Protection from lethal Gram-negative bacterial sepsis by targeting Toll-like receptor 4

Thierry Roger*,†, Céline Froidevaux*,†, Didier Le Roy*,†, Marlies Knaup Reymond*, Anne-Laure Chanson*, Davide Mauri†, Kim Burns†, Beat Michel Riederer†, Shizuo Akira* and Thierry Calandra*,#

*Infectious Diseases Service, Department of Medicine, Centre Hospitalier Universitaire Vaudois and University of Lausanne, CH-1011 Lausanne, Switzerland; †Aptech Biochemicals, CH-1066 Epalinges, Switzerland; ‡Department of Biochemistry, University of Lausanne, CH-1066 Epalinges, Switzerland; †Department of Cell Biology and Morphology, University of Lausanne, CH-1005 Lausanne, Switzerland; and †Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0871, Japan

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Toll-like receptor 4 (TLR4), the signal-transducing molecule of the LPS receptor complex, plays a fundamental role in the sensing of LPS from Gram-negative bacteria. Activation of TLR4 signaling pathways by LPS is a critical upstream event in the pathogenesis of Gram-negative sepsis, making TLR4 an attractive target for novel antisepsis therapy. To validate the concept of TLR4-targeted treatment strategies in Gram-negative sepsis, we first showed that TLR4−/− and myeloid differentiation primary response gene 88 (MyD88)−/− mice were fully resistant to Escherichia coli–induced septic shock, whereas TLR2−/− and wild-type mice rapidly died of fulminant sepsis. Neutralizing anti-TLR4 antibodies were then generated using a soluble chimeric fusion protein composed of the N-terminal domain of mouse TLR4 (amino acids 1–334) and the Fc portion of human IgG1. Anti-TLR4 antibodies inhibited intracellular signaling, markedly reduced cytokine production, and protected mice from lethal endotoxic shock and E. coli sepsis when administered in a prophylactic and therapeutic manner up to 13 h after the onset of bacterial sepsis. These experimental data provide strong support for the concept of TLR4-targeted therapy for Gram-negative sepsis.

The incidence of sepsis is rising, and the mortality remains high, reaching 25%–30% in patients with severe sepsis and 50%–60% in those who develop septic shock (1). Despite initial encouraging results, the benefits of most new antisepsis therapies (e.g., drotrecogin-alpha activated, corticosteroids, intensive insulin therapy, and vasopressin) remain uncertain (2). Thus, encouraging results, the benefits of most new antisepsis therapies (e.g., drotrecogin-alpha activated, corticosteroids, intensive insulin therapy, and vasopressin) remain uncertain (2). Thus, identification of new treatment options for septic patients requires further investigation.

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Regulation of innate immune responses is a delicate balancing act, and dysregulated innate immune reactions, by either default or excess, have dramatic consequences for the infected host, as seen in severe sepsis. Given its central role in the pathogenesis of Gram-negative sepsis, TLR4 is a target of choice for the development of novel antisepsis therapies. Here we report that anti-TLR4 antibodies raised against the ectodomain of TLR4 improved survival in experimental models of Gram-negative bacterial sepsis when administered both prophylactically and therapeutically.

**Results**

**TLR4 and MyD88 Are Critical Effector Molecules in Escherichia coli Sepsis.** To validate the concept of immunomodulation of the TLR4 activation pathway as a treatment strategy for Gram-negative sepsis, we studied cytokine production profiles and survivals of wild-type (WT), TLR4−/−, TLR2−/−, and MyD88−/− mice in a model of lethal peritonitis induced by E. coli, the most common cause of Gram-negative sepsis (15). Given the critical role played by TLR2 in the sensing of Gram-positive bacteria and some Gram-negative bacteria (16, 17), we used TLR2−/− mice as controls. At 4 h after bacterial challenge, very high concentrations of bioactive TNF were detected in the circulation of the WT and TLR2−/− mice (median, 6.5 ng/mL vs. 9.7 ng/mL; P = 0.05) (Fig. 1L). In contrast, TNF was either strikingly reduced or undetectable in the TLR4−/− and MyD88−/− mice (0.5 and 0 ng/mL, respectively; P = 0.002). Likewise, circulating levels of bioactive IL-6 were much higher in the WT and TLR2−/− mice (8.0 and 10.6 ng/mL; P = 0.31) than in the TLR4−/− and MyD88−/− mice (4.2 and 2.0 ng/mL; P = 0.04 and 0.002) (Fig. 1B). Blunted proinflammatory responses were associated with full survival of the TLR4−/− and MyD88−/− mice, whereas all but 1 of the WT and TLR2−/− mice died (P < .001) (Fig. 1C). This indicates that the activation of TLR4, but not TLR2, is critical to the host response to E. coli sepsis.


