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Septic Shock Is Associated with Receptor for Advanced Glycation End Products Ligation of LPS

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Septic shock is a severe systemic response to bacterial infection. Receptor for advanced glycation end products (RAGE) plays a role in immune reactions to recognize specific molecular patterns as pathogen recognition receptors. However, the interaction between LPS, the bioactive component of bacterial cell walls, and RAGE is unclear. In this study, we found direct LPS binding to RAGE by a surface plasmon resonance assay, a plate competition assay, and flow cytometry. LPS increased TNF-α secretion from peritoneal macrophages and an NF-κB promoter-driven luciferase activity through RAGE. Blood neutrophils and macrocytes expressed RAGE, and TLR2 was counterregulated in RAGE−/− mice. After LPS injection, RAGE+/- mice showed a higher mortality, higher serum levels of IL-6, TNF-α, high mobility group box 1, and endothelin-1, and severe lung and liver pathologies compared with RAGE−/− mice without significant differences in plasma LPS level. Administration of soluble RAGE significantly reduced the LPS-induced cytokine release and tissue damage and improved the LPS-induced lethality even in RAGE−/− mice as well as RAGE+/- mice. The results thus suggest that RAGE can associate with LPS and that RAGE system can regulate inflammatory responses. Soluble RAGE would be a therapeutic tool for LPS-induced septic shock. The Journal of Immunology, 2011, 186: 3248–3257.

Septic shock is a systemic response to severe bacterial infection. The shock caused by LPS, the major component of the cell surface of Gram-negative bacteria, is often associated with a high mortality (1). LPS consists of three covalently linked regions: lipid A, core oligosaccharide, and O Ag poly-

Saccharide (2). Lipid A resides at the inner most layer and is responsible for toxicity of LPS, thus having been known as endotoxin (2). LPS can induce massive production of cytokines, such as TNF-α and IL-6, by immune and nonimmune cells, thereby leading to inflammatory tissue injuries and finally multiorgan failure, the clinical hallmarks of septic shock (3–5). It is well known that TLR4, a member of pathogen recognition receptors (PRRs), mediates LPS-induced activation of the innate immune system and subsequent immunostimulation (6). When complexed with CD14 and MD-2, TLR4 has been shown to participate in the cellular recognition of LPS (7). It is also reported that TLR2 could sensitize LPS (8). Other LPS receptors and receptor clusters are recently reported; these include heat shock proteins and 70 and 90, CXCR4 and growth differentiation factor 5 cluster (9), RP105-MD-1 complex (10), CD11c (11), CD55 (12), and scavenger receptor class A (15). On the basis of function, PRRs may be put into two categories of signaling type and endocytic type. TLRs and scavenger receptor class A are regarded as representative of the former and the latter, respectively.

The receptor for advanced glycation end products (RAGE) is a cell-surface receptor belonging to the Ig superfamily. It is composed of an N-terminal extracellular domain with a V region-like domain and two C region-like domains, a single transmembrane domain, and a C-terminal highly charged short cytoplasmic domain, which is essential for signal transduction (16). RAGE is known to be a multiligand receptor participating in a wide variety of physiological and pathological processes, such as diabetic complications, atherosclerosis, cancer, neurodegenerative disorders, and inflammation (16–18). RAGE is engaged not only by advanced glycation end product (AGE) but also by amylophorin/high mobility group box 1 (HMGB1) (19), S100 proteins (20), transhydroxyn (21), β-integrin Mac-1 (22), and amyloid β-proteins (23). Binding of these ligands to RAGE initiates intracellular signaling to activate transcription factors NF-xB (16). Among

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those ligands, HMGB1, S100 proteins, and Mac-1 are reported to yield proinflammatory reactions through RAGE (16, 19, 20, 22). RAGE is now recognized as one of PRRs capable of recognizing pathogen-associated molecular patterns. HMGB1 is a nuclear architectural chromatin binding and a cytosolic protein released from necrotic cells and activated macrophages and has been related to LPS-induced lethality (16, 19). In an LPS-induced septic shock model, administration of anti-HMGB1 neutralizing Ab was shown to prevent the death and RAGE-null mice exhibited a prominent decrease in the lethality compared with wild-type (WT) mice (24). In another septic shock model induced by polymicrobial peritonitis following cecal ligation and puncture, RAGE-null mice were less susceptible to the septic shock than WT mice (25).

These observations have prompted us to investigate whether RAGE could directly bind LPS, mediate LPS-induced signaling and proinflammatory reactions, and eventually lead to the septic shock. The results obtained have clearly defined RAGE as a new receptor of LPS and a decoy form of RAGE (soluble RAGE [sRAGE]) as an effective therapeutic means against LPS-induced septic shock.

