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TLR4/MD-2 Monoclonal Antibody Therapy Affords Protection in Experimental Models of Septic Shock

Bruno Daubeuf,* John Mathison, ‡ Stephan Spiller, ‡ Stephanie Hugues, § Suzanne Herren,* Walter Ferlin,* Marie Kosco-Vilbois,* Hermann Wagner, ‡ Carsten J. Kirschning, †‡ Richard Ulevitch, † and Greg Elson2*

Overactivation of the immune system upon acute bacterial infection leads to septic shock. Specific bacterial products potently stimulate immune cells via toll-like receptors (TLRs). Gram-negative bacteria induce a predominantly TLR4-driven signal through LPS release. To neutralize LPS signaling in experimental models of sepsis, we generated mAbs toward the TLR4/myeloid differentiation protein-2 (MD-2) complex. The binding properties of an array of selected rat mAbs differed in respect to their specificity for TLR4/MD-2 complex. The specificity of one such mAb, 5E3, to murine TLR4 was confirmed by its recognition of an epitope within the second quarter of the ectodomain. 5E3 inhibited LPS-dependent cell activation in vitro and prevented proinflammatory cytokine production in vivo following LPS challenge in a dose-dependent manner. Furthermore, 5E3 protected mice from lethal shock-like syndrome when applied using both preventative and therapeutic protocols. Most notably, in the colon ascendens stent peritonitis model of polymicrobial abdominal sepsis, administration of a single dose of 5E3 (50 μg) protected mice against mortality. These results demonstrate that neutralizing TLR4/MD-2 is highly efficacious in protecting against bacterial infection-induced toxemia and offers TLR4/MD-2 mAb treatment as a potential therapy for numerous clinical indications. The Journal of Immunology, 2007, 179: 6107–6114.

Septic shock caused by acute bacterial infection is a major cause of mortality in intensive care units (1–3). Inflammatory signal amplification upon cellular recognition of bacterial products underlies dysregulation of the innate immune system in sepsis, which is characterized by severe vasodilation, abnormal coagulation, and ultimately organ failure in septic shock (4). Immune-stimulatory microbial and viral products, known collectively as pathogen-associated molecular patterns (PAMPs), are recognized by the immune system via pattern recognition receptors (PRRs). One family of leucine-rich repeat (LRR)-containing proteins, toll-like receptors (TLRs), constitute a subclass of PRRs. TLRs carry an extracellular domain containing an assembly of LRR motifs followed by a characteristic membrane-proximal portion, a transmembrane domain and an intracellular toll-IL-1 receptor (TIR) signaling domain (5, 6). TLR engagement leads to rapid activation of signaling pathways such as those using MAPK, NFκB (NF-κB), and/or IFN responsive factors (IRFs) (7). Massive release of inflammatory mediators into the bloodstream following TLR activation is suspected to cause pathology associated with sepsis, culminating in septic shock (8).

The prototypic PAMP, LPS, is localized in the outer membrane of Gram-negative bacteria. LPS is possibly the most potent microbial stimulator of the immune system and triggers host responses resembling those indicative of septic shock. It has therefore been extensively used to study the mechanisms underlying host responses to infection both in vitro and in vivo (9). Before innate immune responses to Gram-negative bacteria, LPS is bound by the serum-borne acute phase LPS-binding protein (LBP). The LPS: LBP complex is subsequently delivered to the LRR-rich glycoprotein CD14. The ternary LPS:LBP:CD14 complex is consigned/ transfers LPS to another complex consisting of TLR4 and myeloid differentiation protein (MD)-2 to elicit signal transduction (10–14). The TLR4/ MD-2 complex resides at the cell membrane both at the cell surface and endosomally (15). MD-2 is a soluble protein whose interaction with the extracellular domain of TLR4 immobilizes it. MD-2 facilitates intracellular transportation of TLR4 and regulates LPS-induced TLR4 clustering, a mechanism critical for TLR4 signaling (16–19). TLR4 and MD-2 are expressed by cells for which a role in innate immunity has been implicated, such as myeloid, endothelial, and epithelial cells. For instance, the TLR4/MD-2 complex is expressed on lymphocytes such as B cells for which LPS is a strong mitogen, as well as specific T cell subsets to which it might provide a costimulatory signal (20–26).

In this study, we addressed the question of whether neutralization of TLR4 by systemic administration of an antagonistic mAb directed to TLR4/MD-2 might interfere with sepsis pathology induced by Gram-negative bacterial infection. To this end, an antagonistic mAb, 5E3, that recognizes the mouse TLR4/MD-2 complex was obtained by generating hybridomas using B cells of immunized rats. 5E3 was capable of inhibiting TLR4-mediated activation of murine primary cells and cell lines. Administration of

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3 Abbreviations used in this paper: PAMP, pathogen-associated molecular pattern; MD-2, myeloid differentiation protein-2; CASP, colon ascendens stent peritonitis; PRR, pattern recognition receptor; LBP, LPS-binding protein; CBA, cytometric bead array.

