratory demonstrated that LPS tolerance in experimental human endotoxemia and clinical Gram-negative sepsis is associated with elevated levels of IRAK-M, an intracellular negative regulator of MyD88-dependent TLR signaling (10, 15). The present study, which was performed in parallel with the study of van ’t Veer et al. (15), sought to determine whether in vivo exposure of humans to LPS induces hyporesponsiveness of circulating leukocytes to other TLR agonists that rely on MyD88 signaling and therefore are susceptible to inhibition by IRAK-M. In addition, we investigated whether hyporesponsiveness also occurs upon stimulation of TLR3, which signals via TRIF and not via MyD88 and, if so, whether this resulted from up-regulation of the recently identified TRIF inhibitor SARM. We here show that in vivo LPS exposure of human leukocytes induces cross-tolerance to both MyD88-dependent and MyD88-independent TLR ligands. Remarkably, reduced responsiveness to the TLR3 agonist poly(I:C) was associated with reduced rather than increased mRNA and protein levels of SARM in in vivo LPS-exposed blood leukocytes. These data suggest that human endotoxemia results in a hyporesponsiveness of circulating leukocytes to multiple TLR ligands that likely in part but not exclusively is mediated by enhanced expression of IRAK-M.

The capacity of blood leukocytes to release of cytokines in response to most TLR ligands was drastically reduced in the period of 3–8 h after i.v. LPS administration (38, 39). The identical pattern of cytokine secretion in response to LPS, LTA, poly(I:C), and S-27609 suggests the involvement of similar cells types in blood, but this cannot be explained by the known expression of TLR2, TLR3, TLR4, and TLR7 on blood leukocytes. Monocytes and PMN express TLR2 and TLR4 (40–43), lymphocytes and plasmacytoid dendritic cells (pDC) express TLR7 (44–46), and NK cells express TLR3 (44, 47). The release of low levels of TNF and IL-6 in response to flagellin appears to be in accordance with the
limited expression of TLR5 by monocytes (41). Of note, despite expression of TLR9 by B cells, pDC, and neutrophils (43, 44), addition of bacterial DNA (derived from *E. coli*) did not induce secretion of cytokines in human whole blood (data not shown and Ref. 48). The kinetics of our early transient LPS tolerance model differ from classical LPS tolerance models in experimental animals, which are characterized by an early cell-mediated phase, which dissipates after 3–5 days, and a late Ab-mediated phase (6, 49). The difference in the duration of LPS hyporesponsiveness in these models may result from the LPS dosage and possible species differences in LPS receptor signaling pathways (50, 51).

Since TLR3 and TLR4 ligands trigger the release of type I IFNs via TRIF/IRF3-dependent signaling (52–54) and TLR7 and TLR9 ligands via MyD88/IRF7-dependent signaling (55–57), we also analyzed the release of IFN-α, IFN-β, and IFN-ω after whole blood stimulation. IFN-β was detectable in plasma from control (medium) ex vivo-stimulated blood leukocytes obtained before in vivo LPS challenge (89 ± 42 pg/ml), but none of the TLR stimuli augmented the secretion of this cytokine in blood obtained before or after LPS injection (data not shown). Moreover, neither LPS nor poly(I:C) induced the release of IFN-α or IFN-ω in plasma. Although conflicting results have been published regarding the release of type I IFNs in poly(I:C)-stimulated whole blood (58, 59), our observations appear to be in line with those of Yang et al. (60), who found that human PBMC did not produce IFN-α or IFN-β in response to LPS and produced only tiny amounts of IFN-α in response to poly(I:C). Since IFN-α/β is readily produced by murine blood leukocytes and macrophages in response to LPS or poly(I:C) (52–54, 61), these findings indicate that species-specific differences influence the secretion of type I IFNs in response to TLR3 and TLR4 ligands and add to the notion that human and mouse TLR systems are not completely equivalent (62). Furthermore, we found that the TLR7 ligand S-27609, in contrast to LPS and poly(I:C), induced the release of IFN-α and IFN-ω in blood obtained before i.v. LPS challenge. Compared with the *t* = 0 time point, the level of IFN-α was markedly reduced in blood obtained at 3 h after i.v. LPS. Although we did not determine the cell types in the blood activated by each of the TLR ligands, pDC are presumably the source of IFN-α and IFN-ω in plasma after S-27609 stimulation (63, 64). pDC exist in low numbers in the circulation (<0.5% of blood mononuclear cells) and represent a unique cell type which selectively produces high levels of type I IFNs (as well as other cytokines) in response to viral TLR7 ligands (65, 66) as well as TLR9 ligands (46, 67, 68). Interestingly, these cells become refractory to further stimulation after primary TLR ligand exposure (65).