### Materials and Methods

#### LPS

LPS (Escherichia coli 055:B5, E. coli 0127:B8, E. coli 0111:B4, Klebsiella pneumoniae, and Salmonella enterica serotype typhimurium) and 3-deoxy-D-manno-octulosonic acid (KDO)2-lipid A were purchased from Sigma-Aldrich and Avanti Polar Lipids, respectively. Surface plasmon resonance (SPR) assay showed that anti-AGE Abs such as anti-pentosidine Ab (clone PEN-12; TransGenic), anti-pyrraline Ab (clone H-12; TransGenic), and anti-Nε-carboxymethyllysine Ab (clone 6D12; TransGenic) did not react to LPS (Supplemental Fig. 1). Pretreatment of these AGE Abs also did not show any inhibitory effects on LPS-mediated NF-κB activation, suggesting no AGE modification on LPS employed in the assays (Supplemental Fig. 1).

#### Mice and a septic shock model

Male RAGE−/− mice cross bred with the CD-1 strain over seven generations and their WT littermates were used at 8–10 wk of age (26). For induction of septic shock, LPS serotype 055:B5 from E. coli (Sigma-Aldrich) was administered i.p. at 50 mg/kg body weight as described (27). Recombinant mouse sRAGE was prepared for animal experiments to avoid any immunological reactions and kindly provided by Mitsubishi Pharma. Mice received a single i.p. injection of sRAGE protein (35.0 μg/mouse) at 30 min after the LPS challenging. Survival was monitored for 72

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**FIGURE 1.** Binding of LPS to RAGE. **A**, SPR assays of LPS binding to the extracellular domain of RAGE immobilized on a sensor chip. LPS from E. coli 055:B5, E. coli 0127:B8, E. coli 0111:B4, Klebsiella pneumoniae, and Salmonella enterica serotype typhimurium and KDO2-lipid A were used. Kinetic analyses by SPR were done with LPS E. coli 055:B5 (2.5, 5, 10, and 20 ng/ml) and KDO2-lipid A (0.313, 0.625, 1.25, 2.5, and 5 ng/ml). SPR assay was performed as described under Materials and Methods. After the injection (60 s), the mobile phase was changed back to the buffer without LPS. Normalized successive curves are shown from the lowest to highest concentrations. 10, 10 ng/ml; 20, 20 ng/ml. **B**, SPR assay of esRAGE binding to LPS (E. coli 0111:B4) immobilized on a sensor chip. Purified esRAGE (0.5 and 1.0 μg/ml) was injected. **C**, Competition assay by SPR. Five hundred nanograms per milliliter esRAGE was preincubated with indicated concentrations of AGE-BSA for 30 min, and the preincubation mixtures were injected into the sensor chip on which LPS (E. coli 0111:B4) had been immobilized.
**Figure 2.** Binding of LPS to RAGE and LPS activation of NF-κB and secretion of TNF-α. 

A. Plate competition assay. One hundred nanograms per milliliter esRAGE was preincubated with indicated concentrations of LPS (E. coli 055:B5) for 30 min, and the preincubation mixtures were added into wells on which AGE-BSA had been immobilized. The formation of AGE–RAGE complexes was detected with Eu-conjugated anti-RAGE Ab. The ordinate indicates percentage AGE-RAGE binding with the value without LPS being 100%. 

B. Plate competition assay. One hundred nanograms per milliliter esRAGE proteins were preincubated with indicated concentrations of AGE-BSA for 30 min, and the preincubation mixtures were added into wells on which LPS (E. coli 055:B5) had been immobilized. The formation of LPS–RAGE complexes was detected with Eu-conjugated anti-RAGE Ab. The ordinate indicates percentage of LPS-RAGE binding with the value without LPS being 100%. 

C. Flow cytometry. Peritoneal macrophages from RAGE+/+ mice, but not RAGE−/− mice, expressed RAGE protein. RAGE expression was not correlated with TLR2 or TLR4. One representative result from a total three repeats is shown. 

D. TNF-α secretion from peritoneal macrophages. After 24 h incubation of LPS 50 ng/ml, TNF-α level in the cell culture media was assayed. *p < 0.0001 compared with RAGE−/− mice exposed with LPS. 

E. NF-κB promoter assay. C6 glioma cells that had been transformed by a mammalian expression vector pCI-neo with human full-length RAGE cDNA and by a reporter gene carrying an NF-κB promoter and firefly luciferase gene were treated with indicated concentrations of LPS and sRAGE. Immunoblot in the inset shows diminished RAGE proteins under siRNA and expression of dominant-negative RAGE under dnRAGE. Data are presented as mean ± SEM. *p < 0.0001 compared with LPS alone. 

