Toll-like receptors: tools, assays, and implications for in vitro pyrogen tests
Kikkert, R.

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Lipopolysaccharide activation of toll-like receptor 4 is potentiated by human serum and is enhanced in sepsis

Robert Kikkert, Rishi Manoe, Ingrid Bulder, Sacha Zeerleder, and Lucien A. Aarden
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Department of Immunopathology, Sanquin Research, Amsterdam, The Netherlands, and Landsteiner Laboratory, Academic Medical Centre, University of Amsterdam, The Netherlands.

Abstract

Activation of TLR4 by LPS has been generally accepted to be dependent on the presence of LPS-binding proteins as well as the extracellular adaptor molecule MD-2. We noticed that addition of human serum to LPS-stimulated human embryonic kidney (HEK) cells stably-transfected with CD-14 and TLR4 resulted in abundant cell activation and IL-8 production, whereas in absence of serum little IL-8 was produced. As we hypothesized that human MD-2 was involved in the TLR4-potentiating effect, we attempted to purify MD-2 from human serum. Using a combination of size exclusion, anion, and cation chromatography two candidate protein bands were identified. However, mass spectrometry analysis indicated that the candidate protein bands were not MD-2, but suggested that angiostatin, a proteolytic fragment of plasminogen, the expression of which is increased during sepsis, was capable of potentiating TLR4 activation. Follow-up experiments with serum either depleted or enriched for small plasminogen-like molecules, failed to support the hypothesis that angiostatin potentiates the LPS activation of TLR4. Nonetheless, we found that the activation of CD14/TLR4-expressing HEK cells was greatly increased by human serum obtained from septic patients as compared to normal serum, which sheds new light on the findings published by other groups.
Introduction

After the identification of toll-like receptor (TLR) 4 as the cellular lipopolysaccharide (LPS) receptor in 1998, great progress has been made in the understanding of the immune response to Gram-negative bacteria. Following activation of TLR4, several intracellular adaptor molecules such as MyD88, MAL, TIR domain-containing adaptor inducing interferon-beta (TRIF), or TRIF-related adaptor molecule (TRAM) are activated, which in turn orchestrate the activation of several transcription factors ultimately culminating in activation of the transcription factor nuclear factor-kappa B (NF-κB), which controls the expression of an array of inflammatory cytokine genes. An exaggerated response to LPS can contribute to the harmful events of severe sepsis, which include coagulation disorders, (multiple) organ failure, hypotensive shock, and frequently, death. Unravelling of the intracellular signalling components following TLR4 activation has brought the development of therapeutic intervening agents closer, but it has become clear that also extracellular proteins which bind LPS play a crucial role in the activation of TLR4: First, LPS-binding protein (LBP) and CD14 have been long known to enhance LPS responses, and second, the extracellular adaptor molecule MD-2 was claimed to be essential for TLR4 activation.

When human embryonic kidney (HEK) cells stably transfected with CD14 and TLR4 were stimulated with LPS, we surprisingly noticed an almost complete lack of activation by LPS, which was restored when human serum was supplemented. As our group previously observed marked effects of the presence of human serum on LPS stimulation of monocytes, and other groups reported alternative LPS-binding proteins, we set out to purify the serum protein(s) involved in the potentiation of TLR4 activation by LPS. In view of the role of (soluble) CD14, LBP, and MD-2 in the activation of TLR4, the obvious question to answer was whether or not these proteins are involved in the effect of serum.