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Anti-TLR4 Antibodies Inhibit Innate Immune Responses Induced by LPS and Gram-Negative Bacteria. mTLR4-Fc was used to generate high titers of rabbit anti-mouse TLR4 antibodies, which were purified through a 3-step procedure as described in Materials and Methods. Specificity was confirmed by demonstrating that anti-TLR4 antibodies recognized mTLR4-Fc but not an irrelevant chimeric fusion protein (mGITR-Fc) by ELISA (Fig. 2A), and also by the staining of WT but not TLR4°/° mouse peritoneal-derived macrophages by flow cytometry (Fig. 2B). We then studied the capacity of anti-TLR4 antibodies to inhibit responses of innate immune cells stimulated with LPS in vitro. Compared with control antibodies, anti-TLR4 antibodies strongly inhibited LPS-induced intracellular signal transduction, as demonstrated by the luciferase reporter activity driven by NF-κB in RAW 264.7 macrophages (Fig. 2C) and by phosphorylation of ERK1/2 in bone marrow-derived macrophages (Fig. 2D). Anti-TLR4 antibodies also markedly inhibited LPS- and E. coli–induced TNF and IL-6 production by RAW 264.7 macrophages and by mouse whole blood (Fig. 2E–H and data not shown). In contrast, anti-TLR4 antibodies did not affect signal transduction or cytokine production by macrophages or by whole blood stimulated with other TLR ligands, such as Pam3CSK4 (Fig. 2C and D), peptidoglycan (Fig. 2E), and cytosine guanine dinucleotide (CpG) oligonucleotides (ODNs) (Fig. 2F–H). The biological activity of anti-TLR4 antibodies also was demonstrated through a proof-of-principle approach described in Materials and Methods.
Anti-TLR4 Antibodies Protect Against Lethal Endotoxemia. Affording protection against lethal endotoxemia is important in patients with fulminant meningococcal sepsis (19). We explored the protective capacity of the anti-TLR4 antibodies in a model of endotoxin in D-galactosamine–sensitized mice. Consistent with the results observed in vitro, anti-TLR4 antibodies given i.p. 15 min before an LPS challenge almost completely eliminated TNF production (P < .0001) (Fig. 3A) and strongly reduced IL-6 production (P = .005) (Fig. 3B). Of note, the amount of TNF produced by mice treated with anti-TLR4 antibodies was comparable to that produced by TLR4−/− mice. Prevention of cytokine release by anti-TLR4 was associated with improved survival (94% in anti-TLR4 and 92% in TLR4−/− mice; P = .0001) (Fig. 3C). Time-course analyses of the magnitude and duration (up to 60 h) of the inhibition of cytokine production and protection afforded by a single dose of anti-TLR4 antibodies against lethal endotoxemia (Table S1) suggests the possibility that anti-TLR4 treatment also could work when given after the LPS challenge. Indeed, as shown in Fig. 3D, anti-TLR4 treatment remained fully protective when given up to 4 h after LPS exposure (P = .025). Anti-TLR4 antibodies did not protect mice from toxic shock induced by Pam3CSK4, a Gram-positive lipopeptide and activator of TLR1-TLR2 heterodimers (Fig. S3), providing evidence of TLR4 specificity.

Anti-TLR4 Antibodies Protect Against Lethal Live E. coli Sepsis. We studied the impact of anti-TLR4 antibodies in a classical Gram-negative bacterial sepsis model induced by an i.p. injection of live E. coli, the most frequent cause of bacterial sepsis in humans (15). Prophylactic administration of anti-TLR4 antibodies led to a 5-fold reduction in the median circulating TNF level (4.2 ng/mL in controls vs. 0.8 ng/mL in anti-TLR4; P < .005), a 2-fold reduction of IL-6 (11.1 vs. 6.3 ng/mL; P < .005) (Fig. 4A), and a striking increase in survival (0 vs. 80%; P < .0001) (Fig. 4B).