Our finding that LPS challenge induces cross-tolerance to other TLR ligands is in line with previous in vitro and in vivo studies in mice. Pretreatment of monocytes or macrophages with LPS-induced unresponsiveness to LTA (25, 28, 30), flagellin (26), CpG DNA (28), and *S. aureus* (69), and LPS pretreatment of mice induced cross-tolerance to LTA and CpG DNA (25, 28). In this study, we showed that i.v. LPS challenge induced hyporesponsiveness of human leukocytes to the bacterial TLR2 ligands LTA and PGN, the bacterial TLR5 ligand flagellin, and whole bacteria. Moreover, we found that LPS induced tolerance in human leukocytes to the fungal TLR2 ligand zymosan as well as to poly(I:C) and S-27609, mimicking viral ligands for TLR3 and TLR7, respectively. These results suggest that systemic LPS exposure of human blood leukocytes hampers the inflammatory response to various microbial components.

Various mechanisms for the regulation of LPS tolerance have been described, including altered transcription, disruption of chromatin remodeling, altered cell surface receptor expression, expression of anti-inflammatory cytokines, and induction of negative regulators of TLR signaling (18, 70–82). Recently, we found that several intracellular inhibitors of TLR4 signal transduction, including IRAK-M, MyD88short, A20, SHIP-1, and MKP-1, are expressed in blood leukocytes after i.v. LPS challenge (15). In the latter study, increased expression of MyD88short, A20, SHIP-1, and MKP-1 was found within the first 2 h after LPS injection, whereas IRAK-M expression was strongly induced 4–6 h after i.v. LPS challenge when blood leukocytes were unresponsive to subsequent LPS treatment. IRAK-M negatively regulates TLR signaling at the level of IRAK-1 and IRAK-4 (83), which are essential for activation of the TLR2, TLR4, TLR5, TLR7, and TLR9 pathways (21, 84–89). The induction of IRAK-M in blood leukocytes in our endotoxemia model (15), thus may account for the cross-tolerance to TLR2, TLR5, and TLR7 ligands found in the present study. Our observation that i.v. LPS also induced tolerance to the TLR3 ligand poly(I:C) is consistent with the finding that LPS pretreatment of macrophages negatively influenced the activation of both the MyD88- and TRIF-dependent signaling pathways upon LPS restimulation (24). Recently, SARM was identified as a negative regulator of TRIF-dependent TLR signaling (19), but our analysis of SARM in blood leukocytes before and after i.v. LPS challenge revealed that the expression (both at mRNA and protein level) declined during endotoxemia. These results appear to be in
conflict with those of Carty et al. (19) who reported that LPS did not affect SARM mRNA expression, but rapidly increased protein levels in cultured human PBMC. The differences between our and the latter study may be explained by the clear differences in experimental conditions (in vitro cell cultures vs in vivo LPS exposure). Taken together, our data suggest that factors other than SARM are involved in the regulation of cross-tolerance to poly(lC).

In conclusion, our study shows that the secretion of inflammatory cytokines by human blood leukocytes previously exposed in vivo to LPS is markedly reduced when rechallenged with LPS or with bacterial ligands for TLR2 and TLR5, viral ligands for TLR3 and TLR7, or whole bacteria carrying multiple TLR ligands. These results indicate that an i.v. LPS challenge induces cross-tolerance in human leukocytes to multiple bacterial and viral components and suggest that the cytokine response to opportunistic bacterial and viral infections is hampered after systemic exposure to LPS.

Disclosures
The authors have no financial conflict of interest.

References


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