Materials & Methods

Blood/serum

Blood was obtained from healthy volunteers after informed consent in line with the Sanquin Ethical Advisory Council. Blood samples were collected using endotoxin-free blood collection tubes (Greiner Bio-One, Alphen a/d Rijn, The Netherlands). After incubation at 37 °C for 30 min to allow coagulation, blood samples were centrifuged (for 10 min at 1300 x g) after which the supernatant serum was collected and stored at -20 °C until use. For large scale isolation experiments, serum (250 ml) of healthy voluntary blood
donors was provided by Sanquin Blood Bank North West. Sera from septic patients were obtained in a clinical study.\textsuperscript{12}

**TLR-transfected HEK-cells and bio-assay**

Human Epithelial Kidney 293 (HEK) cells stably transfected with CD14, CD14-TLR2 or CD14-TLR4 were a kind gift from Drs. D. Golenbock and E. Latz, Worcester, MA, USA and have been described elsewhere.\textsuperscript{13} Transfected HEK cells were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM, Bio Whittaker, Verviers, Belgium) supplemented with 5% heat-inactivated foetal calf serum (FCS, Bodinco, Alkmaar, The Netherlands), penicillin 100 U/ml / streptomycin 100 μg/ml (Gibco, Merelbeke, Belgium), 50 μM 2-mercaptoethanol (Sigma-Aldrich, Steinheim, Germany), and 5 μg/ml puromycin (Sigma). HEK cells were cultured in 200 µl wells at 37 °C in the presence of 5% CO\textsubscript{2}, in a humidified incubator.

For stimulation experiments cells were seeded at 5 x 10\textsuperscript{4} cells/well in 96-well flat-bottom microtitre plates (Nunc, Roskilde, Denmark) and stimulated the next day. After 16-20 h of stimulation, supernatants were harvested for determination of IL-8 production, as a read out for NF-κB activation. Fractions obtained after gelfiltration (AcA54) or after anion/cation ion exchange chromatography were tested at a concentration of 6.25 – 12.5% (v/v) in this bio-assay. At these concentrations the inhibitory effect of Tween in gelfiltration/ chromatography buffers (0.02% in buffers, resulting in a final concentration of 0.001% - 0.0025% in the assay) on HEK-CD14-TLR4 cell activation was negligible.

**Drugs, stimuli, mAbs**

Recombinant Lipopolysaccharide-binding Protein (rLBP) from R&D Systems, Abingdon, Oxon, United Kingdom was a generous gift from Dr. A.T.J. Tool, Sanquin Research. A human monoclonal antibody against lipopolysaccharide-binding protein (LBP, clone 1C7) was purchased from Hycult Biotechnology, Uden, The Netherlands. Lipooligosaccharide (LOS), derived from *Neisseria meningitidis* was a generous gift from Dr. J. Poolman, RIVM, Bilthoven, The Netherlands. *E. coli* LPS serotype O55:B5 was from Sigma. *Staphylococcus aureus* Cowan I cells (SAC; Pansorbin) was from Calbiochem, San Diego, Ca, USA. Hirudin (15 U/ml) was a gift from Dr. H. te Velthuis, Sanquin Research. MAb anti-C1q-85, which inhibits activation of C1q by immune complexes, was described before.\textsuperscript{14} C3-2 is a C3-inhibitory monoclonal antibody and was a kind gift from Dr. E. Hack. C1-esterase inhibitor (Cetor®) was a gift from Sanquin Plasma Products. Anti-CD3 murine IgE (CLB.T3/4E) was developed at Sanquin\textsuperscript{15} and obtained from Sanquin Reagents. Recombinant human Angiostatin K1-3 was from Sigma. CLB/AP-1, a monoclonal antibody
raised against plasminogen and polyclonal antibodies specific for plasminogen were produced in our laboratory and were described elsewhere.\textsuperscript{16} Recombinant MD-2 was a generous gift from Y. Kruize, LUMC, Leiden, The Netherlands. IMG-539, a polyclonal antibody raised against a KLH-conjugated synthetic peptide corresponding to amino acids 120-133 of human MD-2 was purchased from Imgenex, Huissen, The Netherlands.

