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A Soluble Form of LMIR5/CD300b Amplifies Lipopolysaccharide-Induced Lethal Inflammation in Sepsis

Yoshinori Yamanishi,*† Mariko Takahashi,* Kumi Izawa,* Masamichi Isobe,* Shinichi Ito,* Akiho Tsuchiya,* Akie Maehara,* Ayako Kaitani,* Tomoyuki Uchida,* Katsuhiko Togami,* Yutaka Enomoto,* Fumio Nakahara,* Toshihiko Oki,*† Masunori Kajikawa,† Hiroki Kurihara,† Toshio Kitamura,*‡ and Jiro Kitaura*

Leukocyte mono-Ig–like receptor 5 (LMIR5) is a potent activating receptor that interacts with DNAX-activating protein of 12 kDa; it is predominantly expressed in myeloid cells including neutrophils, macrophages (Mφ), and mast cells (13). We have previously demonstrated that T cell Ig mucin 1 (TIM1) is a crucial myeloid cell receptor for neutrophil-mediated inflammatory responses in sepsis models (22–24). Interestingly, a soluble form of TIM1 (sTIM1) is released in infection and counterregulates TREM-1–mediated inflammatory response in the context of microbial infection (22–26). In contrast, we demonstrate that sLMIR5 is released by neutrophils and that the release is augmented by TLR agonists. Remarkably, LMIR5-Fc administration protects against LPS- or peritonitis-induced death, implicating sLMIR5 in lethal inflammation (22–26). In contrast, we demonstrate that sLMIR5 is amplified in LPS-induced lethal inflammation.

Sepsis is a complex clinical syndrome that results from a harmful host response to infection. The inflammatory response is initiated after detection of microbial infection by pattern-recognition receptors (PRRs) in immune cells; PRRs recognize pathogen-associated molecular patterns that are expressed by microorganisms (1–6). Among PRRs, TLRs play a crucial role in the initiation of inflammatory responses: TLR4 recognizes LPS, a component of the outer membrane of Gram-negative bacteria (7, 8). During sepsis, systemic activation of immune cells by high levels of pathogen-associated molecular patterns causes an imbalanced cytokine response (1–8). In addition, innate immune responses are regulated by receptors that signal through ITAM, ITIM, or both (9, 10). Complete understanding of the immunopathology of sepsis will culminate in the establishment of sepsis-stage–specific therapy (3–5).

Leukocyte mono-Ig–like receptor (LMIR, also called CD300b) (11–15) belongs to a paired activating and inhibitory receptor family (9, 10, 16, 17). Mouse LMIR is also known to be a CMRF-35–like molecule (18) or myeloid-associated Ig-like receptor (19). LMIR5 is an activating receptor that interacts with DNA-activating protein of 12 kDa (DAP12), an ITAM-containing adaptor molecule (13). LMIR5 is predominantly expressed in myeloid cells including neutrophils, macrophages (Mφ), and mast cells (13). We have recently identified T cell Ig mucin 1 (TIM1) and TIM4 as endogenous ligands for LMIR5; the intact Ig-like domain of LMIR5 is required for the binding to TIM1/4 (15, 20, 21). LMIR5 deficiency alleviates mouse ischemia/reperfusion injury in the kidneys in which TIM1 expression is upregulated (15, 21). However, the pathophysiological role of LMIR5 in other types of inflammation models remains elusive.

