Induction of Long-Term Lipopolysaccharide Tolerance by an Agonistic Monoclonal Antibody to the Toll-Like Receptor 4/MD-2 Complex

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Lipopolysaccharide (LPS) is a glycolipid component of the gram-negative bacterial cell wall and induces various host responses, including the production of proinflammatory cytokines. When they are appropriately produced, these cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), activate host immunity to fight off bacteria. The excessive proinflammatory cytokines produced in response to large amounts of LPS, however, can provoke extreme systemic inflammation and often cause lethal endotoxin shock.

Animals pretreated with a sublethal dose of LPS become tolerant to subsequent challenges with a lethal dose of LPS and display reduced mortality. This phenomenon is called LPS tolerance and is defined as the reduced capacity of the host or cultured macrophage/monocyte to respond to LPS following initial stimulation (6, 26). It has also been reported that bacterial or fungal removal is improved during the tolerant state, despite attenuated cytokine production (14, 20). Therefore, LPS tolerance is regarded as a reasonable response that simultaneously manages both the clearance of pathogens and host protection from excess inflammation.

Here we report on the induction of long-term LPS tolerance realized by an agonistic monoclonal antibody (MAb) against the Toll-like receptor 4 (TLR4)/MD-2 complex. Mice pretreated with this MAb showed significant survival advantages compared with the survival of LPS-pretreated mice.

MATERIALS AND METHODS

Mice, C3H/HeN, C3H/HeJ, ddY, and SCID mice were from Japan SLC (Hamamatsu, Shizuoka, Japan). C57BL/6 mice were from Charles River Japan (Yokohama, Kanagawa, Japan). A TLR4-knockout mouse strain with the C57BL/6 background (12) was a kind gift from S. Akira (Osaka University, Osaka, Japan). All animals were maintained in the Center for Laboratory Animals at Saga Medical School and were treated in accordance with the regulations of the Scientists Center for Animal Welfare.

Cell culture. All the cells were cultured in RPMI containing 10% calf serum, 50 μM 2-mercaptoethanol, and penicillin-streptomycin and were incubated at 37°C in an atmosphere of 5% CO2, unless otherwise indicated. For the Ba/F3 cell lines, IL-3 was added as 1,000-fold-diluted culture supernatant of the CHO/IL-3 stable transfectant. Mouse peritoneal exudative cells (PECs) were prepared by washing the peritoneal cavity with cold Hank’s balanced salt solution.

Stable transfectants. A series of Ba/F3 transfectants were described previously (1, 2). Ba/F3/mTLR4/mMD-2/eBluc expresses mouse TLR4-FLAG (TLR4f), MD-2-FLAG (MD-2f), and the nuclear factor eB (NF-eB) promoter-luciferase reporter. Ba/F3/hTLR4/hMD-2/hBluc expresses human TLR4, MD-2f, and the NF-eB promoter-luciferase reporter. To establish Ba/F3/mTLR4/mMD-2, which expressed mouse TLR4f and the tag-free native form of MD-2, Ba/F3/mTLR4f was transfected with pEF-BOS (2) that contained mouse MD-2. Because TLR4 must be associated with MD-2 to function as an LPS receptor (15), we used TLR4/MD-2-cosepressing cells for immunization and analyses.

Antibodies. Two TLR4-deficient mice were intraperitoneally injected four times at weekly intervals with 1 × 105 Ba/F3/mTLR4/mMD-2 suspended in 0.5 ml phosphate-buffered saline (PBS) without adjuvant. Three days after the last injection, the mice were euthanized and their spleens were removed. Splenocytes were dispersed and fused with Sp2/O myeloma cells by using a standard fusion protocol with polyethylene glycol 1500 (Roche, Basel, Switzerland). Hybridomas were selected in hypoxanthine-aminopterin-thymidine medium and were initially screened by flow cytometry with Ba/F3/mTLR4/mMD-2 and parent Ba/F3. Flow cytometry-positive hybridomas were next subjected to NF-eB reporter assays with Ba/F3/mTLR4/mMD-2/eBluc to identify antibodies agonistic to TLR4. UT12 activated NF-eB in the clones obtained, but UT15 did not. To collect immunoglobulin G (IgG)-containing ascites, hybridomas were intraperitoneally injected into SCID mice pretreated with pristane (Sigma, St. Louis, MO). IgG was purified from ascites by using T-GEI MacroPAC (Scipac, Sittingbourne, United Kingdom), a hydrophobic interaction chromatography system. In some experiments, UT12 was further purified by using MAbTrap (Amersham, Piscataway, NJ), a protein G affinity chromatography system. The flowthrough from the protein G column was used as IgG-depleted UT12. The subclasses of UT12 and UT15 were IgG3 and IgG1, respectively. V5606, an isotype control IgG3, and anti-FLAG M2 antibody were from Sigma. MTS510, a rat MAb against the mouse TLR4/MD-2 complex, and Sa2-8, an anti-mouse CD14 MAb, were kind gifts from S. Akashi-Takamura (Tokyo University, Tokyo, Japan). Fluorescein isothiocyanate-conjugated anti-mouse CD14b antibody was from Caltag Laboratories (Burlingame, CA). All the antibodies used for the functional assays were dialyzed in PBS and were sterilized by filtration. Biotinylated antibodies were
prepared by using a ProtOn biotin labeling kit (Vector Laboratories, Burlingame, CA), according to the manufacturer’s instructions.