F. NF-κB promoter activity assay. C6 glioma cells that had been transformed by a mammalian expression vector pCI-neo with human full-length RAGE cDNA and by a reporter gene carrying an NF-κB promoter and firefly luciferase gene were treated with indicated concentrations of LPS and siRNA. Immunoblot in the inset shows diminished RAGE proteins under siRNA and expression of dominant-negative RAGE under dnRAGE. Data are presented as mean ± SEM. *p < 0.0001 compared with LPS alone.
h. Survival as the end point in these experiments was calculated from the time of LPS treatment using the product limit Kaplan-Meier method (28). Animals were treated in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan, and animal experiments were approved by the Committee on Animal Experimentation of Kanazawa University.

**SPR assay**

Purified human endogenous secretory RAGE (esRAGE) proteins having the ligand-binding domain (29) were immobilized to a BIAcore CM5 research-grade sensor chip with the amine coupling kit (GE Healthcare) to a density of ∼5000 response units. LPS (E. coli 0111:B4) was also coupled to the sensor chip by amine coupling. LPS binding to the immobilized RAGE protein or esRAGE binding to the immobilized LPS was examined with the BIAcore 2000 system (GE Healthcare) as described previously (26, 29); the flow buffer used contained 10 mM HEPES (pH 7.4), 0.15 M NaCl, 3 mM Na-EDTA, and 0.005% (v/v) surfactant P-20. Association and dissociation were measured at 25°C in a flow rate of 20 μl/min. The sensor chips were regenerated by washing with 10 mM NaOH and 0.5% (v/v) SDS.

**Plate competition assay**

LPS competition of AGE-RAGE association was assayed with AGE-BSA–coated 96-well plates, human esRAGE proteins, and E. coli–conjugated anti-human RAGE Ab as previously described (26). AGE–BSA competition for LPS–RAGE interaction was also assayed with LPS-coated 96-well plates.

**Peritoneal macrophages**

Thioglycolate-elicited murine peritoneal macrophages were isolated from RAGE+/− and RAGE−/− mice as previously described (30). The macrophages were washed and cultured overnight in DMEM supplemented with 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin and stimulated with LPS 100 ng/ml for 24 h.

**Flow cytometry**

For flow cytometric analysis, peritoneal macrophages were washed and resuspended in staining buffer (PBS containing 2% FCS) containing FcBlock (BD Biosciences), and cells were stained with the following Abs (15 min at 4°C in the dark): CD45-biotin (eBioscience), CD11b–APC-Cy7 (BD Biosciences), TLR4–PE (eBioscience), TLR2–PE-Cy7 (eBioscience), and polyclonal rabbit anti-RAGE Ab (18). Biotinylated Ab and the RAGE Ab were visualized following a second incubation with streptavidin-PE–Texas Red (BD Biosciences) and anti-rabbit IgG–FITC (eBioscience), respectively. Cells were resuspended in 200 μl staining buffer containing 0.2 μg/ml propidium iodide (Sigma-Aldrich), filtered through a 100-μm mesh, and analyzed by FACSaria II (BD Biosciences). Blood were collected with 5 mM EDTA, treated with FcBlock (BD Biosciences), and stained with same Abs above and additional CD3-FLUOR450 (eBioscience), CD8-APC (eBioscience), and CD19-PerCy5-Cy5.5 (eBioscience). For detection of LPS binding to RAGE Ab and anti-rabbit IgG–FITC were replaced with LPS–FITC (Sigma-Aldrich). RBCs were then lysed with FACS lysing solution (BD Biosciences). Cells were resuspended in 200 μl staining buffer, filtered through a 100-μm mesh, and analyzed by FACSaria II (BD Biosciences). For detection of LPS binding to sRAGE in vivo, we used anti-mouse Ig, κ beads of polystyrene particles (BD Biosciences), and the negative control beads (BD Biosciences) with a mouse monoclonal RAGE Ab (clone 278-13G4) (31) to detect the complex of sRAGE with FITC-labeled LPS (Sigma-Aldrich) using flow cytometry. Plasma was obtained after 2 h of i.p. injection of sRAGE (35.0 μg/mouse) and FITC-LPS (50 mg/kg) into mice and then incubated with the RAGE Ab-coupled or the control beads. The beads were washed three times and analyzed with FACSaria II (BD Biosciences). Data were transferred and reanalyzed with FlowJo software (Tree Star).

**Plasma LPS concentration**

Transition of i.p.-injected FITC-labeled LPS (Sigma-Aldrich) into plasma was determined using a Labsystems Fluoroscan Ascent FL fluorescence meter (Labsystems).

**ELISAs for TNF-α, IL-6, endothelin-1, and HMGB1**

 Serum concentrations of TNF-α and IL-6 were measured by ELISA according to the manufacturer’s protocol (R&D Systems, Wiesbaden-Nordenstadt, Germany). Serum endothelin-1 (ET-1) and serum and culture medium HMGB1 levels were measured with Peninsula Laboratories (San Carlos, CA) and Shino test (Tokyo, Japan) ELISA systems, respectively.