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5E3 in mice abrogated LPS-induced production of proinflammatory cytokines. 5E3 protected mice when administered from 6 h before and up to 7 h following systemic application of an otherwise lethal dose of LPS. Most importantly, TLR4/MD-2 blockade upon binding of 5E3 was beneficial with respect to viability in a murine colon ascends stent peritonitis (CASP) model of polymicrobial peritoneal sepsis. Thus, we propose TLR4/MD-2 inhibition as a strategy for therapeutic prevention of septic shock.

Materials and Methods

Cells and reagents

The murine myeloma fusion partner cell line Sp2/0, chinese hamster ovary (CHO) cells, human embryonic kidney 293 fibroblast (HEK 293) cells, and murine macrophage RAW 264.7 cells were purchased from the American Type Culture Collection (ATCC). PEAK cells were purchased from Edge Biosystems. The mouse endothelial cell line SIE-S2 (27) was a gift from Dr. R. Montesano, Centre Médical Universitaire, Geneva, Switzerland.

Mouse macrophages (C57BL/6-derived) were obtained by peritoneal cavity washing 72 h upon intraperitoneal thiglycollate challenge. Mouse splenocytes (C57BL/6-derived) were obtained by collagenase/DNase digestion of spleen. Wistar rats and C57BL/6 mice were purchased from Charles River Laboratories. All in vivo experiments received approval from the appropriate institutional review board. The following mAbs were used for FACS stainings: rat IgG2a (MAAB64; ATCC), rat IgG2b (11G8), rat anti-mouse TLR4 (M155/10, Abcam), anti-human MD-2 (18H10), anti-human TLR4 (HTA125), mouse anti-FLAG™ (Sigma-Aldrich), mouse anti-c-myc, goat anti-mouse IgG-APC (BD Biosciences), and mouse anti-rat IgG Fcγ specific (Jackson Immunoresearch Laboratories) coupled to FMAT Blue (Applied Biosystems). The mouse inflammation cytometric bead array (CBA) kit (BD Biosciences) was used for multicytokine measurement in mouse serum. The following Ab pairs were used for ELISA: anti-mouse IL-6 (BD Biosciences) and anti-mouse TNF-α (BD Biosciences). Streptavidin-HRP (Zymed) was used as secondary reagent for processing tetramethylbenzidine (Sigma-Aldrich) as chromogenic substrate. The following TLR ligands were used in cell stimulation assays: LPS from E. coli strain O111:B4 (Sigma-Aldrich), ultrapure LPS from S. minnesota (List Biologicals), ultrapure LPS from E. coli K12 (Invivogen Life Technologies), and tripalmitoin-cysteinyl-seryl-(lysyl)3-lysine (Pan,CSK; Invivogen Life Technologies).

Generation of Abs against mouse TLR4/MD-2

Rat anti-mouse TLR4/MD-2 mAbs were prepared using stably transfected CHO cells expressing mouse TLR4/MD-2 and recombinant mouse TLR4/MD-2 fusion protein (28) as immunogens. Male Wistar rats were immunized three times at 2 wk intervals with 10⁴ CHO cells expressing mouse TLR4/MD-2 subcutaneously in MPL plus TD7 adjuvant (Sigma-Aldrich), followed by a s.c. hyperboost with either 10⁴ HEK 293 cells expressing high levels of stably transfected MD-2 or 10 μg of the recombinant fusion protein. Three days later, a fusion was performed between lymph node cells and the Sp2/0 myeloma fusion partner as previously described (29). Subsequent screening of hybridomas was performed on mock transfected CHO cells or CHO cells expressing mouse TLR4/MD-2, using the 8200 cellular detection system (Applied Biosystems). Specificity for the TLR4/MD-2 complex was verified by FACS analysis (see below) using mock-transfected or TLR4/MD-2 transfected HEK 293 cells. After two rounds of subcloning, mAbs were purified from hybridoma supernatants expanded in serum-free medium (Hybridoma SFM; Invitrogen Life Technologies) using a Protein G affinity column (GE Healthcare).

Generation of mouse-human hybrid TLR4 mutants

To generate the cross-species TLR4 construct named MHHH containing a subdomain of mouse TLR4 spanning amino acids 23 to 291 substituting for the corresponding human TLR4 domain. A DNA fragment encoding the construct was ligated into the mammalian expression vector pCDNA3.1 (Invivogen Life Technologies), human TLR4, mouse MD-2-FLAG, human MD-2-FLAG, and cloning fragment of human and mouse TLR4 (see above) using the Fugene transfection reagent (Roche). Cells were analyzed 48 h posttransfection.

