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Induction of IRAK-M Is Associated with Lipopolysaccharide Tolerance in a Human Endotoxemia Model

Cornelis van ‘t Veer,1*† Petra S. van den Pangaart,1*† Marieke A. D. van Zoelen,1*† Martijn de Kruif,*† Rakesh S. Birjmohun,‡ Eric S. Stroes,‡ Alex F. de Vos,*† and Tom van der Poll*†

Recent in vitro and murine in vivo studies have identified several potential LPS tolerance factors. In this study, we describe the expression kinetics of these LPS tolerance factors in standardized human endotoxemia models using i.v. LPS bolus administration. Responsiveness to LPS as well as the expression of potential regulators of LPS signaling were determined in peripheral whole blood. Intravenous LPS administration (4 ng/kg) resulted in peak plasma levels of TNF-α at 1.5 h followed by subsequent peaks of the classic negative feedback inhibitors A20 and IL-10 at 2 and 3 h, respectively. Circulating blood monocyte counts decimated during the initial inflammatory response, but normalized in the period between 4 and 8 h post-LPS. The LPS response as determined by ex vivo TNF release per monocyte in whole blood was profoundly decreased at 6–8 h post-LPS injection despite cessation of A20 and IL-10 expression after 4 h. Analysis of MyD88short, IL-1R-associated kinase (IRAK)-1, IRAK-M, ST2, suppressor of cytokine signaling-1 and -3, SHIP-1, and MAP kinase phosphatase-1 expression indicated that the observed LPS tolerance was associated with decreased IRAK-1 and elevated IRAK-M expression in this human model. Interestingly, a lower dose of LPS (1 ng/kg) induced LPS tolerance accompanied with IRAK-M up-regulation but without depletion of IRAK-1. In vitro studies in whole blood showed that IRAK-M up-regulation by LPS is largely dependent on TNF-α. The observed rise of IRAK-M transcription in the human endotoxemia model appeared much greater compared with in vitro-stimulated whole blood. In conclusion, LPS tolerance in human endotoxemia models is associated with IRAK-M up-regulation. The Journal of Immunology, 2007, 179: 7110–7120.

Inhibition of the inflammatory reaction has been the goal of most clinical trials to come to improved treatment strategies of sepsis patients. However, potent inhibition of inflammatory mediators such as TNF-α has failed to increase survival of sepsis patients; in contrast, certain strategies even appeared to worsen the outcome (1). The notion that sepsis patients develop a misbalance between proinflammatory and compensatory anti-inflammatory mechanisms (2–4) has led to a more subtle approach in the last decade with hopeful new approaches emerging (1). In innate recognition of pathogens by TLRs plays a crucial role in the resistance to infections (5–7). LPS (endotoxin) is a pathogen-associated molecular pattern on Gram-negative bacteria that is sensed by the innate immune system through TLR4 (8). Furthermore, LPS is considered to be of major importance in the pathogenesis of Gram-negative sepsis. Activation of TLR4 by LPS triggers binding of MyD88 via Mal to the intracellular portion of the receptor and initiates intracellular signaling that evolves by recruitment of IL-1R-associated kinase 4 (IRAK-4)2 and subsequent association and phosphorylation of IRAK-1 (7). Binding of TNFR-associated factor 6 (TRAF6) to phosphorylated IRAK-1 induces the dissociation of IRAK-1/TRAF6 from the receptor to form a plasma membrane-bound complex with transforming growth factor-β-activated kinase (TAK1) (9). Subsequent phosphorylation of TAK1 and degradation of IRAK-1 at the plasma membrane triggers release of the complex to the cytosol where TAK6/TAK1 activates MAPKs and the IkB-kinase (IKK) complex. The IKK complex phosphorylates IkBα which leads to ubiquitylation and degradation of this NF-κB inhibitor. NF-κB liberated of its inhibitor translocates to the nucleus to induce the expression of its inflammatory target genes (7). In parallel, the induced MAPK pathway may generate phosphorylated p38MAPK involved in RNA stabilization of inflammatory genes and AP-1-dependent transcriptional responses (10). LPS activation of TLR4 also triggers a MyD88-independent response that is mediated by the TIR domain containing adaptor inducing IFN-β (TRIF)/TRIF-relaterd adaptor molecule (TRAM) complex. TRIF/TRAM-mediated signaling induces IKK-α- and TBK1-dependent phosphorylation of IFN regulatory factor 3 and nuclear translocation of IFN regulatory factor 3 resulting in IFN-β release and subsequent expression of type I IFN-inducible genes (7, 10, 11). Moreover, whereas TLR4/MyD88 signaling is found to be responsible for early NF-κB activity, TLR4/TRIF appears to be involved in late NF-κB transcriptional activity after LPS stimulation (7).

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2 Abbreviations used in this paper: IRAK, IL-1R-associated kinase; TRAF, TNFR-associated factor; IKK, IkB kinase; SOCS, suppressor of cytokine signaling; TAK1, transforming growth factor-β-activated kinase; TRIF, TIR domain containing adaptor inducing IFN-β; TRAM, TRIF-related adaptor molecule; MKP-1, MAP kinase phosphatase 1.

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In vivo and in vitro treatment with LPS induces the production of proinflammatory mediators, however, LPS stimulation also induces tolerance to a second LPS challenge (10, 11). The physiological significance of LPS tolerance is best demonstrated by the complete protection by a low-dose LPS against the lethal effects of a subsequent high-dose LPS in animals (12). In recent years, various regulators that inhibit LPS signaling have been identified (10, 11). By the use of knockout mice, it was revealed that ST2, a homolog of the IL-1R, and SHIP, a PI3K inhibitor, are potential mediators in LPS-induced tolerance (12, 13).