In this study, we demonstrate that a soluble form of LMIR5 (sLMIR5) is released by neutrophils, and that the release is augmented by TLR agonists. Remarkably, LMIR5−/− mice are protected against LPS- or peritonitis-induced death, implicating LMIR5 in LPS-induced sepsis. According to recent studies, triggering receptors expressed on myeloid cells-1 (TREM-1) amplifies inflammatory response in the context of microbial infection (22–24). Interestingly, a soluble form of TREM-1 (sTREM-1) is released in infection and counterregulates TREM-1–mediated inflammation (22–26). In contrast, we demonstrate that sLMIR5 is an amplifier of LPS-induced inflammation, highlighting a novel mechanism of sepsis-induced lethality in sepsis.
Materials and Methods

Cells and mice

Murine cell lines used in this study were as follows: RAW264.7, Ba/F3, and BW5147. Bone marrow (BM)-derived mast cell (BMMC) and peritoneal Mb were generated as described previously (12, 15, 27). Neutrophils were isolated from BM by using three-layer gradient as described previously (28). We used LMIR5−/− female mice and their wild type (WT) littermates. LMIR5+/- mice had been backcrossed at least eight generations with C57BL/6 mice (Charles River Laboratories Japan) (15). All the procedures were approved by an Institutional Review Committee of the University of Tokyo (approval no. 20-8).

Abs and other reagents

Anti-LMIR5 polyclonal Ab was obtained from R&D Systems. Anti-LMIR5 mAb (2C12) was from ACTGen. Both anti-LMIR5 polyclonal Ab and anti-LMIR5 mAb (2C12) specifically recognized mouse LMIR5. The recombinant extracellular domain of LMIR5 (aa 18–157) was obtained from R&D Systems. Anti-Flag mAb (M2), goat IgG, human IgG1, and LPS (Escherichia coli 0111:B4) were from Sigma-Aldrich. PE-conjugated donkey F(ab)2 anti-goat IgG and anti-human IgG Abs were from Jackson Immunoresearch Laboratories. Anti–Gr-1 Ab, rat IgG2b, FITC-conjugated anti-mouse F4/80, Gr-1, and CD11b Abs, and PE-conjugated anti–Gr-1 Ab were from eBioscience. Anti-TIM4 mAb (RMT4-54) was a gift from Drs. H. Nomizu and K. Nakamura (Juntendo University, Tokyo, Japan). Oligo-deoxynucleotide (ODN) control, ODN1826, Loxoribine, Flagellin, polyriboinosinic-polycytidylic acid, and peptidoglycan were purchased from InvivoGen. GM6001 (catalog no. 364205) and GM control (catalog no. 364210) were from Calbiochem.

Gene expression analysis

Relative gene expression of LMIR5 or IL-6 among samples was measured by real-time RT-PCR using gene-specific primers as described previously (13, 15).

Generation of Fc fusion proteins

The Fc fusion proteins were purified as described previously (15). To prepare the Fc fragment alone, we used pME18S-hIgG1 Fc vector containing a signaling lymphocyte-activation molecule (SLAM)/CD150 signal sequence (a gift from Dr. H. Arase, Osaka University, Osaka, Japan) (29). Endotoxin levels of Fc fusion proteins used in this study were <0.01 ng/ml protein, when measured by Limulus Amebocyte Lysate (Lonza).

Flow cytometry

Cells were stained as described previously (15). The mice were i.p. injected with 100 µg LMR5-Fc to identify the target cells of LMR5-Fc in vivo. Five minutes after injection, the peritoneal cells were collected and stained with PE-conjugated F(ab′)2 donkey anti-human IgG and FITC-conjugated anti-mouse F4/80. Flow cytometric analysis was performed with FACS Calibur (BD Biosciences) equipped with CellQuest software and FlowJo software.

Transfection and infection

Retroviral transfection and infection were performed as described previously (13, 15, 30). Retroviruses were generated by transient transfection of PLAT-E packaging cells (31).

Biochemistry

Western blotting was performed as described previously (12, 13). In brief, the culture supernatants of RAW264.7 cells or neutrophils were immunoprecipitated with anti-LMIR5 polyclonal Ab (R&D Systems) or goat IgG as control to detect sLMIR5. The immunoprecipitates were immunoblotted with biotinylated anti-LMIR5 polyclonal Ab (R&D Systems) followed by HRP-conjugated avidin (eBioscience).