Transient transfection of PEAK cells

Cells were transfected with combinations of human and mouse TLR4 and/or MD-2. PEAK cells were cotransfected with 0.75 μg of combinations of expression vectors encoding mouse TLR4 (Invivogen Life Technologies), human TLR4, mouse MD-2-FLAG, human MD-2-FLAG, and chimeric fragment of human and mouse TLR4 (see above) using the Fugene transfection reagent (Roche). Cells were analyzed 48 h posttransfection.

FACS analysis

CHO, HEK 293, and PEAK cells were stained at 4°C for 20 min with 5 μg/ml primary Ab in PBS 2% BSA (FACS buffer) and washed twice with FACS buffer. Then, the appropriate secondary Ab was incubated with cells at 4°C for 20 min. RAW 264.7 and SIE-S2 cells were stained as described above for CHO cells except for additional blocking of FcγRs applying 100 μg/ml mouse IgG (Sigma-Aldrich) for 20 min at 4°C before addition of primary Ab. Whole mouse blood from intracardiac puncture was stained as follows: fresh blood was incubated 20 min at room temperature with 10% of heat inactivated mouse serum. Cell staining with Abs was performed as described above. After staining, cells were incubated with 1X FACS lysis solution (BD Biosciences) (10 min in the dark, RT) to remove RBC and washed twice before acquisition. Monocytes were analyzed after gating on CD14+ cells.

Secondary Abs used were goat anti-mouse IgG Fcγ-specific-APC (for HTA125, 18H10, anti-c-myc and anti-FLAG) and mouse anti-rat IgG Fcγ-specific-FMAT Blue (for 5E3 and MTS510).

Persistence of 5E3 binding on RAW 264.7 and SIE-S2 cells was analyzed as follows: 10⁶ cells were stained with primary Ab as described above, then plated in regular medium at 37°C and 5% CO₂ for 1 or 3 h before secondary Ab staining and FACS analysis. All samples were analyzed on a FACScalibur cytometer (BD Biosciences).

Immunoprecipitation and immuno blot analysis

Lysates of 4 × 10⁶ cells were immunoprecipitated or of 5 × 10⁵ cells applied directly to SDS-PAGE and blotting as described (30). Protein A/G agarose beads (Santa Cruz Biotechnology) were used for immunoprecipitation using 5E3, isotype control, or anti-FLAG mAb (M2; Sigma-Aldrich). Immuno blots were incubated with Ab against the FLAG-tag (rabbit anti-Flag; Sigma-Aldrich).

LPS-induced cytokine secretion

RAW 264.7 cells and peritoneal macrophages were plated in 96-well plates 16 h before LPS stimulation. Splenocytes were plated 1 h before LPS stimulation. Abs were incubated with cells 1 h before LPS stimulation. Cells were incubated at 37°C and mouse TNF-α or IL-6 titers in supernatants were determined by ELISA 4 h post LPS-stimulation for RAW 264.7 cells and peritoneal macrophages, as well as 24 h for splenocytes.

Mouse models of endotoxemia

For elicitation of nonlethal endotoxemia, C57BL/6 mice received 100 μg LPS i.p. in PBS 1 h after intraperitoneal Ab injection. 4 h later, blood was drawn by intracardiac puncture and centrifuged. Serum titers of inflammatory cytokines were measured by CBA following the manufacturer instructions. For elucidation of lethal endotoxemia, survival of C57BL/6 mice was monitored during 48 h after i.p. injection of LPS after which no further lethality was noticed in the course of the following week during which mice underlied regular observation. Two models were used. A high dose model encompassed systemic challenge with 48 mg/kg of LPS per mouse. According to a low dose model, 4 mg/kg of LPS and 800 mg/kg D-Galactosamine (D-galN) were applied per mouse. Abs were injected i.p. (40 mg/mouse) at the timepoint indicated.

CASP procedure

Polymicrobial sepsis was induced in C57BL/6 mice according to the established CASP protocol (31) with the following modifications. Using
isoflurane anesthesia and aseptic technique the cecum/ascending colon was brought out through a 1.5 cm midline incision, and a 16G 2 mm stent prepared from a Jelco FEP polymer catheter was placed transmurally 1 cm distal to the iliocecal valve. Gentle pressure was applied to express a 2-mm bead of colon content from the patent stent, and the cecum/colon was returned to the lower left abdominal quadrant. Anti-TLR4 mAb (5E3) or control mAb (11G8), 50 μg in 10 mM HEPES-buffered saline (pH 7.4) was delivered to the upper right abdominal quadrant followed by closure in two layers. Buprenorphine (0.05 mg/kg, 0.4 ml) was administered subcutaneously and the mice were returned to cages for recovery and monitoring. Surface temperature was measured using infrared thermometry several times each day, and animals that were determined to be moribund (surface temperature below 23°C and showing significantly diminished response to challenge and righting reflex) were euthanized.