To test anti-TLR4 antibodies in a condition mimicking their clinical use in patients with sepsis, we administered therapy after the onset of infection in 2 different severity models. In the first model, mice were challenged with a high E. coli inoculum (2 x 10^9 cfu), which caused a fulminant, rapidly lethal sepsis. Delayed (1 h) administration of anti-TLR4 was associated with increased survival rate (30% vs. 10%; P = .02) and prolonged survival time (median time to death, 30 h in anti-TLR4 mice vs. 4 h in control mice; P = .008) (Fig. 4C). In the second model, mice were challenged with a lower E. coli inoculum (2 x 10^7 cfu), which caused an acute but less fulminant course of sepsis. Initiation of anti-TLR4 therapy as much as 13 h after the onset of infection, at which point clinical signs of sepsis were established and circulating levels of endotoxin were elevated (mean ± SD, 13.1 ± 15.2 ng/mL; range, 2.91–45.7 ng/mL; n = 7), remained associated with improved survival (75% vs. 30%; P = .03) (Fig. 4D). Together, these results demonstrate that anti-TLR4 antibodies are highly efficacious as adjunctive therapy for E. coli sepsis, with a window of clinical application including both prophylactic and therapeutic intervention modalities.

Discussion

Major breakthroughs in our understanding of the pathogenesis of Gram-negative sepsis are providing new treatment opportunities for severe sepsis and septic shock. For example, TLR4 and MD-2 have recently emerged as critical sensors of LPS (4–6, 20). As the signal-transducing component of the LPS receptor complex, TLR4 is a very attractive target for new antisepsis therapy. Here we provide compelling experimental evidence supporting the efficacy of anti-TLR4 adjunctive therapy for Gram-negative sepsis. Using a recombinant chimeric fusion protein composed of...
of the N-terminal and central domains (amino acids 1–334) of the extracellular part of TLR4 and the Fc portion of human IgG1, we produced anti-TLR4 antibodies that inhibited LPS-induced intracellular signaling and cytokine production and protected mice from lethal endotoxic shock and E. coli bacterial sepsis, even when treatment was delayed for several hours after endotoxemia or the onset of sepsis. Resolution of the crystal structures of the human and mouse TLR4–MD-2 complexes has provided an explanation for the mode of action of these anti-TLR4 antibodies (12). Based on the identification of the residues implicated in the contact between TLR4 and MD-2, anti-TLR4 antibodies likely impede the binding of the MD-2–LPS complex to TLR4.

The protective effects of the anti-TLR4 therapy were impressive and in some respects unique. Previous studies conducted with anti-LBP or anti-CD14 antibodies in experimental models of endotoxic shock and Gram-negative bacterial sepsis uniformly failed to show protection when treatment was administered after LPS (anti-LBP) or simultaneously with or shortly after bacterial challenge (anti-LBP and anti-CD14) (21–23). In contrast, anti-TLR4 antibodies were found to prevent death from endotoxic shock even when treatment was delayed for as much as 4 h after the LPS challenge (Fig. 3D). These findings provide strong support for an anti-TLR4 treatment strategy in patients with fulminant meningococemia associated with high levels of circulating endotoxin in whom anti-LPS (i.e., recombinant bactericidal/permeability-increasing protein) and anti-sepsis (i.e., activated protein C) therapies have failed (24, 25). Unlike monoclonal antibodies raised against TLR4–MD-2, which work only when administered prophylactically in bacterial sepsis (26, 27), anti-TLR4 antibodies afforded remarkable protection against lethal E. coli sepsis when treatment was delayed for as much as 13 h after the onset of infection (Fig. 4D), offering a much broader window of therapeutic intervention.

Some Gram-negative endotoxin species also are sensed by TLR2 (28–30), and several bacterial components (i.e., peptidoglycan, lipopeptides, flagellin, Cpg DNA motifs) are recognized by other members of the TLR family besides TLR4, including TLR1, TLR2, TLR5, TLR6, and TLR9. These properties support the potential need for combined anti-TLR-therapies. Along these lines, Spiller et al. (27) recently proposed the need for dual blockade of TLR2 and TLR4–MD-2 to protect against Gram-negative sepsis when therapy is initiated after the onset of infection. Challenging the concept of a need for dual TLR2 and TLR4–MD-2 targeted therapy (27), our findings demonstrate that TLR2 clearly was not a key player in the pathogenesis of Gram-negative sepsis. Indeed, unlike the TLR4−/− mice, the TLR2−/− mice produced an abundant amount of cytokines during E. coli sepsis and had a rapidly fatal clinical course identical to that of WT mice (Fig. 1), an observation consistent with recent in vitro data indicating that TLR4–MD-2 is the main recognition system for enterobacteria like E. coli and K. pneumoniae (29). Furthermore, the sole blockade of TLR4 was sufficient to protect against Gram-negative sepsis caused by E. coli, even when therapy was administered long after the start of sepsis. Although somewhat overlooked, prophylactic anti-TLR4 monotherapy also has been shown to be protective against lethal E. coli infection (27), suggesting that administration of repeated doses of anti-TLR4 antibody might increase survival when given therapeutically, as shown in the present study. Other plausible reasons for the divergent results between our study and the study of Spiller et al. (27) could include the much broader antibody repertoire of polyclonal antibodies; the use of different E. coli and mouse strains, bacterial inocula, and antibody classes; and differences in the timing of antibiotic administration.