Measurement of sLMIR5

Neutrophils were incubated with or without LPS in the presence of 100 ng/ml G-CSF (R&D Systems). The concentrations of sLMIR5 in the culture supernatants were measured by ELISA using anti-LMIR5 mAb (2C12; ACTGen) and biotinylated anti-LMIR5 polyclonal Ab (R&D Systems) as capture and detection Abs, respectively.

LPS-induced endotoxinemia in mice

Mice (20–25 g) were i.p. injected with LPS dissolved in 500 µl PBS. In some experiments, 100 µg LMR5-Fc or control Fc was coinjected with LPS. Mice were sacrificed at different time intervals. Peritoneal lavage fluid (PLF; 1 ml PBS) and serum were collected and assayed for sLMIR5, IL-6, and TNF-α by ELISA as described previously (15, 22). Survival was assessed every day for at least 7 d. We used 30 and 4 mg/kg LPS in the experiments for assessing survival and measuring serum cytokines/sLMIR5, respectively.

The colon ascendsent peritonitis model

The surgical procedure of colon ascendsent peritonitis (CASP) was performed as described previously (32). In brief, under complete anesthesia, the abdominal wall was opened. An 18-gauge venous catheter was inserted through into the lumen of the ascending colon and fixed with two sutures. Fluid resuscitation was carried out using 0.3 ml sterile sodium chloride. After surgery, the mice were either observed over a period of 7 d for survival studies or were sacrificed at indicated times after CASP to obtain serum.

Statistical analysis

Data are shown as means ± SD, and statistical significance was determined by the Student t test.

Results

An sLMIR5 is released from the RAW264.7 cell line

First, we found that LPS stimulation reduced the surface expression levels of LMIR5 in RAW264.7 cells in a time-dependent manner (Fig. 1A). Real-time PCR analysis showed that LPS stimulation did not significantly affect LMIR5 transcript levels in RAW264.7 cells, although it quickly increased mRNA for proinflammatory cytokine IL-6 at transcript levels (Fig. 1B). Therefore, we thought it possible that surface LMIR5 in RAW264.7 cells might be shed by LPS stimulation. To test this, we examined whether sLMIR5 was detected by anti-LMIR5 Ab in the culture supernatants of RAW264.7 cells. As reported previously (13), Western blot analysis showed that an N-glycoprotein LMIR5 in RAW264.7 cell lysates was recognized as a broad band (22–38 kDa), including a major band (25 kDa; Fig. 1C). Notably, anti-LMIR5 Ab detected a shorter form of LMIR5 as a broad band (17–24 kDa), including two major bands (17 and 21 kDa) in the culture supernatants of RAW264.7 cells, but not in a control medium. These results indicated that sLMIR5 was constitutively shed from an extracellular LMIR5 in RAW264.7 cells. In fact, sLMIR5 accumulated in the culture supernatants of RAW264.7 cells in a time-dependent manner (Fig. 1D). Interestingly, the release of sLMIR5 in RAW264.7 cells was augmented in the presence of LPS (Fig. 1D). Next, to evaluate the concentrations of sLMIR5 in the culture supernatants of RAW264.7 cells expressing high levels of surface LMIR5 (Supplemental Fig. 1B). In contrast, sLMIR5 was not detected in the culture supernatants of Ba/F3 or BW5147 cells expressing no detectable levels of surface LMIR5 (Supplemental Fig. 1B). Moreover, when LMIR5 was further transduced into RAW264.7 cells, higher concentrations of sLMIR5 were detected in the culture supernatants of LMIR5-transduced cells as compared with mock-transduced cells (Supplemental Fig. 1C). Thus, both the sensitivity and the specificity of this assay system were verified. We then determined the concentrations of sLMIR5 or IL-6 in the culture supernatants of RAW264.7 cells stimulated by various concentrations of LPS. Interestingly, even treatment with 1 ng/ml LPS caused the rapid increase of sLMIR5 as early as 2 h after stimulation (Fig. 1E). Of note, stimulation with 10–1000 ng/ml LPS induced a time- and dose-dependent increase of IL-6 production in RAW264.7 cells, whereas the same stimulation led to a nearly identical time-dependent increase of sLMIR5 (Fig. 1E). To test...
constitutively released by neutrophils of BM (Fig. 2A). In addition, as in RAW264.7 cells, LPS stimulation increased sLMIR5 and downregulated surface LMIR5 expression in neutrophils (Fig. 2A, 2B). However, detectable levels of sLMIR5 were not released by either peritoneal Mφ or BMMC, despite the sufficient expression of surface LMIR5 in these cells (Fig. 2A). These results led us to postulate the involvement of neutrophil-specific proteases in the generation of sLMIR5. To test this, we incubated neutrophils with GM6001, a broad-spectrum inhibitor of matrix metalloproteinases (MMPs), in the presence or absence of LPS because several MMPs were known to be expressed and activated in neutrophils (33, 34). Notably, GM6001 significantly impaired both constitutive and LPS-induced release of sLMIR5 from neutrophils (Fig. 2C). These results indicated that sLMIR5 was released from neutrophils by proteolytic cleavage of surface LMIR5 partly through MMPs. To next examine whether sLMIR5 was indeed released in vivo, we measured the concentrations of sLMIR5 in serum or PLF of C57BL/6 mice that had received i.p. injection of LPS. Remarkably, both serum and PLF levels of sLMIR5, as well as IL-6 and TNF-α, rapidly elevated at 1.5 h after injection and diminished through 6–24 h (Fig. 3A and data not shown). Concurrently, we found a transient decrease followed by a gradual recovery of surface expression levels of LMIR5 in BM neutrophils of mice given an injection of LPS (Fig. 3B). In addition, pretreatment of mice with anti-Gr-1 Ab, which was able to deplete circulating neutrophils (Fig. 3C), substantially inhibited the LPS-induced release of sLMIR5 in serum (Fig. 3D). Taken together, these results showed that neutrophils are the main source of LPS-induced sLMIR5 in vivo.