Results

Generation and characterization of anti-mouse TLR4/MD-2 mAbs

Male Wistar rats were immunized with combinations of TLR4/MD-2 transfected CHO or HEK 293 cells and a soluble TLR4/MD-2 fusion protein to generate hybridomas secreting mAbs specific for the mouse TLR4/MD-2 complex. Of >500 hybridoma clones screened, 32 showed specificity for the TLR4/MD-2 complex as determined by flow cytometry on either mouse TLR4/MD-2 transfected CHO or HEK 293 cells. In each case, a mock transfected cell negative control was included to ensure mouse TLR4/MD-2 specificity. Binding to human or rabbit TLR4/MD-2 transfectants was also performed to detect cross-reactive or anti-“epitope tag” reactive clones. One nonneutralizing cross-reactive human/mouse TLR4/MD-2 clone was identified, whereas no anti-tags mAbs were generated. Fig. 1A shows the binding profile of mAb 5E3, generated by immunization using TLR4/MD-2 transfected CHO cells followed by a final boost with transfected HEK 293 cells. Saturable dose-dependent binding is demonstrated and compared with that of a commercially available anti-TLR4/MD-2 mAb, MTS510 (32).

5E3 was also found to bind native TLR4/MD-2. In a similar fashion to MTS510, 5E3 bound to the murine macrophage cell line RAW 264.7, the murine endothelial cell line SIE-S2 and to primary monocytes in murine whole blood (Fig. 1, B–D, respectively). Furthermore, binding of 5E3 to the surface of both RAW 264.7 and SIE-S2 cells persisted following prolonged incubation at 37°C, suggesting that the Ab is not inducing internalization of TLR4 (Fig. 1, E and F).

To determine the subunit specificity of 5E3 for the TLR4/MD-2 complex, transient transfection was performed in PEAK cells for expression of mouse TLR4/MD-2 transfected CHO cells expressing mouse TLR4/MD-2 as indicated. B, Binding of 5E3 and MTS510 to RAW 264.7 cells analyzed by flow cytometry. Upper panel, Rat IgG2b (filled histogram) and 5E3 (dark gray outline). Lower panel, Rat IgG2a (filled histogram) and MTS510 (gray outline). C, Binding of 5E3 and MTS510 to the SIE-S2 endothelial cell line analyzed by flow cytometry. Upper panel, Rat IgG2b (filled histogram) and 5E3 (dark gray outline). Lower panel, Rat IgG2a (filled histogram) and MTS510 (gray outline). D, Binding of 5E3 on mouse primary monocytes analyzed by flow cytometry. Upper panel, Rat IgG2b (filled histogram) and 5E3 (dark gray outline). Lower panel, Rat IgG2a (filled histogram) and MTS510 (gray outline). E, Persistence of 5E3 binding on RAW 264.7 cells after incubation at 37°C. Rat IgG2b (filled histogram) and 5E3 (gray outline). Upper panel, 1 h incubation. Lower panel, 3 h incubation. F, Persistence of 5E3 binding on SIE-S2 cells after incubation at 37°C. Rat IgG2b (filled histogram) and 5E3 (dark gray outline). Upper panel, 1 h incubation. Lower panel, 3 h incubation.

FIGURE 1. Binding properties of anti-TLR4-MD-2 mAbs. A, Binding of 5E3 (rat IgG2b), MTS510 (rat IgG2a), and their respective isotype controls on stably transfected CHO cells expressing mouse TLR4/MD-2 as indicated. B, Binding of 5E3 and MTS510 to RAW 264.7 cells analyzed by flow cytometry. Upper panel, Rat IgG2b (filled histogram) and 5E3 (dark gray outline). Lower panel, Rat IgG2a (filled histogram) and MTS510 (gray outline). C, Binding of 5E3 and MTS510 to the SIE-S2 endothelial cell line analyzed by flow cytometry. Upper panel, Rat IgG2b (filled histogram) and 5E3 (dark gray outline). Lower panel, Rat IgG2a (filled histogram) and MTS510 (gray outline). D, Binding of 5E3 on mouse primary monocytes analyzed by flow cytometry. Upper panel, Rat IgG2b (filled histogram) and 5E3 (dark gray outline). Lower panel, Rat IgG2a (filled histogram) and MTS510 (gray outline). E, Persistence of 5E3 binding on RAW 264.7 cells after incubation at 37°C. Rat IgG2b (filled histogram) and 5E3 (gray outline). Upper panel, 1 h incubation. Lower panel, 3 h incubation. F, Persistence of 5E3 binding on SIE-S2 cells after incubation at 37°C. Rat IgG2b (filled histogram) and 5E3 (dark gray outline). Upper panel, 1 h incubation. Lower panel, 3 h incubation.
TLR4 were generated to determine the region of mouse TLR4 containing the epitope recognized by 5E3. The extracellular region of TLR4 was nominally divided into four regions. We then constructed four human-mouse hybrids, MHHH, MMHH, MHHM, and MHMH, where H corresponds to a human fragment of TLR4 and M corresponds to a murine fragment of TLR4 (Fig. 2Ci). Each construct was cotransfected into PEAK cells along with human MD-2. 5E3 binding was only detected on cells expressing MMHH construct (Fig. 2Cii). As cells expressing the MHHH construct were negative for 5E3 binding, we conclude that the epitope is contained within the second region of murine TLR4, corresponding to aa 292–371. This conclusion was reinforced by normalization of the fluorescence intensity signals obtained for 5E3 against the TLR4 control mAb (anti-c-myc), showing that 5E3 has a significantly higher affinity to MMHH compared with human TLR4, MHHH, HHHM, and MHMH (Table I).