An anti-TLR4 treatment strategy also is supported by recent data obtained with eritoran (E5564), a synthetic LPS antagonist that binds to MD-2 (12, 31), and TAK-242, a cyclohexene derivative that inhibits TLR4-mediated signal transduction, which prevented lethality in experimental models of LPS shock or bacterial sepsis in rodents (32, 33). At a time when most anti-sepsis clinical trials have yielded frustratingly negative results (2, 34), our experimental data lend strong support to TLR4-targeted therapy (i.e., eritoran and TAK-242) currently under development in patients with Gram-negative sepsis.

Materials and Methods

Mice. Eight- to 10-week-old female OF1, BALB/c, and C57BL6 mice were purchased from Charles River Laboratories. MyD88−/−, TLR2−/−, and TLR4−/− C57BL6 mice have been described previously (4, 17, 35). Mice were bred and housed in specific pathogen-free conditions in groups of 5–10 mice per cage with free access to food and water. All animal procedures were approved by the Office Vétérinaire du Canton de Vaud (authorization numbers 876.5, 877.5, and 1009.4) and performed in accordance with the institutional guidelines for animal experiments.

Cells and Reagents. HEK 293T cells were cultured in OptiMEM medium. RAW 264.7 murine macrophages were grown in RPMI medium 1640 containing 2 mM glutamine. Mouse bone marrow–derived macrophages (BMDMs) were obtained as described previously (36) and cultured with Dulbecco’s modified Dulbecco’s medium containing 2% heat-inactivated fetal bovine serum. All media were supplemented with 10% heat-inactivated FCS (Seromed) and antibiotics. Thioglycollate-elicited peritoneal macrophages were harvested from mice 3 days after i.p. injection of 2 mL of 3% thioglycollate solution (BD Biosciences). Heparinized blood was collected from OF1 mice. Where indicated, cells, or blood were incubated with 1–100 ng/mL of Salmonella minnesota Ultra Pure LPS (List Biological Laboratories), 10 μg/mL of Staphylococcus aureus peptidoglycan (PGN, Sigma), 1 μg/mL of Pam3CSK4 (EMC microcollections), or 0.1 μg Mpg ODN (Coley Pharmaceutical Group).

Soluble Chimeric mTLR4-Fc. A DNA fragment encoding for amino acids 1–334 of mouse TLR4 (mTLR4) was amplified by PCR using the Expand High-Fidelity PCR system (Roche Applied Science) and mT4fc sense (TGCTTCGACGCCACCATGATGCTCCCTGGCTC) and mT4Fc antisense (GGGCCAGCTGATTAGAGGTATGCATTTGAA) oligonucleotides containing a SalI site (indicated in bold). The amplicon was cloned into the pGEM-T Easy Vector (Promega), sequenced, excised with SalI, and subcloned upstream of the sequence encoding for the human IgG1 Fc segment into the pFc plasmid (Apoptech). Recombinant mTLR4Fc-expressing vector was transfected into HEK 293T cells using the calcium precipitation method. The transfected HEK 293T cells were incubated for 3 days in OptiMEM medium (Invitrogen). Supernatant was collected and centrifuged, and soluble recombinant mTLR4-fc fusion protein was purified by protein A (APBiotech) immunoaffinity chromatography. The molecular weight of the recombinant protein was verified by SDS/PAGE analysis, and the presence of the Fc fragment of human IgG was confirmed by Western blot analysis using the mouse GG-7 Fc-specific anti-human IgG antibody (Sigma).