Flow cytometry. Cells were stained with biotinylated antibodies for 1 h and subsequently with phycoerythrin-conjugated streptavidin (BD Biosciences, San Jose, CA) for 45 min at 4°C. For PEC staining, CD11b was additionally stained and only CD11b-positive cells were plotted. FACScan (BD Biosciences) was used for flow cytometry, and the data were analyzed by using WinMDI software.

In vitro cell stimulation. For the luciferase assay, 3 × 10⁵ Ba/F3/mTLR4/ mMD-2/eB cells or Ba/F3/mTLR4/hMD-2/eB cells in 50 μl medium were plated on a 96-well plate and stimulated with LPS or antibodies at 37°C for 5 h. The cells were lysed by adding 50 μl Steady-Glo lysis buffer-substrate solution (Promega, Madison, WI), and luminescence was measured by using an LB907 luminometer (Berthold, Bad Wildbad, Germany). For measurement of cytokine production, the PECs removed from C57Bl/6 No or C57Bl/6 Hcl were plated on a 96-well plate at 1 × 10⁵ cells per 100 μl culture medium containing LPS or antibodies. After 5 h of incubation at 37°C, the culture supernatant was examined by enzyme-linked immunosorbent assay (ELISA) for determination of the concentrations of TNF-α and IL-6. ELISA was conducted by using immunoassay kits for TNF-α or IL-6 (BioSource, Camarillo, CA).

Immunoprecipitation. Cell lystate was prepared with lysis buffer containing 2 mM CaCl₂, 2 mM MgCl₂, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, and Complete protease inhibitor cocktail (Roche). After 30 min of incubation on ice, the whole-cell lystate was centrifuged and the nuclei were removed. The lysate was incubated with 5 μg antibody for 2 h and subsequently with protein G-Sepharose (Amersham) for 1 h at 4°C. After the precipitated proteins were washed, they were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and were subjected to immunoblotting by using the biotinylated anti-FLAG M2 antibody, alkaline phosphatase-conjugated streptavidin (Vector), and 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium color development substrate (Promega).

Induction of lethal LPS shock and LPS tolerance. For lethal LPS shock, mice were intraperitoneally injected with 0.5 μg LPS (from Escherichia coli O55:BS [Sigma]; the LPS had been phenol extracted and purified by ion-exchange chromatography) in 100 μl PBS along with 25 mg d-galactosamine (D-GalN) (Sigma) in 100 μl PBS. Mouse survival was monitored every 6 h for 14 days. For tolerance induction, the mice were intraperitoneally injected with 10 μg UT12 or 1 or 10 μg LPS. No mice were killed by the direct effects of tolerance induction.

Reverse transcription-PCR. PECs from C57BL/6 mice were plated on a 24-well plate at 2.5 × 10⁵ cells/500 μl/well and incubated with 100 ng/ml LPS or 2 μg/ml UT12 for the indicated times. Total RNA was prepared by using TRIzol reagent (Invitrogen, Carlsbad, CA) and transcribed into cDNA by Superscript III reverse transcriptase (Invitrogen). PCRs were performed at 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min for the cytoplasmic negative regulators IRAK-M and SHIP (27 cycles). For the cytoplasmic negative regulator SOCS-1, 35 cycles were carried out under the same conditions used for IRAK-M, except that an annealing temperature of 50°C was used. β-Actin was amplified for 27 cycles at 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s. The sequences of the primers were as follows: for IRAK-M, the sequence of the forward primer is 5'-AGCCCAA GCCATCCATACTT and that of the reverse primer is 5'-GGTGGTCCTAAA CTTGGCCCTC; for SHIP, the sequence of the forward primer is 5'-GTGACCC CAACCTGCTGAC and that of the reverse primer is 5'-AGAGTTGCTTCTT CCAGAAACAA; and for β-actin, the sequence of the forward primer is 5'-A TGGTATGACGATATGC and that of the reverse primer is 5'-ATGAGG TAGTGTGCAGGT.