**Size exclusion chromatography**

Human serum was chromatographed on an AcA54 M column (height 90 cm, diameter 5.3 cm, Amersham Biosciences, Uppsala, Sweden) equilibrated in 140 mM NaCl, 10 mM Hepes (pH 7.2) containing 0.02% Tween (Mallinckrodt Baker, Deventer, The Netherlands). Fractions of 12 ml were collected at 72 ml/h. Aliquots (25µl) of these fractions were analysed for absorption at 280 nm and for TLR4-potentiating activity in the HEK-CD14-TLR4 bioassay. Pooled and concentrated Biogel A 1.5 M fractions containing TLR4-potentiating activity were further processed by high performance liquid chromatography (HPLC).

**Anion-exchange chromatography**

After equilibrating a 2 ml Mono Q-Sepharose column (Tricorn Mono Q\textsuperscript{TM} 5/50 GL, Amersham Biosciences) in starting buffer (20 mM Tris-HCl, 0.02% Tween, pH 8.0), the TLR4-potentiating activity-containing sample (0.5-2 ml) that had been dialysed against starting buffer before, was loaded onto the column. The column was washed with 20 ml starting buffer. Elution was performed using a continuous NaCl gradient from 0–1 M NaCl. The eluted fractions from the anion-exchange chromatography were collected (2 ml/fraction) and stored at -20 °C until use. All fractions were tested for TLR4-potentiating activity, after dialysis against PBS.

**Cation-exchange chromatography**

After equilibrating a 2 ml Mono S-Sepharose column (Tricorn Mono S\textsuperscript{TM} 5/50 GL, Amersham Biosciences) in starting buffer (20 mM Na-Ac, 0.02% Tween, pH 4.6), the TLR4-potentiating activity-containing sample (0.5-2 ml) that had been dialysed against starting buffer before, was loaded onto the column. The column was washed with 20 ml starting buffer. Elution was performed using a continuous NaCl gradient from 0–1 M NaCl. The eluted fractions from the cation-exchange chromatography were collected (2 ml/fraction) and stored at 4 °C until use. All fractions were tested for TLR4-potentiating activity, after dialysis against PBS.
**SDS-PAGE, mass spectrometry**

Fractions containing TLR4-potentiating activity were analysed on SDS/PAGE gels (NuPAGE Novex 4-12% or 10% Bis-Tris polyacrylamide gels, Invitrogen) following the manufacturers’ instructions. After electrophoresis, the gels were stained with silver or Coomassie Blue. For N-terminal sequencing of proteins, fraction samples were concentrated by 5% trichloroacetic acid (TCA) precipitation. After washing with ethanol the samples were separated by SDS/PAGE. N-terminal sequencing of protein bands excised from SDS-PAGE gels was performed at the Proteomics Center VUMC, OncoProteomics Laboratory, Dept. Medical Oncology, Amsterdam, The Netherlands. Tryptic digests of protein samples were analyzed by a mass spectrometer (MALDI TOF/TOF Applied Biosystems 4700 Proteomics Analyzer). MS spectra were searched against the Mascot data base search engine (Matrix Science) to identify the proteins.

**Cytokine measurements**

IL-8 was determined by an ELISA kit (Peli-Kine-compact, Sanquin Reagents, Amsterdam, the Netherlands), according to the manufacturer’s instructions. The plates were read in an ELISA-reader (Labsystems Multiskan Multisoft, Helsinki, Finland) at 450 nm, with 540 nm as a reference.

