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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 other TLR ligands. Recently, we found that LPS tolerance in experimental human endotoxia 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.
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

**FIGURE 1.** Intravenous LPS induces release of TNF in plasma, changes in leukocyte numbers, and LPS tolerance in ex vivo-stimulated blood leukocytes. Volunteers (n = 6) were injected i.v. with LPS (4 ng/kg) at t = 0 and blood was collected at the indicated time points for measurement of plasma TNF levels (A), changes in neutrophils (▼), lymphocytes (●), and monocytes (▲) (B), and assessment of ex vivo cytokine release (C–F). Whole blood collected at the indicated time points after i.v. LPS was mixed with an equal volume of RPMI 1640 medium containing LPS (100 ng/ml end concentration) and stimulated for 24 h. Secretion of TNF (C), IL-1β (D), IL-6 (E), and IL-10 (F) in 1/2 diluted plasma was determined by bead array assay. Asterisks indicate significant differences compared with t = 0 (*, p ≤ 0.05 and **, p ≤ 0.01).
agonist (poly(I: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, hematological 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. Pharmacopeia) 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 (Vacutainer Systems; BD Biosciences) 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 \( \mu \)g/ml end concentration; provided by R. Miller, 3M Pharmaceuticals, St. Paul, MN), PGN (S. aureus; 10 \( \mu \)g/ml end concentration; InvivoGen), flagellin (Bacillus subtilis; 1 \( \mu \)g/ml end concentration; InvivoGen), zymosan (Saccharomyces cerevisiae; 20 \( \mu \)g/ml end concentration; Sigma-Aldrich), poly(I:C) (50 \( \mu \)g/ml end concentration; Sigma-Aldrich), and S-27609 (5 \( \mu \)g/ml end concentration; Sigma-Aldrich) was added to inhibit possible contaminating LPS. All stimuli were used at concentrations previously determined to induce cytokines. After incubation for 24 h at 37°C in 5% CO\(_2\), plasma was obtained by centrifugation (1500 rpm at 4°C, 10 min) and immediately stored at \(-20^\circ\)C.

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-\( \alpha \) (Bender MedSystems), IFN-\( \beta \) (Biosource International), and IFN-\( \omega \) (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-\( \alpha \) was 15 pg/ml, for IFN-\( \beta \) was 6 pg/ml, and for IFN-\( \omega \) 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 \( \mu \)M 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 15 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'-ACTGCAAGGATGGGTTGCAT-3'; antisense, 5'-TCAATGGTGCGACTTCGTGATT-3'. Gene expression is presented as a ratio of the expression of the housekeeping gene \( \beta_2 \)-microglobulin, since we previously found that the expression of \( \beta_2 \)-microglobulin in 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, erythrocytes were lysed and the remaining leukocytes were washed twice with ice-cold PBS. The cells were then lysed in SDS-PAGE sample buffer and heated for 5 min at 95°C and stored at \(-20^\circ\)C. An equivalent of leukocyte equivalents of 3 \( \times \) 10\(^7\) cells was separated by 8% polyacrylamide-SDS gel electrophoreses and transferred to polyvinylidene difluoride membranes (Millipore). Membranes were blocked in TBS (pH 7.4) containing 5% nonfat dry milk proteins and 0.1% Tween 20, washed with 0.1% Tween 20.
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 level in whole blood obtained at 24 h after i.v. LPS, except for IL-10. When corrected for monocyte numbers, the main producers of TNF in LPS-stimulated whole blood (38), LPS-induced plasma TNF levels were significantly reduced by ~70, 90, and 75% at 3, 6, and 8 h, respectively (data not shown). However, since various leukocyte subsets could be responsible for the

![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).](http://www.jimmunol.org/)
secretion of cytokines in response to the TLR ligands studied (39), plasma cytokine levels are presented rather than cytokine levels corrected for a specific cell type. Cytokine levels in plasma after a 24-h incubation with medium alone were at or below detection level of the assays (data not shown), except for IL-6 (1402 ± 461 pg/ml at 3 h after i.v. LPS). Ex vivo stimulation of whole blood obtained before i.v. LPS with LPS did not induce the release of IFN-α, IFN-β, or IFN-ω (data not shown).

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 challenge (Fig. 2). LTA-induced cytokine release returned to baseline level in whole blood obtained at 24 h after i.v. LPS. Hyporesponsiveness of leukocytes at 3, 6, and 8 h after i.v. LPS was also found after stimulation of whole blood with 10 μg/ml PGN, although the decline in cytokine release reached only statistical significance for TNF and IL-6 (Table I). However, in contrast to LTA and LPS, PGN-induced IL-6 release did not return to baseline level and remained low in whole blood obtained at 24 h after i.v. LPS. A similar pattern of tolerance in whole blood obtained at 3, 6, 8, and 24 h after i.v. LPS was also observed after stimulation with the fungal TLR2 ligand zymosan (at 20 μg/ml; Table I).

Intravenous LPS induces hyporesponsiveness of leukocytes to TLR5 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 and IFN-ω into 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*, *S. aureus*, and *S. pneumoniae* (Table II). Stimulation with $1 \times 10^8$ 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 $1 \times 10^8$ 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...
cDNA and determined by RT-PCR. Levels were standardized for β2-microglobulin content. B, Whole blood SARM protein expression after i.v. LPS injection administration (4 ng/kg) as determined by Western blot analysis and quantification of SARM by densitometry of protein bands. Asterisks indicate significant differences compared with \( t = 0 \) (**; \( p \leq 0.01 \)).

FIGURE 8. Intravenous LPS induces tolerance in blood leukocytes to E. coli bacteria. 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 heat-killed E. coli (equivalent of \( 1 \times 10^8 \) CFU/ml) 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 \( t = 0 \) (*; \( p \leq 0.05 \) and **; \( p \leq 0.01 \)).

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 cellular-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-ω 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 are 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 Carter 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(I:C).

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.

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TOLERANCE TO MULTIPLE TLR LIGANDS IN ENDOTOXEMIA


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