**NF-κB luciferase assay**

C6 rat glioma cells were used for this assay according to the procedures as described previously (26, 32). In brief, RAGE-expressing C6 cells stably transformed with NF-κB promoter-driven luciferase construct were pre-incubated for 24 h in DMEM supplemented with 0.1% FBS and then stimulated by LPS serotype 0111:B4 (50 ng/ml) (Sigma-Aldrich) for 4 h. RAGE expression was silenced by the small interfering RNA (siRNA) expression system using the pSilencer 3.0-H1 vector (Ambion, Austin, TX) and a dominant-negative RAGE lacking cytoplasmic domain was over-expressed (26). TLR2 and TLR4 expressions were silenced by Flexitube siRNAs (Qiagen Rn_Tlr2-1, SI02045967, and Rn_Tlr4-2, SI02046002, respectively) according to the manufacturer’s protocol. The luciferase activity was assayed with Luciferase Assay System (Promega). The resultant fluorescent products were measured with a Fluoroscan Ascent FL luminometer (Labsystems).

**Histopathological examination**

Animals were sacrificed for evaluation of tissue damage at 25 h after LPS challenge with or without sRAGE treatment. Excised organs were fixed with 4% paraformaldehyde, embedded in paraffin, and cut into 4-μm-thick sections followed by H&E staining for microscopic analysis. The neutrophil infiltration was assessed by immunostaining for the myeloperoxidase (MPO). Primary Ab of rabbit-polyclonal anti-MPO (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) and peroxidase-conjugated Envision (Envision-PO, Envision System; DakoCytomation) were used. The sections were immersed in DAB solution (Sigma-Aldrich) with H2O2, counterstained with hematoxylin (DakoCytomation), and mounted under coverslips.

**Statistical analysis**

Data obtained by the Kaplan-Meier method were analyzed by the log-rank test. Differences between groups were assessed for statistical significance using the ANOVA test or the unpaired, two-tailed Student t test with Statview software (Abacus Concepts). p < 0.05 was considered to indicate statistical significance.

**Results**

**Physical binding of LPS to RAGE**

To investigate the relationship between RAGE and LPS, we first checked their direct physical binding by SPR. LPS (E. coli 055:B5) was found to bind to RAGE protein immobilized to a sensor chip, and the mean KD value was estimated to be ∼35 nM by using global fitting of monoexponential rate equations derived from the simple 1:1 Langmuir binding model (Fig. 1A). Other LPS from various species and serotypes/strains were also examined and found to bind to RAGE (Fig. 1A). When we tested RAGE binding to LPS that had been immobilized on the chip, positive response signals were also observed between the two (Fig. 1B). Further, the preincubation of AGE-BSA with esRAGE led to a reduction in the binding responses of esRAGE to the LPS in a manner dependent on the dose of AGE-BSA (Fig. 1C). We next examined how chemical degradation of LPS would affect the LPS binding to RAGE. Alkaline degradation of LPS is reported to decrease fatty acid ester content, and acid degradation is reported to result in hydrolysis of LPS, deacylation, and destruction of lipid A (33, 34). The alkaline degradation of LPS did not affect LPS binding to...
RAGE, but the acid degradation canceled the RAGE association (Supplemental Fig. 2). SPR assay revealed that lipid A component of LPS strongly interacted with RAGE with the mean $K_d$ value of 2 nM (Fig. 1A). The LPS-RAGE binding was also confirmed by a plate assay. LPS was found to dose dependently and competitively inhibit AGE binding to RAGE (Fig. 2A). When we used LPS-coated plates, AGE-BSA also competed for LPS-RAGE binding in a dose-dependent manner (Fig. 2B). These suggest that the LPS-binding site on RAGE might overlap with the site to which AGE ligands bind. To identify potential LPS-binding sites of RAGE, we employed synthetic peptides of human RAGE protein. VN1 peptide (KGAPKPPQREWLKLN), but not VN2 peptide (WKNLTRTE0WKLSPQG), was revealed by SPR to bind lipid A as well as various types of LPS (Supplemental Fig. 3).