**Results**

**The effect of serum on TLR4 activation**

When human embryonic kidney (HEK) cells, stably transfected with CD14 and TLR4, were stimulated with the TLR4 ligand LOS (or LPS), IL-8 production was only marginally stimulated. In contrast, SAC, a ligand for TLR2, powerfully stimulated IL-8 production in HEK-CD14-TLR2 cells (data not shown). At the time, only limited knowledge was available as to the role of MD-2 in the stimulation of human TLR4. However, multiple studies reported that serum enhanced the pro-inflammatory cytokine response after LPS stimulation. Therefore, we tested whether human serum was able to potentiate IL-8 production in HEK-CD14-TLR4 cells after LOS (LPS) stimulation. Indeed, LOS powerfully stimulated IL-8 production in HEK-CD14-TLR4 cells in the presence of human serum (figure 1A). In the presence of 10% human serum, LOS dose-dependently induced IL-8 production (figure 1B). Moreover, incubation of LOS-stimulated HEK-CD14-TLR4 with different concentrations of human serum indicated that in the presence of less than 1% serum, IL-8 production in HEK-CD14-TLR4 cells was increased as compared to medium
Figure 1. Serum powerfully potentiates LOS-stimulated IL-8 production in HEK-CD14-TLR4 cells. 
A: HEK-CD14 (white bars), HEK-CD14-TLR2 (hatched bars), and HEK-CD14-TLR4 (black bars) cells were left untreated (control), stimulated with LOS (10 ng/ml), or stimulated with LOS (10 ng/ml) in the presence of 10% human serum (see Materials & Methods for details). After 16-20 h of stimulation supernatants were harvested for measurement of IL-8 production. A representative of at least 5 experiments is shown.
B: Stimulation of HEK-CD14-TLR4 cells with LOS in presence of 10% human serum. A representative of three experiments is shown.
C: Incubation of HEK-CD14-TLR4 cells in the absence (open circles) and presence (10 ng/ml, closed circles) of LOS with different concentrations of human serum.
control (figure 1C). Only sera from humans were capable of potentiating LOS-stimulated IL-8 production in HEK-CD14-TLR4 cells as sera obtained from various mammals (mice, rats, rabbits, pigs, goats, cows, horses, baboons) did not exhibit the TLR4-potentiating effect (data not shown). The effect of serum was lost after heating for 30 min at 56°C (not shown). No differences were found between serum, citrate plasma, and heparin plasma in the TLR4 potentiating effect (data not shown).

**LPS-binding Protein**

An obvious candidate for the serum protein responsible for the increased TLR4 activation was LPS-binding protein (LBP). Therefore, we stimulated HEK-CD14-TLR4 cells with LPS in the absence/presence of LOS, human serum, recombinant LBP, and/or neutralizing antibodies to LBP. LBP failed to potentiate LOS-stimulated IL-8 production and a neutralizing antibody to LBP failed to inhibit the LOS-induced IL-8 production in HEK-CD14-TLR4 cells in the presence of human serum (figure 2).

**Size Exclusion Chromatography**

As LBP was not the serum protein responsible for the TLR4 potentiating effect, MD-2 was the most likely candidate to be involved. However, at that time, no MD-2-recognizing mAbs were available to investigate the serum effect. To identify the molecular size of the TLR4-stimulating bioactivity size, human serum was separated by size exclusion chromatography (SEC) using an Ultrogel AcA54 column, after which the fractions were tested for TLR4-potentiating activity (figure 3). Fractions containing TLR4-potentiating activity (typically fraction 72 – 95) were pooled, concentrated and subjected to another round of SEC which separated the fractions containing TLR4-potentiating activity from fractions predominantly containing human albumin. SEC using both an AcA54 column and a Superdex 200HR 10/30 column, in the presence of molecular weight markers, indicated that the TLR4-potentiating activity eluted at approximately 30-33 kDa.

**Ion Exchange chromatography**

To further characterize the serum protein(s) with TLR4-potentiating activity, pooled and concentrated AcA54 fractions were separated by mono Q anion exchange chromatography (see materials & methods). HEK-TLR4-potentiating activity eluted in one peak. As expected, the mono Q fractions showing activity (fraction 20 and 21, fig. 4B) still contained several proteins (see fig. 4A). In addition, the elution profile of none of the protein bands coincided with the biological activity (not shown). Therefore, mono Q fraction 20 and 21 were combined and separated by cation exchange using a Mono S column (figure 5A). Mono S fraction 20, and to a lesser extent fractions 19 and 21, showed activity in the assay.
Potentiation of LPS-activated TLR4 by human serum

Figure 2. The role of LBP in the potentiation of LOS-stimulated IL-8 production in HEK-CD14-TLR4 cells.