**FIGURE 1.** The release of sLMIR5 from RAW264.7 cells. (A) RAW264.7 cells were incubated with or without 100 ng/ml LPS for 12 h (left panel) or for indicated hours (right panel). Staining with anti-LMIR5 Ab (bold line histogram) or with control Ab (shaded histogram) (left panel). The mean fluorescent intensity (MFI) of LMIR5 staining (right panel). (B) Relative gene expression levels of LMIR5 or IL-6 in RAW264.7 cells at different time intervals after stimulation with 100 ng/ml LPS. (C) The cell lysates or culture supernatants of RAW264.7 cells were immunoprecipitated with anti-LMIR5 Ab or control Ab and then immunoblotted with anti-LMIR5 Ab. (D) RAW264.7 cells were incubated with or without 100 ng/ml LPS for indicated hours. The bands correspond to sLMIR5 in the culture supernatants. (E) RAW264.7 cells were incubated with different concentrations of LPS for indicated hours. The concentrations of sLMIR5 in the culture supernatants were measured by ELISA. (F) RAW264.7 cells were stimulated with different concentrations of indicated stimuli for 12 h. The concentrations of sLMIR5 in the culture supernatants were measured by ELISA (upper panel). The MFI of LMIR5 staining (lower panel). (A–F) Data are presented as mean ± SD (*p < 0.05) and represent three independent experiments.

whether stimulation with other TLR ligands induced the release of sLMIR5, we incubated RAW264.7 cells with different concentrations of CpG ODN, Loxoribine, Flagellin, polyriboinosinic-polyribocytidylid acid, or peptidoglycan. ELISA and flow cytometric analysis demonstrated that dose-dependent increase of sLMIR5 as well as the reciprocal reduction of cell-surface LMIR5 levels were caused by stimulation with all the TLR ligands tested (Fig. 1F). Altogether, TLR stimulation led to the increase of sLMIR5 in RAW264.7 cells.