Important regulators of TLR4 signaling are A20, a classical negative feedback regulator of NF-κB (14, 15) and IRAK-M, an inhibitor of IRAK-1/IRAK-4 (16, 17). Furthermore, a LPS-inducible splice variant of MyD88, MyD88short, which lacks the entire second exon may also attenuate IRAK-1 signaling (18). Importantly, IRAK-1 protein and mRNA expression are found to be down-regulated in vitro LPS stimulation experiments (19, 20). Also MAP kinase phosphatase (MKP)-1, a phosphatase involved in regulation of MAPK activity, was identified as a potential effector of LPS tolerance (21). Moreover, suppressor of cytokine signaling (SOCS)-1-deficient mice are highly susceptible to LPS (22) and SOCS-1 may mediate the degradation of Mal (23), the adaptor protein used by TLR4 and TLR2 in MyD88-dependent signaling. IRAK-1 pro-

\[
\text{MyD88 (full length plus short)} \\
\text{Forward, } TGAATCTGGAGGACAGTCAAT \\
\text{Reverse, } CGGGGCTGAGCAAAACATT \\
\text{Fragment Size: 421}
\]

\[
\text{MyD88 (short specific)} \\
\text{Forward, } GGAGGACAGTCAATGGGAG \\
\text{Reverse, } GAGGAGGGTTCAGTAGGTGG \\
\text{Fragment Size: 250}
\]

\[
\text{SHIP-1} \\
\text{Forward, } AGATTTCCCTTCTGCTGAGG \\
\text{Reverse, } ACCCTTGGCGCTGGAGTCA \\
\text{Fragment Size: 246}
\]

\[
\text{SOCS-3} \\
\text{Forward, } GAGTTGACGCAAAAGAACCT \\
\text{Reverse, } CAGTTGCTGGAAAGCAACCT \\
\text{Fragment Size: 176}
\]

\[
\text{ST2 (membrane plus soluble)} \\
\text{Forward, } TCATAGGACTGGATCCTTG \\
\text{Reverse, } GCCCTGTACCTTGATCCTTG \\
\text{Fragment Size: 202}
\]

\[
\text{B}_{2}-\text{microglobulin} \\
\text{Forward, } CTAGGGCCATACCTTCTCTTTCT \\
\text{Reverse, } GCTCCACCACCTTTTCTACTCT \\
\text{Fragment Size: 185}
\]

Table I. Primer sequences used for real-time PCR using the Light Cycler

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<tr>
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<tr>
<td></td>
<td>Reverse, AGTTGCTCTGGGTCCTTTCTC</td>
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<tr>
<td>IRAK-M</td>
<td>Forward, GTACACTAGACAGGAGGAAACTTT</td>
<td>167</td>
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<tr>
<td></td>
<td>Reverse, GCAGGAAGAGCAGCCCTCCT</td>
<td></td>
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<tr>
<td>MKP-1</td>
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<tr>
<td></td>
<td>Reverse, CTTCCCTTCTGCTTTACCAA</td>
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<tr>
<td>MyD88 (full length plus short)</td>
<td>Forward, TGAATCTGGAGGACAGTCAAT</td>
<td>421</td>
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<tr>
<td></td>
<td>Reverse, CGGGGCTGAGCAAAACATT</td>
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<tr>
<td>MyD88 (short specific)</td>
<td>Forward, GGAGGACAGTCAATGGGAG</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>Reverse, GAGGAGGGTTCAGTAGGTGG</td>
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<tr>
<td>SHIP-1</td>
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<td>Reverse, ACCCTTGGCGCTGGAGTCA</td>
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<td>Reverse, CAGTTGCTGGAAAGCAACCT</td>
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</tr>
<tr>
<td>ST2 (membrane plus soluble)</td>
<td>Forward, TCATAGGACTGGATCCTTG</td>
<td>202</td>
</tr>
<tr>
<td></td>
<td>Reverse, GCCCTGTACCTTGCTCTTTCT</td>
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<tr>
<td>B_{2}-microglobulin</td>
<td>Forward, CTAGGGCCATACCTTCTCTTTCT</td>
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</tr>
<tr>
<td></td>
<td>Reverse, GCTCCACCACCTTTTCTACTCT</td>
<td></td>
</tr>
</tbody>
</table>

**Human endotoxemia models**

The studies were approved by the institutional scientific and ethics committees and written informed consent was obtained from all volunteers. Medical history, physical examination, routine laboratory examination, and electrocardiogram were all normal. The subjects did not participate, use any medication, or have any febrile illness in the month preceding the study. The subjects fasted overnight before LPS administration. On the study day, blood was collected from i.v. cannulas and the subjects were challenged afterward with LPS (Escherichia coli LPS, lot G; U.S. Pharmacopeia) as a bolus i.v. injection as described above (24–27). In total, 24 healthy male subjects with a normal medical history were studied; 8 subjects (age 25–35 years) received LPS at a dose of 4 ng/kg whereas 16 subjects (age 39.9 ± 8.3 years) received LPS at a dose of 1 ng/kg. The latter group consisted of healthy subjects recruited from a Dutch population-based study to identify genes that control high-density lipoprotein cholesterol (HDLc) levels.

**Blood collection**

Blood for cytokine assays, soluble ST2 measurement, leukocyte counts, and differentials were collected in K3-EDTA-containing tubes at the time points indicated. Plasma was prepared by centrifugation at 3000 rpm for 15 min at 4°C and stored at −20°C until assays were performed. Blood for FACS analysis, Western blotting, and ex vivo LPS stimulation was drawn in sodium heparin-containing vacutainer tubes. Peripheral blood mRNA was obtained using the PAXgene tube and PAXgene RNA Isolation System (PreAnalytiX, Qiagen) as described by the manufacturer.