HEK-CD14-TLR4 cells were stimulated with LOS (10 ng/ml), LOS + LBP (250 ng/ml), LOS + 5% human serum, or LOS + 5% human serum in the presence of a LBP neutralizing antibody (10 µg/ml). Data represent mean IL-8 production ± SEM of duplicate samples.

(figure 5B). SDS-PAGE showed that this activity co-eluted with a protein band with an estimated molecular weight of 35 kDa under non-reducing conditions (fig. 5C). Reduction led to a shift towards a slightly higher molecular weight. These bands were excised. N-terminal mass spectrometry analysis revealed that the excised protein bands from both the reduced and non-reduced gel were identical and that the N-terminal sequence corresponded to that of angiostatin (figure 6), an internal fragment of plasminogen.

Angiostatin

Mini-plasminogen molecules such as angiostatin have been observed in septic patients. In addition, it was reported that TLR4-potentiating activity is present in sera from septic patients, and not in sera from healthy volunteers. Therefore, sera derived from 40 well-characterized septic patients were tested for the TLR4-potentiating effect. Although serum from healthy controls powerfully stimulated IL-8 production in HEK-CD14-TLR4 cells, we found that the sera from septic patients were more active as compared to sera from healthy controls (figure 7). Because we surmised that cleavage from plasminogen of small plasminogen-like molecules such as angiostatin may occur during coagulation, and that this
Figure 3. Partial purification of TLR4-potentiating serum fractions by gel filtration.

Human serum (40 ml) was chromatographed on a Ultragel AcA54 column (10 X 60 cm) equilibrated in 140 mM NaCl, 20 mM Hepes (pH 7.2) containing 0.02% Tween. Fractions of 12 ml were collected at 72 ml/h. Aliquots of these fractions were analysed for absorption at 280 nm (left Y-axis, open circles) and activity (as defined by potentiation of LOS-stimulated IL-8 production in HEK-CD14-TLR4 cells, right Y-axis, closed circles). Activity was determined by stimulation of HEK-CD14-TLR4 cells with LOS (10 ng/ml) in the presence of 25% v/v of AcA54 Ultragel fractions. The void volume eluted at fraction 48. A representative of six AcA54 runs is shown.

may be increased in septic sera, we depleted human serum from plasminogen using a previously described method. However, no differences could be found in the potentiation of LPS-induced HEK-CD14-TLR4 activation between normal human serum and plasminogen-depleted serum (data not shown). We also stimulated HEK-CD14-TLR4 cells with LPS in the presence of a recombinant fragment (K1-3) of angiostatin or serum. Whereas serum potentiated IL-8 production in LPS-stimulated HEK-CD14-TLR4 cells, angiostatin K1-3 at concentrations ranging from 1 to 10,000 ng/ml did not (data not shown).
Discussion

Responses to LPS of TLR4-expressing cells such as monocytes were shown to be enhanced by human serum,\textsuperscript{17,18} which was presumed to be caused by serum proteins such as LBP and soluble CD14. Muta & Takeshige\textsuperscript{21} reported that human serum potentiated LPS-stimulated NFκB-driven luciferase activity in CD14- and TLR4-transfected HEK cells, a cellular system similar to the one used in our studies. Moreover, in this study the effect of incubation with 5% human serum on LPS-stimulated HEK-CD14-TLR4 cells was comparable to incubation with 250 ng/ml LBP. However, in our experiments (figure 2) we were unable to demonstrate a role for LBP in the potentiation of TLR4. Perhaps this is not surprising as LBP merely functions to enhance the presentation of LPS to CD14, a co-receptor which is highly expressed on the CD14-TLR4-transfected HEK cells used. Moreover, LBP was reported to be stable at high temperatures,\textsuperscript{17} and because we found a marked loss in the TLR4-potentiating activity of serum after incubation at 56 °C (not shown), it is improbable that in our assays LBP is the serum factor conferring the TLR4-potentiating effect.