**Induction of TNF-α secretion and NF-κB activation**

Next, we isolated peritoneal macrophages from RAGE$^{+/+}$ and RAGE$^{-/-}$ mice and examined LPS-induced TNF-α secretion into the culture media. Flow cytometry showed the RAGE protein expressed on the cell surface of the macrophage from RAGE$^{+/+}$ mice (Fig. 2C). TLR2 and -4 expressions were also observed in macrophages from both and were independent of RAGE expression (Fig. 2C). LPS stimulation significantly increased TNF-α secretion in RAGE$^{+/+}$ mouse-derived macrophages when compared with that in RAGE-null macrophages (Fig. 2D). We further checked whether LPS could induce a post-RAGE signaling by using an RAGE-expressing C6 glioma cells carrying a luciferase reporter gene under a control of NF-κB promoter. Addition of LPS in the cell-culture media markedly increased NF-κB–driven luciferase activity (Fig. 2E). This activation was significantly inhibited in the coexistence of sRAGE, although the inhibition was partial in the conditions employed. RAGE dependency of the LPS-induced NF-κB activation was also demonstrated with RAGE siRNA and dominant-negative RAGE lacking the intracellular domain. The C6 glioma cells expressing RAGE siRNA or dominant-negative RAGE showed significant reduction in the LPS-induced luciferase activity. We performed gene knockdown experiments with RAGE siRNA, TLR2 siRNA, TLR4 siRNA, or the all siRNAs in the C6 rat glioma cells. The LPS-induced NF-κB luciferase activity in the C6 glioma cells were downregulated by the treatment with RAGE siRNA, TLR2 siRNA, TLR4 siRNA, or the combination of three siRNAs by 37.2, 17.6, 41.1, and 57.1%, respectively (Fig. 2F). These observations suggest that RAGE and TLR4 contribute in a comparable manner to the LPS-induced NF-κB activation in this cell line.

**RAGE expression on blood neutrophils and monocytes**

We also checked RAGE expression levels in mouse circulating neutrophils and monocytes by flow cytometry. The neutrophils and monocytes were defined as CD45$^+$CD11b$^+$Gr1$^+$CD3$^-$ and CD45$^+$CD11b$^+$CD3$^+$CD19$^-$, respectively. Both cell types from RAGE$^{+/+}$ mice expressed RAGE protein (Fig. 3A). Neutrophils were found to have more abundant cell-surface RAGE protein than monocytes (Fig. 3B). FITC-labeled LPS efficiently bound to RAGE$^{+/+}$ mouse-derived neutrophils and monocytes (Fig. 3C–E). In addition, this ligation was significantly lowered in RAGE$^{-/-}$ mouse-derived cell and by the pretreatment of unlabeled LPS (Fig. 3D, 3F), indicating sufficient evidence of RAGE-specific LPS binding on the inflammatory cell surface. We next examined relationship between RAGE and TLR2 and -4 on blood neutrophils and monocytes. TLR2-positive population was significantly and compensately increased in neutrophils from RAGE$^{-/-}$ mice when compared with RAGE$^{+/+}$ mice (Fig. 3G, 3H). This tendency was also seen in monocytes (Fig. 3G, 3H). However, we could not observe such an increase in TLR4 in RAGE$^{-/-}$ mice (Fig. 3G, 3H).

**LPS-induced septic shock in RAGE$^{+/+}$ and RAGE$^{-/-}$ mice**

To examine in vivo role of LPS–RAGE interactions in an LPS-loading septic shock model, RAGE$^{-/-}$ and RAGE$^{+/+}$ mice were i.p. injected with LPS and observed. All of the mice that received LPS showed apparent signs of distress such as apathy, fur ruffling, and diarrhea. These symptoms were more prominent in RAGE$^{+/+}$ mice. All RAGE$^{+/+}$ mice succumbed to shock within the period from 24–38 h after LPS injection (Fig. 4A). However, RAGE$^{-/-}$ mice began to die after 18 h of LPS injection, and 10% of the animals still survived at 72 h ($p < 0.003)$ (Fig. 4A), indicating that RAGE$^{-/-}$ mice are relatively resistant to septic shock. Circulating LPS concentrations did not differ between RAGE$^{+/+}$ and RAGE$^{-/-}$ mice during the initial absorption phase of LPS and at the peak (Supplemental Fig. 4).