5E3 inhibits LPS-induced activation of several cell types in vitro

The ability of 5E3 to block LPS responses in a variety of relevant cell types was determined. Using RAW 264.7 cells, dose-dependent inhibition of cell activation upon challenge with LPS (E. coli O111:B4) at a dose of 10 ng/ml was analyzed. 5E3 completely inhibited LPS-induced TNF-α production within a range between 10 μg/ml and 100 ng/ml, with an IC50 of 9.4 ng/ml (Fig. 3A). Under these conditions, MTS510 had no neutralizing effect on LPS-dependent TNF-α production. A similar inhibition profile was seen for 5E3 when using an alternative LPS species (S. minnesota R595, data not shown). When analyzing 5E3 effects at a fixed concentration of 10 μg/ml whereas applying LPS at consecutively increasing amounts, complete inhibition of TNF-α production was observed if 100 ng/ml LPS was applied. Inhibition was still substantial if 10 μg/ml was used for challenge (Fig. 3B). 5E3 potently inhibited LPS-induced IL-6 production in mouse peritoneal macrophages if applied at concentrations as low as 10 ng/ml (Fig. 3C). Higher concentrations of 5E3 (1 μg/ml and above) were required indicated. HTA125 binds specifically to human TLR4, 18H10 binds specifically to human MD-2 and the anti-FLAG mAb recognizes both human and mouse MD-2. B, Lysates of stably transfected HEK293 cells overexpressing FLAG-tagged murine (m) TLR4 and mMD-2 were incubated with agarose beads and Abs (i.e., isotype control; α-FLAG, FLAG specific M2 mAb). Precipitates were subjected to SDS-PAGE, blotted, and resulting membranes incubated with FLAG-specific polyclonal antiserum (kDa). C, i, Schematic representation of the human/mouse TLR4 hybrids. Red blocks represent human sequence while blue blocks represent mouse sequence. C, ii, Transiently transfected PEAK cells expressing various versions of TLR4 human-mouse hybrids were prepared. The cells were then incubated with an anti-c-myc Ab to reveal the level of expression, an anti-FLAG Ab for MD-2-FLAG expression and 5E3 and analyzed by flow cytometry. WT, Wild type; M, mouse; H, human.

Table 1. Normalization of the fluorescence intensity signals obtained for 5E3 on cells expressing human/mouse hybrids of TLR4

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<tr>
<td>Human WT</td>
<td>0.72***</td>
<td>0.14</td>
<td>0.10</td>
</tr>
<tr>
<td>MHHH</td>
<td>0.99***</td>
<td>0.18</td>
<td>0.17</td>
</tr>
<tr>
<td>HHHM</td>
<td>0.95***</td>
<td>0.21</td>
<td>0.18</td>
</tr>
<tr>
<td>MMHH</td>
<td>1.01***</td>
<td>0.58***</td>
<td>0.15</td>
</tr>
<tr>
<td>MHMH</td>
<td>0.98***</td>
<td>0.31</td>
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Two-way ANOVA test between MD-2/TLR4 or 5E3/TLR4 and iso/TLR4, ***p < .001.
to completely inhibit LPS-induced IL-6 production in mouse splenocytes (Fig. 3D). This difference could be explained by the fact that the splenocyte assay required a longer LPS exposure time to obtain significant amounts of cytokines in culture supernatant (24 h vs 8 h for the peritoneal macrophage assay described above).

We next determined the specificity of 5E3 for cell activation via TLR4/MD-2. Mouse peritoneal macrophages were stimulated via TLR4/MD-2 using LPS or via TLR2 using Pam3CSK4. LPS-dependent cell stimulation was efficiently blocked in a dose-dependent manner using 5E3, whereas no inhibition was seen with the TLR2-specific blocking mAb, T2.5 (30) (Fig. 4A). Conversely, Pam3CSK4-dependent cell activation was unaffected by 5E3, and efficiently blocked in a dose-dependent manner by application of T2.5 (Fig. 4B).