Because of the high expression of CD14 on HEK-CD14-TLR4 cells it is also difficult to envisage that soluble CD14 in human serum would be responsible for the TLR4-potentiating effect in our assays. Indeed, we observed that very high concentrations of anti-CD14 mAbs only slightly inhibited LPS-induced IL-8 production, indicating that additional soluble CD14 is not required for TLR4 activation. Moreover, there is general consensus that LBP and CD14 are not absolutely required for LPS responses.\textsuperscript{22}

Apart from these serum proteins we also investigated the role of the coagulation/complement system. TRL4 was originally discovered as the human homolog of the \textit{Drosophila} Toll protein\textsuperscript{23} which is activated by Spätzle in response to pathogens via particular serine proteases.\textsuperscript{24,25} Remarkably, there is considerable evolutionary homology in the serine protease cascades of the coagulation, complement, and innate immune system.\textsuperscript{26,27} The pro-inflammatory role of coagulation\textsuperscript{28,29} together with reports demonstrating that 1) activation of human TLR4 was inhibited by a serpin (serine protease inhibitor),\textsuperscript{30} and 2) Drosophila mutants lacking particular serpins exhibited increased Toll activation,\textsuperscript{31} prompted us to investigate the involvement of serine proteases in the activation of TLR4. However, protease inhibitors of coagulation or inhibitors of the complement system such as hirudin, tissue factor plasminogen inhibitor (TFPI), C1-esterase inhibitor, anti-C1q-85 mAb, and anti-C3-2 mAb failed to inhibit the serum-mediated activation of TLR4 by LPS (data not shown).

Thus, because no role for LBP, soluble CD14, or for the coagulation/complement system in the potentiation of TLR4 could be demonstrated, we next sought to isolate the
**Figure 4. purification ion of TLR4-potentiating activity by Mono Q**

A: Mono Q chromatography of pooled AcA54 fractions containing TLR4 potentiating activity (solid line = OD280; dashed line = % buffer B). The fractions containing TLR4-potentiating activity are indicated by arrows).

B: TLR4-potentiating activity of fractions after mono Q chromatography. Fractions were dialysed against PBS and tested (12.5% v/v) in duplicate.
responsible factor(s), possibly being MD-2, from human serum. As no mAbs raised against MD-2 were available to study the TLR4-potentiating effect, we isolated the TLR4-potentiating activity by size-exclusion chromatography. Because the activity eluted from the column at a molecular mass of 30-33 kDa (fig. 3), it was clear that LBP and soluble CD14, with a molecular mass of 60 kDa and 55 kDa, respectively, were not involved in the TLR4-potentiating effect. Data on the molecular mass of MD-2 are less clear-cut. Recombinant monomeric MD-2 has a molecular mass ranging from 18-25 kDa, dependent on its glycosylation.32,33 However, several research groups found that MD-2, when recombinant expressed, exists as heterogeneous large disulfide-linked oligomers ranging in molecular mass from 30 – 250 kDa.32,34,35 Only the monomeric form of MD-2 is able to facilitate TLR4 activation by LOS/LPS.34 Using mAbs raised against recombinant MD2, human normal plasma MD-2 was reported to exist in oligomers. However, MD-2 in septic sera was apparently also present in monomers.36 In contrast, Pugin et al.,20 found that, also by using size exclusion chromatography, MD-2 activity in sera from sepsis patients eluted at a molecular weight of approximately 60 kDa. Thus, the finding that in our experiments, TLR4-potentiating activity in human normal serum eluted at 30-33 kDa, was in conflict with both Wolfs (monomeric MD-2 only present in septic serum)36 and Pugin (MD-2 activity eluted at 60 kDa).20