Next, we investigated serum levels of acute inflammation markers and a vascular tone regulator closely related to LPS-induced septic shock. Serum levels of TNF-α and IL-6 were significantly lower in RAGE$^{-/-}$ mice than in RAGE$^{+/+}$ mice at 1 and 2 h and at 6 h, respectively, after injection of LPS (Fig. 4B, 4C), the point times at which the serum concentrations of the respective cytokines are known to peak (5, 27). Moreover, serum ET-1 level was also significantly decreased in RAGE$^{-/-}$ mice compared with RAGE$^{+/+}$ mice (Fig. 4D). The difference in the release of acute-phase cytokines, TNF-α and IL-6, and vascular constrictor ET-1 could influence the difference in the survival rate. Additionally, we checked serum level of HMGB1, a late-phase cytokine associated with the survival in septic shock and known as a bioactive molecule binding to RAGE. Serum HMGB1 increased at 24 h after LPS injection in RAGE$^{+/+}$ mice but remained unchanged in RAGE$^{-/-}$ mice (Fig. 4E). We also measured serum levels of S100A8/A9 and S100B, other RAGE ligands, by ELISAs. S100B level before LPS injection (background level) was found to be significantly lower in RAGE$^{-/-}$ mice than in RAGE$^{+/+}$ mice (Supplemental Fig. 5). After LPS injection, both serum levels of S100A8/A9 and S100B were dramatically increased (Supplemental Fig. 5). However, serum S100A8/A9 level at 24 h after LPS injection was 2-fold higher in RAGE$^{+/+}$ mice than in RAGE$^{-/-}$ mice (Supplemental Fig. 5). After LPS injection, both serum levels of S100A8/A9 and S100B were dramatically increased (Supplemental Fig. 5). However, serum S100A8/A9 level at 24 h after LPS injection was 2-fold higher in RAGE$^{-/-}$ mice than in RAGE$^{+/+}$ mice (Supplemental Fig. 5); this suggests that increased serum S100A8/A9 and S100B might not settle the severe inflammation by LPS in RAGE$^{+/+}$ mice, and RAGE deficiency elicited a significant increase in serum S100A8/A9 level after LPS loading.

**sRAGE treatment attenuated LPS-induced septic shock**

We next examined whether the treatment with sRAGE, which acts as a decoy receptor for RAGE ligands, could improve the survival of the LPS-injected RAGE$^{+/+}$ mice as well as RAGE$^{-/-}$ mice. In this experiment, we reproduced the same survival difference seen in Fig. 4A between RAGE$^{+/+}$ and RAGE$^{-/-}$ mice. However, the mean survival time of RAGE$^{+/+}$ or RAGE$^{-/-}$ mice was longer in Fig. 5A than in Fig. 4A; this might be caused by the difference in body weight (41.1 ± 1.3 and 36.0 ± 0.5 g in Figs. 4A, 5A, respectively; $p < 0.0001)$. As a result, the treatment of sRAGE was found to attribute significantly longer survival rate not only in RAGE$^{+/+}$ mice but also in RAGE$^{-/-}$ mice (Fig. 5A). For detection of LPS–sRAGE complex formed in vivo, we established a new assay system with the anti-mouse Ig, κ beads, and the RAGE Ab, by which sRAGE is visualized when complexed with FITC-LPS (Fig. 5B). Negative control beads or depletion of sRAGE in this reaction mixture did not show positive signals (Fig. 5B). After 2 h...
FIGURE 3. Flow cytometry. A, RAGE expressed on neutrophils and monocytes. Dark shading, cells from RAGE+/+ mice; gray shading, cells from RAGE−/− mice. One representative result from a total three repeats is shown. B, Neutrophils have more abundant cell-surface RAGE than monocytes. Dark shading, neutrophils from RAGE+/+ mice; gray shading, monocytes from RAGE+/+ mice. *p < 0.013. C–F, LPS binding to immune cell surface. Whole blood was incubated with 50 μg/ml FITC-LPS or FITC alone for 2 h at 4°C. Dark shading, FITC-LPS incubation with cells from RAGE+/+ mice; gray shading, FITC alone incubation with cells from RAGE+/+ mice (C). Whole blood was incubated with 50 μg/ml FITC-LPS for 2 h at 4°C. Dark shading, cells from RAGE+/+ mice; gray shading, cells from RAGE−/− mice (D). Arrow indicates a neutrophil cluster highly associated with FITC-LPS. One representative result from a total three repeats is shown. Mean fluorescence intensity (MFI) of FITC-LPS signals from neutrophils and monocytes. *p < 0.05 compared with RAGE+/+ monocytes (E). Neutrophil population (%) highly associated with FITC-LPS as indicated in D with the arrow (F). Two hours incubation of 50 μg/ml FITC-LPS and whole blood with or without pretreatment of 50 μg/ml unlabeled LPS at 4°C. *p < 0.05 compared with RAGE+/+ without pretreatment of unlabeled LPS. G, Relationship between RAGE and TLR2 or -4 in neutrophils and monocytes. One representative result from a total three repeats is shown. H, TLR2- and -4–positive percentage in neutrophils and monocytes. Data are presented as mean ± SEM. *p < 0.05.
FIGURE 4. LPS-induced septic shock in mice (9.00 ± 0.25 wk of age and 41.1 ± 1.3 g in body weight). A, Mortality after LPS administration. LPS was injected i.p. at the dosage of 50 mg/kg body weight. Closed circle, RAGE+/+ mice (n = 18); open circle, RAGE−/− mice (n = 13). Statistical analysis was performed using log-rank test. *p < 0.0001 between RAGE+/+ and RAGE−/− mice. Serum concentrations of TNF-α (B), IL-6 (C), ET-1 (D), and HMGB1 (E) determined by ELISA. Serum samples were taken at the indicated time points after LPS administration. Closed circle, RAGE+/+ mice (n = 12); open circle, RAGE−/− mice (n = 6); closed bar, RAGE+/+ mice (n = 8); open bar, RAGE−/− mice (n = 5). Data are presented as mean ± SEM. **p < 0.05 between RAGE+/+ and RAGE−/− mice.