Ab-mediated inhibition of TLR4-dependent systemic induction of shock-like syndromes

The effect of 5E3 treatment on the induction of lethal shock was investigated using a high-dose LPS model. Administration of a sublethal (100 μg) dose of LPS i.p. in wild-type mice led to rapid induction of proinflammatory cytokines in the serum that can be readily detected systemically 1 h postadministration. Toxemia-dependent lethality is typically observed from 24 h later. We investigated the ability of 5E3 to inhibit LPS-induced shock by comparing LPS-treated mice with mice treated with 5E3 and LPS.

**FIGURE 4.** 5E3 neutralizes TLR4-ligand but not TLR2-ligand induced cytokine responses in vitro. LPS (O111:B4; 10 ng/ml) (A) or Pam3CSK4 (10 ng/ml) (B) were added to mouse peritoneal macrophages following a 30 min incubation of 5E3, T2.5 or isotype control mAbs at the indicated concentrations and incubated for 4 h. IL-6 levels in the supernatants were measured by ELISA.

**FIGURE 5.** Effect of 5E3 administration on LPS-induced cytokine production in vivo. LPS (O111:B4) was injected i.p. into C57BL/6 mice (2 mice/group) 1 h post injection of mAbs. Four hours later, IL-6 levels were measured by CBA. A, 100 μg (4 mg/kg) LPS and variable mAb concentration (as indicated). B, 250 μg (10 mg/kg) of mAb and variable LPS concentration (as indicated). Two-way ANOVA test, ***, p < 0.001; *, p < 0.05.
post-LPS treatment onwards. In an initial set of experiments, we focused on analyzing an effect of 5E3 pretreatment on LPS-induced proinflammatory cytokine production. 5E3 inhibited systemic IL-6 production in a dose dependent manner (Fig. 5A). The average serum level of IL-6 in the isotype control group was 39.4 ng/ml. Partial inhibition of serum IL-6 was observed at concentrations as low as 1 mg/kg 5E3, with almost total abrogation of IL-6 production at the highest concentration tested (10 mg/kg). We also performed experiments where the mAb concentration administered was fixed (10 mg/kg) and the LPS dose varied from 4 mg/kg (100 μg) to 20 mg/kg (500 μg). 5E3 remained very effective at inhibiting IL-6 production at all LPS doses tested (Fig. 5B). In addition to IL-6, we found that serum levels of a variety of cytokines and chemokines produced systemically following LPS administration were affected by 5E3 treatment (Table II).

Applying 5E3 according to a protective protocol (administration before LPS challenge) and according to a therapeutic protocol (administration after LPS treatment) on mouse survival from lethal toxemia was investigated. 5E3 treatment protected mice if administered up to 4 h before LPS treatment (Fig. 6A). A substantial protection (~75%) was evident already at the earliest time-point tested (6 h before LPS administration). In contrast, 5E3 protected mice when administered 4 h post-LPS administration (Fig. 6B). This protection steadily declined with increasing length of the respective time period from 4 to 7 h post-LPS administration (100 to 40% survival, respectively). Application of 5E3 at 8 h post-LPS administration, however, did not protect mice. These results define a therapeutic window for protection of mice in LPS-induced toxemia from at least –7 h to 4 h (total protection) or –14 h to +7 h (80 and 40% protection, respectively).

5E3 was also found to protect mice from lethal toxemia in a low LPS dose model involving D-GalN sensitization when mAb was administered 2 h before LPS injection (data not shown).

Ab-mediated protection of mice to polymicrobial peritoneal sepsis

Intending to exploit a biologically complex and clinically relevant infection model, we investigated the protective role of 5E3 in a mouse model of intestinal perforation. Applying the established CASP procedure (33), we treated mice by applying 2 mg/kg (50 μg) 5E3 upon CASP surgery which resulted in a significantly increased survival rate as compared with members of the group treated with control mAb (Fig. 7). At 72 h postsurgery, survival in the 5E3 group was above 80% whereas in the control, survival was slightly >40%. These results clearly show that treatment with a TLR4 antagonistic mAb before bacterial insult is beneficial to mice in experimentally induced polymicrobial peritoneal sepsis.

Discussion

LPS is the major inducer of inflammatory host responses contributing to the adverse effects of Gram-negative bacterial infection on the host organism during infection. LPS-induced signal transduction is known to be initiated by the type I receptor TLR4 associated to MD-2. Using an antagonistic anti-TLR4/MD-2 mAb, 5E3, we show here its protective function in mice challenged with an otherwise lethal dose of LPS and in an experimental model of polymicrobial peritoneal sepsis. To our knowledge, this is the first report on generation of an antagonistic TLR4/MD-2 mAb capable of exerting an LPS-TLR4 neutralizing effect in vivo confirming the pivotal role of cell surface TLR4 in acute Gram-negative infections.