**Neutrophils are the main source of LPS-induced sLMIR5 in mice**

We next determined whether sLMIR5 was released in the culture supernatants of primary myeloid cells. Notably, sLMIR5 was

**FIGURE 2.** Soluble LMIR5 is released by neutrophils in vitro. (A) Surface expression of LMIR5 in BM neutrophils, peritoneal Mφ, or BMMC. Staining with anti-LMIR5 Ab (bold line histograms) or with control Ab (shaded histogram; upper panel). BM neutrophils, peritoneal Mφ, or BMMC were incubated in the presence or absence of 100 ng/ml LPS for indicated hours. The concentrations of sLMIR5 in the culture supernatants were measured by ELISA (lower panel). (B) Raw BM neutrophils were incubated with or without 100 ng/ml LPS for 24 h. Staining with anti-LMIR5 Ab (bold line histograms) or with control Ab (shaded histogram). (C) BM neutrophils were preincubated with 10 μM of indicated MMP inhibitors for 30 min before incubation with or without 100 ng/ml LPS for 24 h. The concentrations of sLMIR5 in the culture supernatants were measured by ELISA. Data are presented as mean ± SD (*p < 0.05, **p < 0.01) and represent three independent experiments.
**FIGURE 3.** Neutrophils are the main source of sLMIR5 in vivo. (A and B) WT mice were i.p. injected with 4 mg/kg LPS. Serum and BM cells were collected from the mice at 1.5, 6, or 24 h after injection (n = 6 per group). (A) The concentrations of sLMIR5, IL-6, or TNF-α in serum were measured by ELISA. (B) Surface expression levels of LMIR5 and Gr-1 in BM cells derived from the mice before or at 6 h after injection (left panel). The MFI of LMIR5 staining in Gr-1<sup>high</sup> BM neutrophils derived from the mice at different time intervals after injection (right panel). Data are presented as mean ± SD and represent three independent experiments. (C and D) WT mice were i.p. injected with 500 μg of either anti-Gr-1 Ab (n = 4) or control Ab (n = 6) 24 h before i.p. injection of 4 mg/kg LPS. Serum was collected from the mice at 1.5 h after LPS injection. (C) Percentages of CD11b<sup>high</sup>Gr-1<sup>high</sup> neutrophils in peripheral blood were examined by flow cytometry. (D) The concentrations of sLMIR5 in serum were measured by ELISA. All bars correspond to the means (*p < 0.05).

LMIR5-Fc binds to peritoneal M<sub>φ</sub>, resulting in the production of proinflammatory cytokines

To investigate the in vivo role of sLMIR5, we gave the mice an i.p. injection of LMIR5-Fc, a surrogate for sLMIR5. Notably, administration of LMIR5-Fc, but not control Fc, led to transient but significant levels of IL-6 and TNF-α production in both serum and PLF, although it did not cause lethal inflammation (Fig. 4A and data not shown). To determine the in vivo target of sLMIR5, we examined what types of the cells were bound by LMIR5-Fc after i.p. administration of LMIR5-Fc. Flow cytometric analysis showed that LMIR5-Fc strongly bound to F4/80<sup>+</sup> M<sub>φ</sub> in BM cells derived from the mice before or at 1.5, 6, or 24 h after injection were measured by ELISA (n = 6 per group). Data are presented as mean ± SD. (B) Five minutes after i.p. injection with 100 μg LMIR5-Fc, the mice were sacrificed. The peritoneal cells were collected and stained with PE-conjugated donkey F(ab′)2; anti-human IgG Ab and FITC-conjugated anti-mouse F4/80 Ab. (C) Staining of peritoneal M<sub>φ</sub> or BMMC with LMIR5-Fc (bold line histograms) or with control Fc (shaded histogram). Data represent four independent experiments. (D) Peritoneal M<sub>φ</sub> or BMMC were incubated with 20 μg/ml LMIR5-Fc or human IgG1 for 24 h. The concentrations of IL-6 or TNF-α in the culture supernatants were measured by ELISA. (E and F) The concentrations of IL-6 or TNF-α in the culture supernatants were measured by ELISA. (E) Peritoneal M<sub>φ</sub> were incubated with various concentrations of LMIR5-Fc, human IgG1, or control Fc for 24 h. (F) After pretreatment of LMIR5-Fc (10 μg/ml) with indicated concentrations of anti-LMIR5 Ab or goat IgG as control, peritoneal M<sub>φ</sub> were stimulated with LMIR5-Fc for 24 h. (C–F) Data are presented as mean ± SD (*p < 0.01) and represent four independent experiments.