Discussion

The response to bacterial LPS provides a superb illustration of innate immune function. In this study, we have demonstrated for the first time, to our knowledge, that LPS and its lipid A component directly interacts with a cell-surface receptor RAGE, which may in turn induce intracellular NF-κB activation and TNF-α secretion (Figs. 1, 2). Frecer et al. (35) reported that a common structural motif in LPS-binding proteins and peptides would be the amphipathic cationic binding pattern BHPHB (B, basic; H, hydrophobic; P, polar residue). For example, LPS-binding protein from Bombyx mori and TLR4 have this type of sequences, RVQGR (residues 111–115) and KLTLR (residues 190–194), respectively. We found RLEWK sequence matched with this pattern in the residues 48–52 of human RAGE within the Ig V-type ligand-binding domain. The VN1 peptide (KGAPKPPQRLWKLHN) having RLEWK sequence actually bound to lipid A as well as LPS in SPR assay (Supplemental Fig. 3). These suggest that LPS may associate with the V-type region of RAGE as do AGE ligands, being consistent with the present result that showed the LPS competition of AGE–RAGE binding (Figs. 1C, 2A, 2B). Structural-based analysis such as nuclear magnetic resonance or cocrystallization experiment can draw an ultimate conclusion of LPS-RAGE association in the future. LPS caused a marked increase in NF-κB activity in a RAGE-expressing C6 glioma cell line, and this was significantly inhibited by sRAGE, RAGE siRNA, and dominant-negative RAGE (Fig. 2E). However, the inhibition was partial, suggesting that LPS receptors other than RAGE might work in this system. In fact, it is reported that C6 glioma cells expresses TLR4 (36). We also actually detected both expressions of TLR2 and TLR4 in this cell line (data not shown). According to our gene knockdown experiments with RAGE siRNA, TLR2 siRNA, TLR4 siRNA, or the all siRNAs (Fig. 2F), RAGE and TLR4 contribute in a comparable manner to the LPS-induced NF-κB activation in this cell line. In comprehensive view of immune system and physiology, it is also important that TLR2 level was compensatedly upregulated when RAGE was completely deleted in mice (Fig. 3G, 3H).