**Ex vivo whole blood LPS tolerance after LPS infusion**

Whole blood anticoagulated with sodium heparin was mixed with an equal volume of RPMI 1640 medium (Invitrogen Life Technologies) and incubated in polypropylene tubes with 100 ng/ml LPS from *E. coli* serotype 0111:B4 (Sigma-Aldrich) for 24 h in a humidified incubator at 37°C and 5% CO₂. Plasma was obtained by centrifugation (1500 rpm at 4°C, 10 min) and stored at −20°C.

**In vitro stimulation of whole blood with LPS**

For in vitro experiments, whole blood of healthy volunteers anticoagulated with sodium heparin was mixed with an equal volume of RPMI 1640 medium (Invitrogen Life Technologies) and incubated in polypropylene tubes with the indicated concentrations of standard preparations of LPS from *E. coli* serotype 0111:B4 (Sigma-Aldrich), or the ultra-pure preparation of this LPS (InvivoGen) for the indicated time in a humidified incubator at 37°C and 5% CO₂. Plasma was obtained by centrifugation (1500 rpm at 4°C, 10 min) and stored at −20°C. In some experiments, the remaining cell pellet was immediately incubated with 9 volumes of ice-cold isotonic erythrocyte lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA (pH 7.4)) for 10 min and centrifuged for 5 min at 1500 rpm at 4°C. Part of the cells were washed with ice-cold PBS, pelleted, and taken up in RLT lysis buffer for RNA isolation according to the RNeasy Mini kit.
protocol (Qiagen). The other part of the cells was washed twice with PBS and taken up in FACS buffer (PBS supplemented with 0.5% BSA, 0.01% NaN₃, and 0.35 mM EDTA) for FACS analysis.

**ELISA and cytometric bead array**

TNF-α and IL-10 levels were measured by CBA multiplex assay (BD Biosciences). Soluble ST2 was determined using a human IL-1 R4/ST2 DuoSet ELISA (R&D Systems).

**FACS analysis and Western blotting**

Whole blood samples were incubated for 10 min with 9 volumes ice-cold erythrocyte lysis buffer and centrifuged at 1500 rpm for 10 min at 4°C. Remaining cells were washed twice with ice-cold PBS. For Western blotting, cells were lysed in SDS-PAGE sample buffer and heated for 5 min at 95°C and stored at −20°C. For FACS analysis, 0.5 × 10⁶ cells were incubated in 25 μl of FACS buffer and mixed with an equal volume of Ab diluted in FACS buffer. All subsequent procedures in the FACS analysis were performed at 4°C, and all FACS reagents were titrated to obtain optimal results as recommended by the manufacturers. Cell surface staining was performed with FITC-labeled mouse anti-human ST2L (clone B4E6; MD Biosciences) and PE-labeled anti-human CD14 (clone M6P9; BD Biosciences). After washing the cells with FACS buffer, at least 10⁴ cells were analyzed using a FACS Calibur flow cytometer (BD Biosciences).

**Evaluation of mRNA levels by quantitative RT-PCR**

Total RNA obtained with the PAXgene System or the RNeasy Mini kit was treated with RQ1 RNase-Free DNase (Promega) and reverse transcribed using oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies) according to recommendations of the suppliers. RT-PCR were performed on cDNA samples that were 4-fold dilutions. The other part of the cells was washed twice with PBS and taken up in FACS buffer (PBS supplemented with 0.5% BSA, 0.01% NaN₃, and 0.35 mM EDTA) for FACS analysis.

**FIGURE 1.** Intravenous LPS administration (4 ng/kg) induces whole blood LPS tolerance in human volunteers. Plasma levels of TNF-α (A) and blood monocyte numbers (B) after i.v. administration of LPS. Ex vivo whole blood LPS tolerance after IV LPS administration (C) expressed as TNF-α production per monocyte after 24 h stimulation with 100 ng/ml LPS. Data are expressed as mean ± SEM. Asterisks indicate significant differences compared with t = 0 (*, p ≤ 0.05; **, p ≤ 0.01).

**FIGURE 2.** Expression of A20. Whole blood A20 mRNA expression after LPS administration (4 ng/kg i.v.) as determined by quantitative RT-PCR. Levels were standardized for β₂-microglobulin content. Data are expressed as mean ± SEM. Asterisks indicate significant differences compared with t = 0 (**, p ≤ 0.01).

**FIGURE 3.** Activation of the IL-10 system. Plasma IL-10 levels (A) and Western blot analysis of STAT-3 phosphorylation (Tyr705) in whole blood leukocytes (B) after LPS administration (4 ng/kg IV). Data are expressed as mean ± SEM. Asterisks indicate significant differences compared with t = 0 (***, p ≤ 0.01). Phospho-STAT-3 Western blots are representative for all eight individuals tested.
the indicated time in hours (of whole blood with 100 ng/ml LPS for indicated cells after in vitro stimulation of whole blood with 100 ng/ml LPS for the indicated time in hours (A), or after in vivo LPS administration (4 ng/kg i.v.) (B). ST2 expression after LPS exposure (black line) was compared with whole blood incubated without LPS (dashed lines in A) and to the expression before LPS injection (dashed line in B). Profile in B is representative for all eight individuals tested. Plasma levels of soluble ST2 (C) and ST2+ST2L mRNA levels in whole blood (D) after in vivo LPS administration (4 ng/kg i.v.). mRNA levels were determined by quantitative RT-PCR and standardized for \( \beta_2 \)-microglobulin content. Data are expressed as mean \( \pm \) SEM. Asterisks indicate significant differences compared with \( t = 0 \) (\( * \), \( p \leq 0.05 \); ++, \( p \leq 0.01 \)).