dose-dependently suppressed IL-6 production of peritoneal M<sub>φ</sub> stimulated by LMIR5-Fc (Fig. 4F). Collectively, these results indicated that LMIR5-Fc activated peritoneal M<sub>φ</sub> through the interaction of an extracellular domain of LMIR5 with its ligand expressed by peritoneal M<sub>φ</sub>. Because LIMIR5 bound to TIM4 that was highly expressed in peritoneal M<sub>φ</sub> (15), we asked whether TIM4 was involved in LMIR5-Fc–induced cytokine production of peritoneal M<sub>φ</sub>. To this end, we used LIMIR5 del4-Fc lacking both the C-terminal portion of an Ig-like domain and an extracellular stalk region; unlike LMIR5-Fc, LMIR5 del4-Fc fails to bind TIM1 or TIM4 (15). Indeed, LMIR5-Fc, but not LMIR5 del4-Fc, stained TIM1- or TIM4-expressing Ba/F3 cells (Supplemental Fig. 2B). Notably, LIMIR5 del4-Fc significantly stained peritoneal M<sub>φ</sub> expressing TIM4, albeit at weaker levels than LMIR5-Fc (Supplemental Fig. 2B). Because peritoneal M<sub>φ</sub> did not express significant levels of TIM1 (15), it is possible that in peritoneal M<sub>φ</sub>, LMIR5-Fc bound to TIM4 as well as to an unknown ligand other than TIM1/4 via different regions of LMIR5. Importantly, stim-
ulation with LMR5 del4-Fc caused cytokine production of peritoneal Mφ at levels comparable with stimulation with LMR5-Fc (Supplemental Fig. 2C). Taken together, these results suggested that the interaction of LMR5-Fc with an unidentified ligand other than TIM1/4 was involved in LMR5-Fc–induced cytokine production of peritoneal Mφ.

LMR5−/− mice are protected against LPS- or peritonitis-induced lethal inflammation

To delineate the in vivo significance of sLMIR5, we compared LPS responses between WT and LMR5−/− mice. First, we confirmed that irrespective of LPS administration, sLMIR5 was absent both in the culture supernatants of LMR5−/− neutrophils and in serum and PLF of LMR5−/− mice (Supplemental Fig. 3). We then measured levels of proinflammatory cytokines (IL-6 and TNF-α) in the serum or PLF of WT and LMR5−/− mice before and after i.p. injection of LPS. Intriguingly, when compared with WT mice, LMR5−/− mice exhibited lower levels of LPS-induced cytokine production in both serum and PLF (Fig. 5A). Importantly, LMR5 deficiency decreased the mortality induced by LPS administration: all the WT mice died within 4 d after a lethal dose of LPS injection, whereas 6 of 12 LMR5−/− mice survived even after 7 d (Fig. 5B). These results suggested that LMR5 deficiency improved LPS-induced death by suppressing proinflammatory cytokine production in mice. In contrast, we found no significant difference in LPS-induced cytokine production in vitro between WT and LMR5-deficient myeloid cells, including peritoneal Mφ, indicating that LMR5 was not intrinsically involved in LPS-TLR4 signaling in these cells (Supplemental Fig. 4). Based on these observations, we speculated that the reduced mortality and cytokine production in LPS-injected LMR5−/− mice might be explained by the absence of sLMIR5. To test this possibility, we administered LMR5-Fc or control Fc together with a sublethal dose of LPS to LMR5−/− mice, and found that the addition of LMR5-Fc, but not control Fc, accelerated LPS-induced death in LMR5−/− mice (Fig. 5C). These results suggested that sLMIR5 played a critical role in LPS-induced lethality. Next, we induced CASP as a murine abdominal sepsis model in WT and LMR5−/− mice. The increase of sLMIR5 in serum of WT mice was confirmed in this model (Fig. 6A). Remarkably, LMR5−/− mice were resistant to death in a CASP model (Fig. 6B). Serum levels of TNF-α at 4 h after the induction of a CASP model were lower in LMR5−/− mice in comparison with WT mice (Fig. 6C). In addition, the peritoneal administration of LMR5-Fc, but not control Fc, accelerated septic death in LMR5−/− mice that had been subjected to a CASP model (Fig. 6D). Taken together, sLMIR5 was implicated in lethal inflammation induced by a pathophysiological sepsis model.