5E3 binds to the TLR4 portion of the TLR4/MD2 complex and is a specific and potent inhibitor of LPS-mediated cell activation both in vitro and in vivo. The mechanism of inhibition of LPS-dependent signaling is at present unclear. The Ab inhibits the effects of large amounts of LPS both in vitro and in vivo. Furthermore, LPS binding to cells of the myeloid lineage is unaffected by preincubation with 5E3, indicating LPS binding to CD14 is unaffected by mAb binding (data not shown). In addition, 5E3 does not induce internalization of

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**Table II. Percentage Inhibition of LPS-induced Proinflammatory Cytokine Production by 5E3 Treatment**

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<th>Dose (mg/kg)</th>
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*a* 5E3 was injected 1 h after LPS and blood was drawn for cytokine determination 4 h after LPS injection.

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**FIGURE 6.** Effect of 5E3 administration on viability after lethal LPS challenge (high dose model). mAbs (1.2 mg/mouse) were injected in C57BL/6 mice before (A) or after (B) injection of a lethal dose (48 mg/kg) of LPS (O111:B4) at times indicated. Numbers in brackets show survivors per group.

**FIGURE 7.** Effect of 5E3 administration on survival of mice undergoing CASP surgery. Control mAb (11G8) or 5E3 (50 μg/mouse) was injected i.p. in C57BL/6 mice (16 mice/group) following CASP surgery using 16G gauges. Survival was monitored during a subsequent 72-h period. *, p = 0.01, log rank analysis.
TLR4 (Fig. 1, E and F) and there was no evidence of 5E3-mediated apoptosis induction in mouse whole blood or on mouse endothelial cells following prolonged incubation at 37°C (data not shown). Most likely, 5E3 inhibits TLR4 signaling by blocking LPS-TLR4 binding or by inhibiting TLR4/MD-2 receptor clustering/aggregation, known to be essential for subsequent signal transduction events (19).

We mapped the epitope of 5E3 to a 79 amino acid region of the extracellular domain of TLR4. This region has previously been shown to be functionally important in determining the species-specific recognition of certain types of LPS, and has been described as the “hypervariable region” of TLR4 (34). These data indicate that 5E3 binds to a functionally important epitope of mouse TLR4. Further studies are underway to define the precise role of this region in TLR4 signaling.

TLRs protect hosts from bacterial infection (35, 36). Under certain circumstances, however, TLR activation elicits harmful effects through induction of a state of hyperinflammation, such as upon acute infection (37). Inhibition of TLR activation via several different mechanisms has been shown to be protective in a variety of models of TLR-dependent lethal shock. For example, inhibition of LPS signaling following various strategies was shown to provide protection to the adverse effects of systemic LPS or Gram-negative bacterial challenge. Accordingly, anti-LBP mAbs protected mice from LPS/D-galN-induced endotoxin shock (11). Anti-CD14 mAbs attenuated the toxic effects of LPS in rabbits, primates and humans (12–14, 38, 39). In addition, anti-CD14 mAbs protected against the deleterious systemic effects caused by live Gram-negative bacteria in a rabbit model of lung infection (40). Synthetic LPS antagonists have also been used systemically to inhibit TLR4 activation and afford therapeutic benefit in both experimental disease models and in human diseases (41, 42). Furthermore, inhibition of TLR2 signaling with an antagonistic mAb can protect mice against TLR2-driven toxemia induced either by Pam3CSK4 or heat-inactivated Gram-positive B. subtilis (30). These data demonstrated that TLRs and related accessory proteins are potential targets for therapeutic intervention in inflammation caused by confrontation with microbial products.

The antagonistic anti-TLR4/MD-2 mAb, 5E3, is highly efficient at preventing LPS-driven hyperactivation of the innate immune system in experimental models. It is probable that the relatively low expression of the TLR4/MD-2 complex on the relevant LPS-responsive cells (Fig. 1, C and D and data not shown) explains the high efficacy of mAb application both in vitro and in vivo even at relatively low doses. Application of the mAb before LPS administration was highly efficient in protecting mice from lethal shock (100% and 80% survival at 7 and 14 h post-LPS injection, respectively), supporting the notion for a prophylactic usage of TLR4 blocking mAbs in certain clinical settings where endotoxin-related shock episodes are prevalent. In addition, administration of mAb 4 h or up to 7 h post-LPS administration protected between 100% and 40% of mice from the otherwise lethal effects of an LPS challenge. Complement-mediated depletion of TLR4/MD-2-expressing cells could be largely excluded by using an isotype-matched nonblocking anti-TLR4/MD-2 mAb which was not protective if lethal LPS challenge was performed (data not shown). Again, delay of shock onset by 5E3 administration was evident in mice as long as 7 h post-LPS challenge certainly indicating relative largeness of a therapeutic time-window. Notably, CD14 blockade up to 4 h post-LPS administration protected rabbits against organ injury and prevented death (13). Both of these findings indicate the gradual and reversible effect of an LPS-induced sepsis pathogenesis in contrast to abruptness and irreversibility, such attributes having been assigned to it previously. It remains possible, however, that the time-window of therapeutic opportunity can be extended further in the clinical context within which invaders causing acute infection are prone to attack by host cells. This would hinder instant proliferation and dissemination toward intrinsic limitation which is not, however, operative upon punctual administration of high-dose LPS for instant onset of shock.