Expression kinetics of A20 after i.v. LPS

In attempts to unravel the kinetics of the major anti-inflammatory and/or LPS tolerance mediators induced by LPS in the present human endotoxemia model, we first determined the mRNA expression of A20. A20 is a classic negative feedback inhibitor induced directly by NF-\( \kappa \)B. A20 inhibits the generation of active NF-\( \kappa \)B by blocking at the TRAF/IKK level (14, 15). Importantly, experiments in mice indicate that A20 is required for termination of TLR responses (15). A20 mRNA expression was induced strongly in peripheral blood leukocytes as a burst at 2 h after LPS infusion (Fig. 2). It is noteworthy that the A20 peak coincided with the decrease in TNF plasma concentration (see Fig. 1B), and the kinetics of A20 expression in this endotoxemia model are consistent with an early role of this inhibitor in the termination of LPS mediated responses. A20 mRNA levels immediately declined at 4 h reaching near baseline levels at 6 h. The lack of significant A20 expression in the LPS tolerant phase does not indicate a role of A20 in the observed LPS tolerance. Indeed, although A20-deficient cells are hyperreactive to LPS, the lack of A20 does not prevent LPS tolerance induction (15).

Results

Peripheral blood TNF-\( \alpha \), monocyte levels and LPS tolerance after i.v. LPS

Intravenous injection of healthy volunteers with 4 ng/kg LPS resulted in significant release of TNF-\( \alpha \) within 1 h as described before (26, 27) with plasma peak levels of 1507 \( \pm \) 403 pg/ml at 1.5 h and a rapid decline afterward, returning to baseline levels at 3 h after injection (Fig. 1A). The systemic inflammatory response induced by the 4 ng/kg LPS injection was accompanied by a drop in circulating monocytes to 5–10% of the initial count between 1 and 3 h postinjection (Fig. 1B). After 4 h, monocyte numbers started to rise again reaching \( \sim \)50% of the initial values at 6 h and monocyte counts returned to preinjection levels at 8 h. LPS tolerance was monitored by ex vivo released TNF-\( \alpha \) in hepatisized peripheral whole blood stimulated with LPS. Because monocytes are the main producers of TNF-\( \alpha \) in this type of assay (2, 30, 31), the released TNF-\( \alpha \) was standardized for monocyte counts. The release of TNF per monocyte in whole blood was drastically diminished in the period 3–8 h after LPS injection (Fig. 1C). After 24 h, the ex vivo TNF release in whole blood was restored to a level that was no longer significantly different from pre-LPS levels.

FIGURE 4. Expression of SOCS-3. Whole blood SOCS-3 mRNA expression after LPS administration (4 ng/kg IV) as determined by quantitative RT-PCR. Levels were standardized for \( \beta_2 \)-microglobulin content. Data are expressed as mean \( \pm \) SEM. Asterisks indicate significant differences compared with \( t = 0 \) (\( * \), \( p \leq 0.05 \)).

FIGURE 5. Membrane-bound ST2 and soluble ST2 expression. FACS analysis of expression of the membrane bound form of ST2 (ST2L) on CD14\( ^{hi} \) cells after in vitro stimulation of whole blood with 100 ng/ml LPS for the indicated time in hours (A), or after in vivo LPS administration (4 ng/kg i.v.) (B). ST2 expression after LPS exposure (black line) was compared with whole blood incubated without LPS (dashed lines in A) and to the expression before LPS injection (dashed line in B). Profile in B is representative for all eight individuals tested. Plasma levels of soluble ST2 (C) and ST2+ST2L mRNA levels in whole blood (D) after in vivo LPS administration (4 ng/kg i.v.). mRNA levels were determined by quantitative RT-PCR and standardized for \( \beta_2 \)-microglobulin content. Data are expressed as mean \( \pm \) SEM. Asterisks indicate significant differences compared with \( t = 0 \) (\( * \), \( p \leq 0.05 \); ++, \( p \leq 0.01 \)).
Kinetics of the IL-10 system after i.v. LPS

Next, we determined the activity of the IL-10 system in our human model. The release of IL-10 is a major anti-inflammatory pathway that is involved in down-regulation of LPS-initiated processes (32). IL-10 release upon i.v. LPS challenge, as measured in plasma, started at 1 h and peaked at 3 h after LPS injection (512 ± 52 pg/ml; Fig. 3A). Subsequently, IL-10 levels declined rapidly at 4 h and had returned to baseline levels at 6 h. IL-10 mediates its anti-inflammatory potential mainly by STAT-3 phosphorylation (33, 34), and, in agreement, STAT-3 phosphorylation in peripheral blood leukocyte lysates (Fig. 3B) was associated with the observed IL-10 peak at 2–3 h after LPS injection in the present study. Furthermore, IL-10 and STAT-3 phosphorylation had ceased at 6 h, consistent with an early transient activation of STAT-3 by IL-10 in our model. As such, IL-10 and STAT-3 signaling appears associated with the observed LPS tolerance in this model at 3 h post-LPS, and not with the later stage (6–8 h). However, it should be noted that traces of phospho-STAT-3 remained detectable up to 24 h.

Kinetics of SOCS mRNA expression after LPS i.v.