Statistics. Survival curves were analyzed by the log rank test. To compare cytokine concentrations, the two-sided, unpaired Student t test or the two-sided Welch test was performed. Differences were judged to be statistically significant when the P value was less than 0.05.

RESULTS AND DISCUSSION

Establishment of UT12. We immunized TLR4-deficient mice (12) with Ba/F3 cells expressing mouse TLR4 and MD-2. Hybridomas were screened by using NK-b Mab reporter assays to obtain antibodies that induced LPS-like signals through the TLR4/MD-2 complex. One antibody activated NK-b as strongly as LPS did and was named UT12. UT12 immunoprecipitated the TLR4/MD-2 complex from Ba/F3 cells expressing TLR4/MD-2 (Fig. 1A). UT12 could not immunoprecipitate TLR4 or MD-2 alone from cells expressing each molecule. UT12 reacted with PECs derived from wild-type mice but not with PECs from TLR4-deficient mice (Fig. 1B).

UT12 induces an LPS-like signal in vitro. When UT12 was added to reporter cells expressing mouse TLR4/MD-2, NF-κB activity increased in a dose-dependent manner, comparable to that induced by LPS (Fig. 2A). The potency per weight of UT12 for NF-κB activation, as estimated from the titration curves, was approximately 1/15 to 1/20 that of LPS. UT12 did not activate NF-κB in reporter cells expressing human TLR4/MD-2, although LPS activated the cells well (Fig. 2B). This finding excludes the possibility that there was detectable contamination by LPS or other impurities in our UT12 preparation. UT15, an anti-TLR4/MD-2 MAb obtained from the same fusion experiment as UT12, or Y5606, an IgG3 isotype control, showed no activation of NF-κB in either mouse or human TLR4/MD-2-expressing cells (Fig. 2A and B).

The activation of NF-κB by LPS was strongly suppressed in serum-free medium (Fig. 2C), as reported previously (8). Interestingly, UT12 retained its activation ability in the absence of serum, suggesting serum-independent (presumably CD14-independent) signaling by UT12. To examine the effects of CD14, we neutralized soluble CD14 by using an anti-CD14 MAb. The activation by LPS was suppressed by the neutralization of CD14 (Fig. 2D), whereas the activation by UT12 was not suppressed, indicating CD14-independent signaling by UT12. Thus, UT12 could be a useful tool for studying TLR4 signaling because we can remove any influence of CD14 in the experimental system. This also indicated that the NF-κB activation achieved by our UT12 preparation was not attributable to LPS contamination.

To further eliminate the possibility of LPS contamination, we inactivated our UT12 preparation by boiling. Heat-treated UT12 completely lost its activity, in contrast to LPS, which was still active after heat treatment (Fig. 2E), confirming negligible LPS contamination. We further purified our UT12 preparation using protein G affinity chromatography. The new preparation was almost pure, as judged by Coomassie staining (data not shown). The new preparation still demonstrated a similar ag-
onistic activity both in vitro (Fig. 2F) and in vivo (data not shown). Furthermore, the depletion of IgG from the UT12 preparation resulted in the abrogation of the stimulating activity (Fig. 2F). These results clearly indicate that UT12 is responsible for the activation and eliminate the possibility of contamination with other bioactive substances and alternative TLR4 agonists.

We next investigated whether the NF-κB activation induced by UT12 was linked to the production of proinflammatory cytokines. PECs prepared from C3H/HeN mice or LPS-insensitive C3H/HeJ mice (19) were stimulated with LPS or UT12 in vitro. Treatment of HeN PECs with UT12 resulted in the secretion of large amounts of TNF-α and IL-6, as is observed upon LPS stimulation. On the other hand, LPS-insensitive HeJ PECs did not respond to treatment with either LPS or UT12 (Fig. 2G). These results indicate that UT12 induces NF-κB activation and the subsequent production of proinflammatory cytokines in vitro as efficiently as LPS does specifically through the TLR4/MD-2 complex.

Recognition of TLR4/MD-2 by UT12 is not affected by LPS. It is conceivable that UT12 exerts LPS-like effects by recognizing the LPS-binding site on the TLR4/MD-2 complex. To examine this possibility, we pretreated Ba/F3/mTLR4f/mMD-2f/mCD14 with LPS, followed by staining with UT12 or MTS510. MTS510 binding to TLR4/MD-2 was abrogated, as reported previously (1), whereas UT12 binding was not affected by LPS pretreatment at either 4°C or 37°C (Fig. 3A). These data suggest that the epitope for UT12 does not overlap with the LPS binding site and that the epitope is not affected by the putative conformational change of the TLR4/MD-2 molecules during LPS recognition (1). In addition, UT12 and LPS did not exhibit significant functional antagonism or synergistic potentiation (Fig. 3B). It is presumed that UT12 can recognize LPS-bound TLR4 molecules that have undergone the conformational change. Epitope analyses might help uncover changes to the conformation and/or the formation of TLR4 molecules during signal induction.