The effect of TLR4 blockage in polymicrobial peritoneal sepsis was investigated as well. Experimental peritoneal sepsis can be induced either by intraperitoneal injection of LPS or forced extravasation of bacteria from the gut into the peritoneal cavity through puncture of the ileum, using for example CASP procedure. The former model is used widely to resemble septic shock. The latter, however, is considered to more closely resemble a major clinical cause of sepsis. Both procedures elicit specific symptoms of systemic inflammatory response syndrome (SIRS) (31). The severity of disease in CASP is determined by the size of the gauge used for the surgical procedure while LPS dosage determines the extent of LPS toxemia. A recent study has shown that the CASP procedure follows the pattern of diffuse peritonitis resulting in an early and progressive infection that leads to systemic inflammation (31).

We show that TLR4/MD-2 antagonism significantly enhanced mouse survival during the 72-h period post-CASP surgery. This result indicates that the host is able to defend itself effectively against an overwhelming polymicrobial challenge in the absence of TLR4-mediated sensing. Not denying a beneficial role for TLR4 in sensing localized and lower burden infection, our data suggest that TLR4 signaling is a major inducer of the adverse effects of the host response during the course of the infection studied in this model. CASP-associated pathology is induced simultaneously by a variety of different Gram-negative as well as Gram-positive bacterial species residing in the host intestine. LPS-TLR4/MD-2 signaling would appear to be a major driving force of hyperinflammation in polymicrobial abdominal sepsis promoting lethal shock in the context of presumably multiple PRR activation. Recently, we have reported on the relative contribution of TLR4 and TLR2 to the recognition of Gram-negative and Gram-positive bacteria by human cells (43), showing that host immune cells were highly sensitive to even low doses of Gram-negative bacteria (10⁸ cfu/ml), and that this recognition was exclusively TLR4/MD-2-dependent. Significantly higher doses of Gram-negative bacteria (10⁹ cfu/ml), were required to induce a response via TLR2. In contrast, Gram-positive bacteria only activated host cells at high inoculum (>10⁶ cfu/ml), largely but not exclusively through TLR2.

TLR4/MD-2 blockade in sepsis potentially holds advantages over other previously evaluated anti-inflammatory approaches, such as LPS, TNF-α, or IL-1 blockade (44). Given that TLR4/MD-2 has been reported to recognize other bacterial and inflammatory products than just LPS, inhibition of this receptor might result in more broad protection than application of LPS specific/binding compounds (45, 46). Furthermore, blockade of TLR4/MD-2 aims at inhibiting the release of proinflammatory cytokines by inhibiting cell activation, which should have a broader effect than TNF-α or IL-1β inhibition. These other approaches have their own merits and are approved for treatment of chronic inflammation, but TLR4/MD-2 blockade follows a different objective as outlined above.

Together, these results suggest that host cell TLR responses are generally dependent not only on the type, but also the dosage and time of exposure to the invading bacteria. It would therefore seem plausible that in the CASP model of polymicrobial sepsis, where not only the dose of bacteria leading to infection, but also the kinetics of infection might be markedly different to that seen with the other peritoneal sepsis models (31), the contribution of each TLR or pathogen recognition receptor to the induction of lethal shock is different. The benefit of TLR4 blockade in therapeutic regimes, and indeed the blockade of other TLRs (for example, TLR2) in combination with TLR4 blockade in both preventative
and therapeutic regimes following CASP surgery, remains to be determined. Future studies should also address the effects of TLR blockage on shock resulting from polymicrobial sepsis when coupled to antibiotic treatment, which can result in the rapid release of high levels of PAMPs from degrading microbes (34, 47). Our approach aimed at analyzing the effect of TLR4 blockage in models of Gram-negative and polymicrobial infection. This positive result points to TLR4 triggering rather than bacterial virulence as the causative agent of lethality in this model offering TLR4 blockage as protective strategy even in the absence of antibiotics.

In summary, our results demonstrate that in vivo TLR4/MD-2 blockage mediated by specific mAb administration is a promising strategy for potential prevention of the lethal effects of severe inflammation upon acute bacterial infection.

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Disclosures

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2. Beutler, B., and A. Poltorak. 2001. Toll-like receptors and therapeutic regimes following CASP surgery, remains to be determined. Future studies should also address the effects of TLR blockage on shock resulting from polymicrobial sepsis when coupled to antibiotic treatment, which can result in the rapid release of high levels of PAMPs from degrading microbes (34, 47). Our approach aimed at analyzing the effect of TLR4 blockage in models of Gram-negative and polymicrobial infection. This positive result points to TLR4 triggering rather than bacterial virulence as the causative agent of lethality in this model offering TLR4 blockage as protective strategy even in the absence of antibiotics.