STAT-3 activation will induce expression of SOCS-3, an inhibitor of the IL-6 receptor (35). The feedback inhibition by SOCS-3 of IL-6 signaling, which also uses STAT-3, plays an important role in the differential outcome of IL-10 vs IL-6 signaling (35). Moreover, in septic mice SOCS-3 is expressed mainly in monocytes and granulocytes (36). SOCS-3 mRNA expression in peripheral blood leukocytes increased at 2 h after LPS injection (Fig. 4), in agreement with STAT-3 and IL-10 signaling at that time point. However, despite the cessation of IL-10 release and decline of STAT-3 activation at 6 h, SOCS-3 mRNA levels maintained to be high in the LPS tolerant phase up to 8 h after LPS injection. The role of SOCS-3 in the anti-inflammatory action of IL-10 has been refuted (35) and SOCS-3 does not display functions which are involved in the down-regulation of LPS signaling (37). Thus, although SOCS-3 mRNA is elevated during the LPS tolerant phase in the current LPS i.v. model it is probably not directly involved in the tolerance phenomenon, but may reflect a relative high stability of the SOCS-3 mRNA or a prolonged transcription factor activity of traces of STAT-3 in the present model.

Another member of the SOCS family, SOCS-1, an inhibitor of the JAK/STAT pathway and involved in the down-regulation of IFN signaling, has been described to play a major role in LPS tolerance in mice (22). Using several primer pairs we were unable to detect SOCS-1 mRNA expression by RT-PCR in our blood leukocyte cDNA after LPS injection indicating a lack or...
extremely low expression of SOCS-1 in our endotoxemia model. As human SOCS-1 may be a hard to amplify gene by PCR, we attempted to determine SOCS-1 expression by Western blotting using Abs published to be suitable for recognition of SOCS-1 by this technique, but failed to detect SOCS-1 in leukocyte lysates from our LPS i.v. model. Because we obtained only negative data in regard to SOCS-1 expression, our findings argue against an important role for SOCS-1 in LPS tolerance in the present human LPS i.v. model.

**Kinetics of membrane anchored and soluble ST2 expression after LPS i.v.**

An important LPS tolerance factor in murine LPS endotoxemia models is ST2 (12). The observed LPS tolerance induced by i.v. LPS in our human model was however not associated with up-regulation of membrane anchored ST2L on monocytes (Fig. 5B). In contrast, ST2L was up-regulated on monocytes that were activated by 100 ng/ml LPS in ex vivo whole blood incubations (Fig. 5A). Soluble ST2 was released in plasma of the endotoxemic volunteers from 3 to 24 h after LPS injection, with peak values observed at 8 h (Fig. 5C). The level of soluble ST2 induced by the endotoxemia remained remarkably high at 24 h and was, for instance, at that point not significantly different from the levels observed at 6 h post-LPS. The soluble ST2 observed in plasma appears to originate from the surrounding tissues and not from the blood itself, because LPS did not stimulate soluble ST2 expression in ex vivo-stimulated whole blood (data not shown).

In agreement, mRNA levels of transcripts of the ST2 gene, as evaluated by RT-PCR with a primer pair that recognizes both the ST2 and ST2L, did not increase in blood leukocytes after i.v. LPS injection (Fig. 5D).

**Kinetics of IRAK-M and IRAK-1 expression after LPS i.v.**

Another factor that may cause LPS tolerance is IRAK-M, an inhibitor of IRAK-1/IRAK-4 signaling (17). Importantly, IRAK-M is produced exclusively by monocytes/macrophages (16). IRAK-M mRNA was strongly induced in blood leukocytes of the endotoxemic subjects at 4–6 h after LPS injection (Fig. 6A). At 24 h, IRAK-M levels had normalized to pre-LPS levels. The IRAK-M up-regulation was also observed at the protein level by Western blotting if whole blood leukocyte lysates were loaded corrected for monocyte numbers (Fig. 6, B and C). Less stringent statistical testing with two-tailed paired t tests revealed also significant up-regulation of IRAK-M mRNA ($p < 0.01$) and IRAK-M protein ($p < 0.05$) at 8 h after LPS injection. IRAK-1 transcription was down-regulated in whole blood leukocyte mRNA samples from 4 up to 8 h after infusion of 4 ng LPS/kg (Fig. 6D), this down-regulation of IRAK-1 in blood leukocytes was also observed at the protein level by Western blotting and standardization for $β$-actin content (Fig. 6, E and F). Thus, at 4 ng of LPS/kg an up-regulation of IRAK-M is accompanied by a drop in IRAK-1 levels in whole blood (Fig. 6A), indicating that LPS tolerance is potentially ruled by a change in the IRAK-1/IRAK-M ratio. However, in subjects challenged with a lower LPS dose (1 ng/kg), we observed whole blood LPS tolerance (Fig. 7A) that was associated with IRAK-M up-regulation (Fig. 7C) but without the drop in IRAK-1 mRNA levels (Fig. 7B). The latter indicates that depletion of IRAK-1 mRNA in whole blood is not a prerequisite for LPS tolerance in this human endotoxemia model.

**Kinetics of MyD88short mRNA expression after LPS i.v.**

An inhibitory splice variant of MyD88 lacking exon 2 has been postulated to be involved in LPS tolerance (18). This splice vari-
**FIGURE 9.** SHIP mRNA expression. SHIP expression in blood leukocytes after LPS administration (4 ng/kg i.v.) as determined by quantitative RT-PCR and standardized for β2-microglobulin content. Data are expressed as mean ± SEM. Asterisks indicate significant differences compared with \( t = 0 \) (**, \( p \leq 0.01 \)).

**FIGURE 10.** Expression of MKP-1. Whole blood MKP-1 mRNA expression after LPS administration (4 ng/kg i.v.) as determined by quantitative RT-PCR. Levels were standardized for β2-microglobulin content. Data are expressed as mean ± SEM. Asterisks indicate significant differences compared with \( t = 0 \) (**, \( p \leq 0.01 \)).

Kinetics of SHIP expression after LPS i.v.