**UT12 induces LPS-like shock.** When large amounts of LPS are administered, mice fall into endotoxin shock, followed by death (26). To investigate whether UT12 could function in vivo, we tried inducing LPS-like shock using UT12. C3H/HeN or C3H/HeJ mice were intraperitoneally injected with LPS along with β-GalN (9), and then the survival of the mice was monitored. As expected, all LPS-sensitive HeN mice died within 12 h after UT12 or LPS injection (Fig. 4A), concomitant with the remarkable increase in serum TNF-α and IL-6 levels (Fig. 4B). LPS-insensitive HeJ mice survived lethal challenges with LPS or UT12, with no induction of TNF-α or IL-6 (Fig. 4A and B). UT12 displayed a similar lethality for C57BL/6 and...
D-GalN is a well-established reagent that sensitizes mice to the lethal effect of LPS by perturbing hepatic metabolism. The lethal effect is mainly mediated by the TNF-α produced upon LPS stimulation of macrophages. In the D-GalN-sensitized mouse model, the sensitivity of the host to TNF-α is increased without altering the actual mechanisms of toxicity compared to those in the nonsensitized model (13). That is, the fundamental lethality caused by LPS and UT12, which induce TNF-α production, can be reflected in the D-GalN-sensitized mouse model. Thus, the D-GalN-sensitized mouse model is applicable in this study for comparison of lethal shock by LPS and UT12. We determined the 50% lethal doses (LD_{50}) of LPS (0.8 to 4 ng/mouse) and UT12 (16 to 80 ng/mouse) for D-GalN-sensitized mice (data not shown). The ratio of LD_{50,LPS}/LD_{50,UT12} was approximately 1:20, which, intriguingly, corresponded to the potency ratio of UT12/LPS for NF-κB activation in vitro. These results may support the possibility that the effects detected in the D-GalN-sensitized mouse model reflect the basic properties of LPS or UT12.

**UT12 induces long-term LPS tolerance in vivo.** The administration of LPS to animals at a sublethal dose causes an LPS-hyporesponsive state called LPS tolerance (26). The LPS-like function of UT12 prompted us to investigate whether UT12 induced LPS tolerance. When the mice were challenged with a lethal dose of LPS 24 h after tolerance induction by 1 μg LPS or 10 μg UT12 per mouse, none of the mice died in the 14-day monitoring period (Fig. 5A). Three days after tolerance induction, however, all of the mice made tolerant by LPS died within 18 h after the lethal challenge. Mice made tolerant by LPS and challenged at day 6 or day 9 did not survive for even 12 h (Fig. 5A, lower panel). Surprisingly, the tolerance induced by UT12 was maintained much longer than the tolerance induced by LPS. Most of the mice made tolerant by UT12 survived the day 3 lethal challenge throughout the monitoring period. Sixty percent of the mice challenged at day 6 were alive at the end of the monitoring period. Mild but obvious tolerance remained even 9 days after tolerance induction; 50% of the mice were alive 30 h later, and 78 h was required for all mice to die (Fig. 5A, upper panel). Thus, UT12 could induce a significantly more prolonged LPS tolerance than LPS could. Mice pre-treated with Y5606 showed no increased survival upon lethal challenge (data not shown).

For mice made tolerant by either UT12 or LPS, the production of TNF-α and IL-6 by lethal challenge was suppressed in...
the early phase of tolerance and increased in proportion to the time after tolerance induction (Fig. 5B). The suppression of cytokine production in mice made tolerant by UT12, however, was significantly stronger than that in mice made tolerant by LPS. Moreover, the recovery of cytokine production was markedly delayed in mice made tolerant by UT12 compared to that in mice made tolerant by LPS. Serum proinflammatory cytokines in mice made tolerant by UT12 were attenuated to a very low level until day 6 and started to increase from day 9. On the other hand, in mice made tolerant by LPS, the serum cytokine concentration was increased from day 3 and at day 9 reached the level for mice not made tolerant.

We also investigated whether larger amounts of LPS could induce LPS tolerance more efficiently. Ten micrograms of LPS was administered to each mouse to induce LPS tolerance. Even when the mice were pretreated with 10 μg LPS, all mice died within 48 h after lethal challenge at day 3. No mice challenged at day 6 survived for 12 h (Fig. 5C).