In our experiments, following a combination of size exclusion, cation, and anion chromatography, one single fraction with TLR4-potentiating activity was obtained. When this particular fraction was compared with neighbouring chromatography fractions on SDS-gels, a distinct protein band was identified as a candidate for the TLR4-potentiating effect. N-terminal mass spectrometry analysis revealed that the candidate protein band was angiostatin. As small plasminogen-like molecules such as angiostatin have been reported to be increased in the serum of septic patients19 and because in other studies, TLR4-potentiating activity was reported to be present in serum of septic patients, but not of healthy controls,20 we compared both types of serum. In contrast with Pugin et al. we found TLR4-potentiating activity also in normal sera, but in agreement with Pugin et al.20 this was greatly increased in septic sera. Serum depleted for plasminogen (>99% depletion) exhibited similar TLR4 potentiation as normal serum. Also the use of a recombinant fragment of angiostatin/plasminogen (K1-3) did not support the hypothesis that the TLR4-potentiating effect of human serum was mediated by angiostatin. Angiostatin, which comprises four so-called Kringle domains (K1-4), is cleaved from plasminogen, which contains five Kringle domains (K1-5), by the action of neutrophil elastase.37 The 4th Kringle domain (K4) contains five lysines that constitute a positively charged area,37 which may be involved in the binding of negatively charged LPS. Also lysine residues in a particular region of the MD-2 molecule have been shown to play a crucial role in the LPS-signalling
Figure 5. purification ion of TLR4-potentiating activity by a combination of Mono Q and mono S-
sepharose chromatography.
A: Mono S chromatography of pooled mono Q fractions containing TLR4 potentiating activity (straight line
= OD280; dashed line = % buffer B). The fraction containing TLR-potentiating activity (fraction 20) is
indicated by an arrow.
B: Bioassay of fractions after a combination of mono Q and mono S chromatography. Fractions were
dialysed against PBS and tested 12.5% v/v.
C: Mono S fractions were TCA precipitated (see materials and methods) and separated by SDS-PAGE.
Protein bands indicated by arrows were excised for mass spectrometry analysis.
and TLR4-activating properties of MD-2.\textsuperscript{38,39} Because the epitope of the mAb used for the depletion of plasminogen from human sera has not (yet) been mapped, it is conceivable that it binds to the 5\textsuperscript{th} Kringle domain (K5) of plasminogen and therefore is unable to deplete angiostatin (K1-4) from human serum. Similarly, the angiostatin fragment (K1-3) used may exert no effect on TLR4 potentiation if the 4\textsuperscript{th} Kringle domain of angiostatin is required for the potentiating effect. Future experiments will be required in order to elucidate the possible involvement of the 4\textsuperscript{th} Kringle domain in the TLR4-potentiating effect.

We have also considered the possibility that the serum factor responsible for the TLR4-potentiating effect is co-purified with angiostatin without being detectable in SDS-PAGE. It was reported that picomolar concentrations of endotoxin and recombinant MD-2 at nanogram per millilitre doses, stimulate TLR4.\textsuperscript{40} In our experiments, detection of the presence of native MD-2 in serum and active fractions is prohibited by a lack of available MD-2-recognizing mAbs. Like Visintin \textit{et al.},\textsuperscript{22} we were also unable to demonstrate the presence of native MD-2 either in human serum or in active serum fractions by Western-blotting with a commercially available polyclonal antibody against MD-2 (data not shown). Although it is highly unlikely that in our studies, MD-2, co-purified with angiostatin, exerts the TLR-4 potentiating effect, other research groups have proposed that MD-2 is a biologically active serum constituent.\textsuperscript{22,38} Using soluble human TLR4-chimeric molecules (sTLR4-Fc) Visintin \textit{et al.}\textsuperscript{22} were able to deplete “MD-2 activity” from human serum, which was restored by supplementation of recombinant MD-2. Although these experiments suggested that MD-2 is present in human serum, the authors acknowledged that their evidence was indirect.\textsuperscript{22} After all, the sTLR4-Fc molecules would deplete any (other)
Figure 6. The amino acid sequence of human angiostatin. (NCBI gi|21465835). Boxed areas indicate fragments of angiostatin that were identified by mass spectrometry.