SHIP is a phosphatase involved in dephosphorylation of PI3K, and involved in the induction of LPS tolerance in mice (13). We determined the induction of SHIP after LPS i.v. in our subjects at the mRNA and protein level. SHIP transcripts in blood leukocytes were elevated shortly (1–2 h) after LPS infusion, dropped below pre-LPS levels at 4–8 h after LPS and returned to baseline levels at 24 h (Fig. 9). Because SHIP protein may be long lived, at least in in vitro cell cultures (38), we determined whether the observed early SHIP mRNA peak induced a stable protein expression by Western blotting. Multiple forms of 145, 135, and 110 kDa due to C-terminal truncation have been described for SHIP (38). No up-regulation was observed in the period 3–8 h after LPS infusion of any of these species of SHIP using an Ab which recognizes all forms of SHIP (results not shown).

Kinetics of MKP-1 mRNA expression after LPS i.v.

Another phosphatase described to be involved in LPS tolerance in murine cells is MKP-1, an inhibitor of p38MAPK (21). p38MAPK activation is of major importance for TNF release, also in the used human i.v. LPS model (27). However, apart from a modest, though significant, doubling of the MKP-1 mRNA level in the peripheral blood leukocytes at the 2 h time point after LPS injection, no elevation of MKP-1 transcription was observed at later time points (Fig. 10).

**FIGURE 8.** MyD88short mRNA expression. Analysis of MyD88short splice variant in leukocytes after in vitro stimulation of whole blood of two donors with 100 ng/ml LPS for the indicated time in hours (A and B), or after in vivo LPS administration (4 ng/kg i.v.) (C and D). MyD88short lacking exon 2 was detected by RT-PCR qualitatively using sense and antisense primers annealing in exons 1 and 3 (A and C), and quantitatively using a primer specific for the exon 1/3 connection (B and D). MyD88short mRNA levels were standardized for β2-microglobulin content. Data are expressed as mean ± SEM. Asterisks indicate significant differences compared with \( t = 0 \) (**, \( p \leq 0.01 \)).

LPS and TNF dependence of LPS tolerance factors after in vitro whole blood stimulation

The data obtained indicated that IRAK-M expression is one of the major factors to be associated with ex vivo LPS tolerance in the human “low endotoxemia” models. To test whether this could be explained by the dependence of the expression of the different LPS tolerance factors on the triggering LPS concentration, we performed in vitro whole blood stimulations and titrated the LPS to the concentrations of 0.014 or 0.056 ng/ml obtained in the human endotoxemia models if the injected bolus of, respectively, ~70 ng (1 ng of LPS/kg) or ~280 ng (4 ng of LPS/kg) is expected to distribute over the entire blood volume of ~5 L. Neither SHIP nor MKP-1 displayed prolonged up-regulation of mRNA expression in whole blood upon exposure to LPS, consistent with the in vivo data (Fig. 11, F and G). Both IRAK-M and MyD88short mRNA are induced in a similar concentration-dependent manner in whole blood leukocytes by concentrations between 0.01 and 0.1 ng/ml LPS (Fig. 11, A and B), indicating that the observed induction of IRAK-M and lack of induction of MyD88short in vivo is not just a function of the LPS concentration. To test whether the induction of IRAK-M and MyD88short was influenced by cytokines we blocked TNF-α during the whole blood incubations with inhibitory anti-TNF Abs. These incubations showed that LPS-induced IRAK-M expression in whole blood depends largely on exposed TNF-α activity while the expression of MyD88short appears to be TNF-α independent (Fig.
It is striking that MyD88short expression is induced ~10-fold in vitro and stimulation in the in vivo model is virtually absent, while IRAK-M up-regulation is moderate in vitro (~3-fold, Fig. 11A) and fierce (~10-fold, Fig. 6A) in vivo. Apparent from these studies is that IRAK-M expression is differentially regulated in in vivo models compared with other LPS tolerance factors. From our data, it follows that LPS-stimulated IRAK-M production is dependent on TNF-α activity and that IRAK-M production appears to be accelerated in vivo. In the in vitro whole blood stimulations, IRAK-1 down-regulation was not overt and did not display an obvious concentration dependence with regard to the stimulating LPS concentration (Fig. 11E).

Discussion

In this study, we determined the expression of potential LPS tolerance factors in a human standardized endotoxemia model. The study opted to gain more insight in the LPS tolerance that develops in humans following intravascular activation of the innate immune system.
system by a bolus i.v. LPS injection. Identification of the involved LPS tolerance factors in this model might aid in future design and interpretation of this experimental approach in humans (24–27, 39–41). The refractory state of the peripheral blood to produce TNF in response to LPS was established by determination of the amount of TNF released per monocyte because these cells generate the vast majority of cytokines after LPS stimulation of whole blood (2, 30, 31). A bolus i.v. injection of 4 ng of LPS/kg body weight, cleared in healthy volunteers within 45 min from the circulation (42), and induces a hyperinflammatory phase with TNF levels peaking in the peripheral blood at 1.5 h post-LPS injection. This phase is characterized by a drop in monocyte counts up to 4 h, with peak transcription levels of A20 in peripheral blood at 2 h, and IL-10 production and concomitant STAT-3 phosphorylation being raised at 2–3 h after LPS. These observations are in full agreement with the classic direct negative feedback of the NF-κB pathway by A20 (15) and limitation of the innate inflammatory response by IL-10 signaling (32). The monocytes left in the circulation during this phase are hyporesponsive to LPS (Fig. 1C, t = 3 h). After 4 h post-LPS injection, the intravascular inflammatory process resolves in this model as indicated by decline of plasma cytokine levels to basal values and return of monocytes in the peripheral blood. The normalization of monocyte counts in the bloodstream is not associated with significant elevations of A20 and IL-10 expression or STAT-3 phosphorylation in whole blood, whereas the monocytes present in the blood 6–8 h after the bolus LPS injection are notoriously LPS tolerant (Fig. 1). Although expression of membrane-anchored ST2 (ST2L) and MyD88 is short, both known LPS tolerance factors, could be readily induced in in vitro-stimulated whole blood with high LPS concentrations (Figs. 5 and 8), we did not observe increased expression of these inhibitors 6–8 h after the bolus LPS i.v. in vivo challenge. Interestingly, plasma levels of soluble ST2 were dramatically increased during the LPS tolerant phase 6–8 h post-LPS. However, while the intracellular portion of membrane bound ST2L may inhibit TLR4 signaling by binding to Mal or MyD88, (12) this function is not expected of excreted soluble ST2. Rather, soluble ST2 may function as a released scavenger of IL-33 (43). Moreover, at 24 h post-LPS injection plasma levels remained relatively high of soluble ST2 (Fig. 5) while LPS tolerance already had faded.