Anti-TLR4 Antibodies. Anti-TLR4 antibodies were produced in New Zealand White rabbits by repeated immunization with 100 μg of purified mTLR4-Fc fusion protein in Splev. Anti-TLR4 antibody titers were measured by ELISA as described below. Rabbits were bled when anti-TLR4 antibody titers reached a plateau. Nonimmune and anti-mTLR4-Fc antibodies were isolated from rabbit serum by protein A affinity chromatography following the manufacturer’s recommendations (GE Healthcare). Affinity-purified anti-mTLR4 antibodies used in some experiments were isolated from anti-mTLR4-Fc sera using a 3-step procedure that included IgG purification using protein A chromatography, followed by anti-Fc and anti-GST affinity chromatography coupled to a coupled affinity column and a final step of mTLR4-specific antibody purification using a mTLR4–Fc–coupled affinity Hi-trap NHS-activated column (APBiotech). The endotoxin content of the purified antibodies was 100 pg per mg of antibodies as measured by the limulus amebocyte lysate assay (Charles River Laboratories).

ELISA for Measurement of Anti-TLR4 Antibodies. First, 96-well plates were coated overnight at 4 °C with 1 μg/mL of mTLR4-Fc or mGITR-Fc fusion protein as a negative control. After washing, the plates were incubated for 1 h at 37 °C with PBS containing 5% FCS and then with serial dilutions of preimmune or immune rabbit serum, before a final incubation step with HRP-conjugated...
goat anti-rabbit IgG (Pierce). Peroxidase activity was assessed using the TMB reaction. The concentrations of TNF and IL-6 in cell culture supernatants were measured by ELISA assays (Promega). Results are presented as mean ± SEM.

Flow Cytometric Analysis. The 24-well plate assay was performed using the FACSCalibur flow cytometer (BD Biosciences) and FlowJo 8.5.3 software (FlowJo).

**Cytokine Measurements.** RAW 264.7 murine macrophages were plated at a density of 2 × 10^6 cells per well in 96-well culture plates (Costar). A whole blood stimulation assay was performed in the 96-well plates in a total volume of 200 μL of RPMI medium (1640). Cells and whole blood were stimulated for 4 h with LPS, PGN, Pam3CSK4, or CpG ODN and then cultured for 18 h with 10 ng/mL of LPS. Luciferase and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega). Results are expressed as relative luciferase activity (ratio of luciferase activity to Renilla luciferase activity).

**Western Blot Analyses.** BMDMs were plated at a density of 2 × 10^6 cells per well in 6-well culture plates and incubated as described in Fig. 2D. Cells lysates were fractioned through 12% SDS/PAGE gels and then transferred onto nitrocellulose membranes. Membranes were incubated overnight at 4°C with antibodies specific for ERK1/2 (Cell Signaling Technology). After washing, membranes were incubated for 1 h with secondary HRP-conjugated goat anti-rabbit IgG. Signals were measured using the ECL Western blot analysis system (GE Healthcare). Membranes were then stripped and reprobed with anti-rabbit IgG. Blots were scanned and quantified by densitometry.

### Endotoxin and Bacterial Sepsis Models

Two models of endotoxin shock were used. In the low-dose LPS model, mice were sensitized with an i.p. injection of 40 μg of D-galactosamine (Sigma) 15 min before an i.v. injection of 50 ng of E. coli ultra pure O111:B4 LPS (List Biological Laboratories). In the high-dose model, mice were injected i.p. with 1 mg of E. coli O111:B4 LPS. In the bacterial sepsis models, bacterial peritonitis was induced by an i.p. injection of either 2 × 10^8 or 2 × 10^9 cfu of E. coli O18. Mice were treated with ceftriaxone (100 mg/kg i.p.) plus gentamicin (20 mg/kg i.p.) given at + 15 min (ceftriaxone), + 4 h (ceftriaxone and gentamicin) and then every 12 h in mice inoculated with 2 × 10^9 cfu of E. coli O18 and + 12 h (ceftriaxone) and + 24 h (ceftriaxone and gentamicin) and then every 12 h in mice inoculated with 2 × 10^9 cfu of E. coli O18. Anti-TLR4 and control antibodies were administered either prophylactically or therapeutically, as described in Fig. 4. Mice were monitored at least twice daily until death or complete recovery occurred. Blood samples were harvested from the tail vein for quantification of circulating bacteria and measurements of serum TNF and IL-6 concentrations.

### Statistical Analyses

Comparisons among treatment groups were performed using Fisher’s exact test for categorical data and the Mann-Whitney test for continuous variables. The Kaplan-Meier method was used for survival, and differences were analyzed by the log-rank sum test. All analyses were performed using GraphPad Prism. All reported p values are 2-sided, and values ≤ 0.05 are considered to indicate statistical significance.

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