TLR4-binding serum protein and recombinant MD-2 may constitute one of several proteins involved in TLR4 potentiation. As in our studies, but in conflict with the study by Pugin et al., Visintin et al. also reported potentiation of LPS-stimulated TLR4 activation by normal human serum. Pugin et al. used HEK293 cells transfected with TLR4 only, and found strong effects of neutralizing mAbs against LBP and CD14 on TLR4 activation, indicating that the effects of septic serum might be caused by up-regulated levels of soluble CD14 and LBP. Nonetheless, Pugin also reported increased MD-2 mRNA levels in sera from septic (shock) patients as compared to serum from healthy controls. Using the same HEK-TLR4 cells and using mAbs raised against recombinant MD-2, Wolfs et al. recently also reported TLR4 potentiation by sera from septic, but not from normal donors. Also using mAbs raised against recombinant MD-2, Viriyakosol et al. demonstrated that MD-2 was greatly increased in the serum of septic patients as compared to the serum of healthy controls. Nonetheless some problems remain: first, Pugin et al. and Wolfs et al. reported potentiation of TLR4 activation only by septic sera whereas Visintin et al. and our group additionally found potentiation of TLR4 by normal serum. Second, only indirect evidence has been presented to support the presence of MD-2 in human serum, and the possibility of other serum proteins involved in the potentiation of LPS-induced TLR4 activation cannot be ruled out and merits further research. Moreover, Visintin et al. concluded that MD-2 is present in human serum with a concentration of 50 nM, corresponding to 1.9 µg/ml. If MD-2 were present in such high concentrations it is highly surprising that we and other groups were unable to isolate MD-2 from human serum. Remarkably, the anti-MD2 mAbs used by Viriyakosol and Wolfs demonstrated a MD-2 protein with a molecular weight of 18 – 25 kDa, whereas size exclusion and ion exchange chromatography performed by Pugin revealed that the TLR4-potentiating fractions eluted at approximately 60 kDa. This is particularly surprising because a number of studies have indicated that LPS activates TLR4 only when (recombinant) MD-2 is present in monomeric form, suggesting that other serum proteins may be involved. Future experiments will be necessary.
Potentiation of LPS-activated TLR4 by human serum

Figure 7. The potentiating effect of serum on IL-8 production in LOS-stimulated HEK-CD14-TLR4 cells is increased in sepsis patients.
HEK-CD14-TLR4 cells were stimulated with LOS (10 ng/ml) in the presence of serum (5%) from healthy individuals or from sepsis patients. Indicated is individual and median IL-8 production. (** P<0.0001, Mann-Whitney U-test).

to formally demonstrate the presence of native MD-2 in human serum and to elucidate the role of angiostatin in the potentiation by human serum of LPS-stimulated TLR4.

To summarize, we found that activation by LPS of TLR4 is potentiated by human normal serum and even further enhanced by serum from septic patients. Chromatography data suggested that angiostatin derived from human serum was involved in the TLR4-potentiating effect, but this remains to be corroborated. Meanwhile, recent developments in TLR research suggest an important role for serum proteins in the regulation of multiple TLRs. Notably, the serum protein vitronectin in collaboration with integrin β3 receptors expressed on monocytes was recently reported to potentiate bacterial lipoprotein (BLP) activation of TLR2. Thus, several as yet undiscovered serum proteins might play a role in the regulation of TLR activation, and angiostatin might constitute one of the proteins involved in HEK-TLR4 activation.
References