Of the other potential LPS tolerance factors, we observed up-regulation of SHIP-1 and MKP-1 transcripts in whole blood in the initial 2 h after LPS injection, but not in the later LPS tolerant phase. Of these two, SHIP-1 up-regulation was most profound, and because SHIP-1 protein may have a long half-life in cells (38), we evaluated SHIP-1 protein levels in total blood leukocytes lysates after LPS injection. However, Western blotting of SHIP-1 indicated a lack of SHIP-1 protein expression in the phase 6–8 h after LPS injection, consistent with the observed lowered transcription levels at that time (Fig. 9). MKP-1 levels were only increased 2-fold at 2 h after LPS injection, but had returned to baseline levels after 4 h. This expression profile of MKP-1 does not indicate a major role of MKP-1 in the observed LPS tolerance at 6–8 h post-LPS in the human endotoxemia model. Consistently, recent reports (44–46) unraveled MKP-1 as a dynamic inhibitor of p38MAPK after LPS activation of macrophages, with transcript and protein levels peaking after 1 h LPS stimulation and return of MKP-1 protein levels to baseline within 3 h of stimulation. Based on the above, we do not surmise a role of SHIP-1 or MKP-1 in the present human model.

In the 4 ng of LPS/kg endotoxemia model, we observed that the LPS tolerance phase 6–8 h post-LPS injection was associated with increased whole blood transcript levels of IRAK-M, an inhibitor of IRAK-1/IRAK-4 signaling specifically expressed in monocytes/macrophages, and decreased transcript levels of IRAK-1, a prerequisite in TLR signaling. Depletion of blood leukocytes of IRAK-1 was confirmed at the protein level in the present model. At 24 h after LPS injection, IRAK-M and IRAK-1 levels had normalized (Fig. 6), and whole blood LPS tolerance had faded (Fig. 1C). Interestingly, in a 1 ng of LPS/kg i.v. bolus model, we observed an identical whole blood LPS tolerance at 6 h post-LPS as compared with the 4 ng LPS/kg model, which was associated with IRAK-M up-regulation but not with IRAK-1 depletion. Thus, while 1 ng of LPS/kg i.v. is sufficient to induce LPS tolerance and IRAK-M expression in human endotoxemia models, it appears that 1 ng of LPS/kg is below the threshold to induce IRAK-1 depletion of blood leukocytes in this model, while other mediators such as IRAK-M and A20 (data not shown) showed similar expression kinetics as in the 4 ng of LPS/kg study. We observed only an association of IRAK-M expression with whole blood LPS tolerance in human endotoxemia models, and to our knowledge the tools are presently lacking to scrutinize the functional involvement of IRAK-M in the present model. Whether IRAK-M is involved or not in the induced observed LPS tolerance, IRAK-1 depletion determined at the whole blood level does not seem to be crucially involved in this phenomenon. In monocytes of sepsis patients, IRAK-M is expressed more rapidly upon exposure to LPS compared with those of healthy controls (47), and IRAK-M has just recently been shown to be crucially involved in the fatal effects of pneumonia in mice with abdominal sepsis (48). As such, it appears that antagonizing IRAK-M, although complicated by its intracellular localization, could be a fruitful target to boost the immune system in sepsis patients.

Although we observed IRAK-M expression to be associated with whole blood LPS tolerance in the present human endotoxemia model, it should be noted that other factors than the ones studied herein, such as the NF-κB p50 homodimer mechanism, may have contributed to the observed LPS tolerance phenomenon (49, 50). In this respect, we reported earlier that transcription of the NF-κB p50 gene was unchanged in the human endotoxemia model (29). It should also be noted that other molecules may be involved in the LPS tolerance that are part of a cascade and only expressed transiently for a brief time period. Moreover, it cannot be excluded that residual activity of for instance A20 or phosphorylated STAT-3 influenced the observed LPS tolerance in cooperation with other factors. In this respect, it is noteworthy to mention that SOCS-3 transcription, potentially driven by STAT-3 activation (35) was observed up to 8 h after LPS infusion (Fig. 4), and traces of phospho-STAT-3 remained detectable in whole blood leukocyte lysates up to 24 h post-LPS (Fig. 3).