Additionally, LPS pretreatment of the mice induced cross tolerance to the lethal shock caused by UT12 (Fig. 5D). This suggests that the signaling pathways for UT12 and LPS are in part identical or that they “cross-talk.”

In most LPS tolerance studies, researchers use several hundred micrograms of LPS per mouse, often with multiple administrations. In these cases, on the basis of the functional activities per weight of agonists, we would have to use milligrams of UT12 per mouse, which may not be physiologically possible and/or practical in either the clinical or the experimental setting. Although large amounts of LPS may induce prolonged tolerance states, it is possible that they elicit unwanted side effects, such as excessive inflammation. We therefore used reduced amounts of LPS and UT12 for the induction of tolerance. Because UT12 induces a tolerance longer than that induced by LPS, we could lower the amount of UT12 to be administered, possibly reducing side effects.

Interestingly, the induction of tolerance with UT12 resulted in a much longer tolerance in vivo, despite its slightly lower potency than LPS in vitro. This may be explained by the differences in the in vivo clearance rates between UT12 and LPS. LPS in blood is rapidly neutralized by binding to high-density lipoprotein particles and is excreted by the liver (15). When LPS is injected intravenously into mice, 60 to 75% is eliminated from the circulation in 8 min (22). Intraperitoneally administered LPS may also be cleared at a rapid rate. In contrast, IgG is a natural, relatively stable protein in the animal body. The half-life of mouse IgG is approximately 7 days (18, 25). A similar half-life could be expected in the case of UT12. Therefore, newly synthesized TLR4/MD-2 complex molecules were upregulated upon stimulation by UT12. In LPS-treated PECs, the levels of expression of IRAK-M, SHIP, and SOCS-1 were augmented, as reported previously (23). As expected, incubation of PECs with UT12 upregulated the negative regulators, demonstrating kinetics similar to that of LPS treatment (Fig. 6). These results suggest that UT12 shares the same cytokine signaling pathways with LPS for the induction of tolerance.

Concluding remarks. We have reported here that UT12 is an agonist of TLR4 signaling and in vivo induces tolerance much longer than the natural ligand, LPS, does. In addition to UT12, two MAbs agonistic to TLR4 have been reported. One is an anti-human TLR4 MAb that induces marginal stimulatory signals (4). The other is an anti-mouse TLR4/MD-2 MAb, Sa15-21 (3). Sa15-21 upregulates NF-κB activation and the expression of antiapoptotic genes in the liver, and mice pretreated with Sa15-21 survive the hepatic failure triggered by LPS. However, pretreatment of mice with Sa15-21 enhances LPS-induced TNF-α production, although Sa15-21 alone induces minimal TNF-α production. These properties of Sa15-21 are noticeably distinct from those of UT12, which induces TNF-α production by itself and which suppresses LPS-induced TNF-α production when it is administered before LPS is. Because UT12 rescues mice from endotoxin shock for a more prolonged period with smaller required amounts than those of Sa15-21, TNF-α may play an important role in the full LPS tolerance induced by UT12.

Besides LPS, other structural components of pathogens, e.g., lipoteichoic acid, peptidoglycan, and bacterial lipoprotein, have been used to induce tolerance (26). These materials extracted from pathogenic microorganisms may be contaminated with other physiologically active substances (10, 11, 16). Due to the nature of MAb, UT12 induces signals only through TLR4, eliminating any unexpected host responses. The potent effect of UT12 suggests that more specific and efficient control of tolerance induction can be accomplished with small amounts of UT12, avoiding excessive cytokine production. In some infectious states, however, the use of short-acting or easily reversible reagents may be preferable. It is important to use UT12 and/or such reagents to suit individual conditions. Alternatively, reducing the amount of UT12 could shorten the period of tolerance.

It has been reported that LPS pretreatment improves the clearance of bacteria and fungi in infection experiments (14, 20) and increases survival in cecal ligation and puncture-induced sepsis (5, 24) and in colon ascendens stent peritonitis (7). Therefore, it is highly expected that UT12 will result in
similar improved effects in in vivo sepsis models. These effects should be investigated in a future study.

Taken together, the induction of tolerance by using MAbs against TLR4 could be a novel potentially therapeutic strategy for endotoxin shock. Moreover, UT12 might be used as an immune activator, because TLR4 agonists are used for such purposes as adjuvants of vaccination and antitumor reagents (21). Accordingly, UT12 provides the prospect for the use of agonistic anti-TLR4 MAbs for clinical applications.

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