In animal experiments, the elevation of serum TNF-α, IL-6, and ET-1 and the tissue damage induced by LPS was significantly attenuated in RAGE−/− mice when compared with RAGE+/+ mice (Figs. 4, 5). This would endow RAGE−/− mice with relative resistance to LPS-induced septic shock, resulting in the higher survival rate (Figs. 4, 5); this is compatible with the results using a different mouse genetic background model by Abeyama et al. (24). Because plasma LPS concentrations were not different between RAGE+/+ and RAGE−/− mice (Supplemental Fig. 4), RAGE should rate-limit the production of inflammatory cytokines and the development of septic shock. Previous studies reported that all TNF-α, IL-6, and ET-1 are NF-κB–dependent effector molecules in the downstream of RAGE signaling (16, 37, 38), which could be closely associated with the development of RAGE-related diseases. In septic shock, ET-1–mediated vasoconstriction can cause deterioration in the regional microcirculation, leading to tissue damage (39). Being consistent with a notion that TNF-α is an initiator of the cascade of proinflammatory responses to LPS loading (5), circulating TNF-α level was observed to peak as rapidly as 1 h after LPS challenge (Fig. 4B). An
FIGURE 5. sRAGE protection against LPS-induced septic shock in mice (8.2 ± 0.07 wk of age and 36.0 ± 0.5 g in body weight). A, Mortality after LPS administration. LPS was injected i.p. at the dosage of 50 mg/kg body weight. Closed circle, RAGE+/+ mice without treatment ($n = 11$); open circle, RAGE−/− mice without treatment ($n = 14$). Statistical analysis was performed using log-rank test. Thirty-five micrograms sRAGE per mouse was administered for the treatment. Closed triangle, RAGE+/+ mice with sRAGE treatment ($n = 15$); open triangle, RAGE−/− mice with sRAGE treatment ($n = 14$). *$p < 0.02$ between sRAGE-treated and untreated RAGE+/+ mice, **$p < 0.01$ between sRAGE-treated and untreated RAGE−/− mice. B, Detection of LPS–sRAGE complex in plasma. Anti-mouse Ig, κ beads (Beads) of polystyrene particles (51-9006274, BD Biosciences), or the negative control beads (C-Beads) (51-9006227, BD Biosciences) with anti-RAGE Ab (aRAGE; clone 278-13G4) were used. The anti-mouse Ig, κ beads (Beads), but not control beads (C-Beads), incubated with the RAGE Ab (aRAGE) and sRAGE could make a complex with FITC-LPS (upper panel). Depletion of sRAGE in this reaction mixture did not show positive signals (upper panel), indicating the usefulness of this detection system. sRAGE–FITC–LPS complex was detected in the plasma from mice with i.p. injection of FITC-LPS and sRAGE (lower panel). C, Histopathological findings. H&E stain of lung (original magnification ×100 and ×400) and liver (original magnification ×100 and ×400) of RAGE+/+ and RAGE−/− mice treated or not treated with sRAGE. Tissues were removed and fixed at 25 h after LPS injection. D, Neutrophil infiltration evaluated by immunohistochemistry with anti-MPO Ab. Brown signal indicates neutrophil. Serum concentrations of TNF-α ($E$) at 1 h (*$p < 0.05$, **$p < 0.037$), IL-6 ($F$) at 6 h (*$p < 0.023$, **$p < 0.014$, $p = 0.051$ between sRAGE-treated or nontreated RAGE−/− mice), ET-1 ($G$) at 24 h and HMGB1 ($H$) at 24 h after LPS injection. Results are presented as mean ± SEM. Liver+/+, liver from RAGE+/+ mice; Liver−/−, liver from RAGE−/− mice; Lung+/+, lung from RAGE+/+ mice; Lung−/−, lung from RAGE−/− mice.
elevation of the late mediator HMGB1, which is also a RAGE ligand, was marked at 24 h after LPS loading (Fig. 4E). HMGB1 is known to be released readily from necrotic or damaged cells and serves as a local signal for inflammation. Such paraconi- like actions of HMGB1 should also be taken into account even in this systemic inflammatory model. These findings suggest that LPS-mediated RAGE signaling accelerates acute inflammatory reactions and vascular dysregulation leading to tissue damages, which then mediate HMGB1 release, resulting in a pernicious cycle of RAGE-dependent lethality in septic shock.

This study has also shown that sRAGE, a truncated soluble form carrying extracellular ligand-binding domain of RAGE, has protective effects against LPS-induced septic shock in mice. Previous studies using sRAGE demonstrated its beneficial effects on RAGE-associated diseases such as atherosclerosis and inflammation (20, 40, 41). The treatment with sRAGE reduced the LPS induction of TNF-α and IL-6 and of liver and lung damages and significantly improved survival rate (Fig. 5). It may be reasonable to posit that sRAGE trapping of LPS may also inhibit non-RAGE LPS receptor signaling as well. In addition, sRAGE may also neutralize late-phase HMGB1 function, leading to prevention of LPS-induced septic shock. Liliensiek et al. (25) have also shown that RAGE-mediated RAGE signaling accelerates acute inflammatory reactions of HMGB1 by necrotic cells triggering inflammation.

In conclusion, the results obtained indicate that RAGE participates in inflammatory responses. Upon LPS stimulation, sRAGE seems to antagonize ligand binding not only to RAGE, but also to TLR2 and TLR4, resulting in inhibition of LPS-induced inflammation. sRAGE may become an effective remedy for treating septic shock.

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Disclosures

The authors have no financial conflicts of interest.

References

19. Scaffidi, P., T. Misteli, and M. E. Bianchi. 2002. Release of chromatin protein HMGB1 by necrotic cells triggered by using sRAGE. Further studies are needed to reveal whether sRAGE directly can block other receptors than RAGE or sRAGE can an- tagonize ligand/LPS binding to the other receptors.
20. A recent report using the alive E. coli infection model in contrast to this LPS shock model demonstrated that intact RAGE signaling contributed to an effective antibacterial defense such as inhibition of bacterial outgrowth and dissemination, and RAGE deficiency resulted in enhanced organ injuries such as liver necrosis (42).
21. In general, the innate immune response to severe bacterial infection can act as a double-edged sword, on the one hand protecting the host against invading pathogens, and on the other hand poten-
22. tially destroying cells and tissues. Even though the LPS shock model is an extreme exaggerated inflammation model, RAGE can participate in sensing pathogens and controlling a delicate balance between clearance of invading pathogens and exaggerated inflamma-
23. tion. In conclusion, the results obtained indicate that RAGE participates in inflammatory responses. Upon LPS stimulation, sRAGE seems to antagonize ligand binding not only to RAGE, but also to TLR2 and TLR4, resulting in inhibition of LPS-induced inflammation. sRAGE may become an effective remedy for treating septic shock.