In our studies, we have chosen to determine the involved parameters in a whole blood environment. This to prevent loss of factors involved in the LPS tolerance phenomena and to prevent aberrations of mRNA expression or protein expression induced by fractionation of blood components. The changes of parameters at the whole blood level in our study will however be affected by the change in leukocyte differentials in the blood induced by endotoxemia (26, 27). Furthermore, the different factors are not always specific for a certain cell type in the blood, or the expression may vary greatly among the different cells. Choosing a minimally altered model system in attempts to reach high biological significance will render lower specificity in contrast to model systems with purified components with great specificity but potentially compromised biological significance. This issue remains a challenge, certainly in the research involving sepsis. With respect to the whole blood LPS tolerance in our study, we attempted to reduce the specificity problem by determination of the amount of TNF released per monocyte, the main producer of this cytokine in
whole blood LPS stimulation assays (2, 30, 31). The choice to determine TNF as target of LPS tolerance is instigated by the crucial role of this cytokine in the resistance to infections and in the pathophysiology of septic shock. The above, and the notion that the majority of the knowledge on factors involving LPS-induced LPS tolerance has been gathered using cells of the monocyte/macrophage lineage (10, 11), has prompted the focus on LPS tolerance of monocytes in our study. Absolute monocyte counts normalized in the endotoxia model between 6 and 8 h post-LPS injection; however, because of the neutrophilia induced by the endotoxia the relative amount of monocytes remained below basal values. Because of this, the parameters specific for monocytes and standardized for amount of total cells (Western blots) or housekeeping gene (RT-PCR) will be underestimated in our study. The observed lack of increased membrane-anchored ST2 expression on monocytes in the human endotoxemia model was assessed by FACS on CD14-positive cells and was not complicated by the above issues. Furthermore, because IRAK-M is specifically expressed by monocytes/macrophages, (16) and because the expression of specific monocyte factors is most likely underestimated in our whole blood mRNA detection assays, we are confident that IRAK-M is significantly up-regulated in monocytes in the LPS tolerant phase in the endotoxia model. To further characterize the IRAK-1 depletions of blood leukocytes in the human endotoxia model, we attempted to stain intracellular IRAK-1 in permeabilized cells and double staining with cell markers, but these experiments are ongoing. Interestingly, we observed that LPS tolerance may develop in the human endotoxemia model in the absence of IRAK-1 depletion in a low-dose LPS study (Fig. 7). We observed no up-regulation of SHIP-1 protein levels in whole blood leukocyte lysates in the LPS tolerant phase 6–8 h after LPS in the endotoxemia model; this finding may argue against a role of SHIP-1 in the observed LPS tolerance, but it should be noted that this determination may not be representative for or may have underestimated the SHIP-1 levels in monocytes.

Two studies have reported changes in the transcriptome by microarrays on RNA of total blood leukocytes of healthy volunteers challenged in the present standardized endotoxemia model (40, 51). Our data on IRAK-M up-regulation and IRAK-1 depletion at the mRNA level after a 4 ng of LPS/kg bolus are largely in agreement with the study of Calvano et al. (40) which used a 2 ng of LPS/kg challenge. The Calvano study reports the transcriptome at 0, 2, 4, 6, 9, and 24 h after the LPS challenge. By sampling at an earlier time point (t = 1 h) and potentially by using a higher LPS dose, we observed a significant up-regulation at an early stage (1–2 h post-LPS) of MKP-1 and SHIP-1 expression that was not reported by Calvano (40). Furthermore, we evaluated SHIP-1 protein expression because this may have a long half-life in cells, and observed no up-regulation of SHIP-1 protein expression at the whole blood level in the LPS tolerant phase (6–8 h), accompanied by down-regulation of SHIP-1 mRNA transcription. Down-regulation of SHIP-1 mRNA at the later time points is in agreement with the Calvano study (40). Our data describing the protein expression of membrane-anchored ST2 and soluble ST2 in this standard endotoxemia model are to our knowledge unique, the two transcriptome studies (40, 51) do not report on the different transcripts of the ST2 gene. Neither of the two microarray studies reports up-regulation of SOCS-1, consistent with the lack of transcripts of SOCS-1 in our RT-PCR assays. With respect to MyD88, we observed no increase of the inhibitory splice variant MyD88short in the LPS tolerant phase in our study, while full-length MyD88 mRNA levels were found to be increased in the Calvano study (40).

Our results indicate that IRAK-M is readily expressed in vivo after low-grade endotoxia in humans; this apparently in contrast to membrane-anchored ST2, SOCS-1, MyD88short, and SHIP expression, factors that may require more or longer exposure to LPS than the challenge used and tolerated in the present human model. In this respect, we found that in vitro-stimulated IRAK-M expression by LPS in whole blood is dependent on induced TNF-α activity (Fig. 11C). Furthermore, the amplitude of IRAK-M gene transcription appeared to be 3- to 4-fold greater after the i.v. LPS bolus in human volunteers compared with that observed after in vitro stimulation of whole blood (compare Figs. 6A and 11A). Our observations implicate that LPS-induced IRAK-M expression is mediated by TNF and can be further accelerated by other factors in the circulation such that the IRAK-M expression in vivo may exceed the IRAK-M expression that can be intrinsically generated in whole blood. Interestingly, del Fresno et al. (52) have shown previously that IRAK-M expression can be induced in vitro in isolated human monocytes by the NO donor GNSO in a TNF-dependent manner. Also, endothelial-derived NO synthase appears crucial in the reactions observed in mice injected with LPS, and indeed NO may enhance TNF-α production by human mononuclear cells (53, 54). Thus, inflammatory reactions dependent on NO may be accelerated through endothelial-derived NO production. Based on the above, it may be hypothesized that the vigorous IRAK-M production obtained by low-dose LPS infusion in humans is caused by TNF-mediated and NO-accelerated processes.

This study indicates that IRAK-M is one of the most likely candidates to be involved in the observed LPS tolerance of monocytes that reappear in the blood circulation after an i.v. LPS bolus challenge of healthy volunteers. Our data further characterize the present human standard LPS model for intravascular activation and deactivation of the innate immune system (24–27, 39–41).

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Disclosures
The authors have no financial conflict of interest.

References