Discussion

The ideal therapy for sepsis is to suppress the excessive inflammatory responses in its early phase. Therefore, understanding the precise mechanism of the sepsis progression is indispensable to improving therapy. In this study, we delineated a novel mechanism as to how inflammatory responses were accelerated in LPS-induced sepsis: sLMIR5 was an inflammatory mediator linking the initial activation of LPS-stimulated neutrophils with lethal inflammation in sepsis. The precise analysis revealed that neutrophils were the main source of LPS-induced sLMIR5 in mice (Figs. 2, 3). The reason why sLMIR5 is released by RAW264.7 cells (Mφ cell lines), but not by peritoneal Mφ, is uncertain. Similar to the release of sLMIR5 by neutrophils, that by RAW264.7 cells was significantly, but not completely, suppressed by treatment with GM6001 (data not shown). Real-time PCR

**FIGURE 5.** The resistance of LMR5-deficient mice to LPS-induced mortality was decreased by LMR5-Fc administration. (A) WT or LMR5−/− mice were i.p. injected with 4 mg/kg LPS or PBS (WT/PBS: n = 4; knockout [KO]/PBS: n = 3; WT/LPS: n = 10; KO/LPS: n = 9). The concentrations of IL-6 (upper panel) or TNF-α (lower panel) in serum (left panel) or PLF (right panel) of the mice at 1.5 h after injection were measured by ELISA. All the bars correspond to the means. (B) WT or LMR5−/− mice were i.p. injected with 30 mg/kg LPS and monitored for survival (n = 12 per group). (C) LMR5−/− mice were i.p. injected with 30 mg/kg LPS together with 100 μg LMR5-Fc or control Fc, and monitored for survival (n = 8 per group).

**FIGURE 6.** LMR5−/− mice are resistant to death induced by CASP. (A) WT mice were subjected to CASP model and sacrificed at 1.5, 6, or 24 h after the surgery. The concentrations of sLMIR5 in serum of the mice (n = 4 per group) were measured by ELISA. Data are presented as mean ± SD. (B) WT or LMR5−/− mice were subjected to CASP model and monitored for survival (n = 12 per genotype). (C) The concentrations of IL-6 (left panel) or TNF-α (right panel) in serum of WT mice (n = 5) or LMR5−/− mice (n = 4) at 4 h after the induction of CASP were measured by ELISA. All bars correspond to the means (*p < 0.05). (D) LMR5−/− mice that had been subjected to CASP model were further i.p. injected with 100 μg of either LMR5-Fc or control Fc and monitored for survival (n = 5 per group).
analysis of expression of MMP1, MMP2, MMP3, MMP8, and MMP9 in RAW264.7 cells or peritoneal Mφs showed that MMP3 and MMP9 were expressed in RAW264.7 cells, whereas several MMPs, including MMP3 and MMP9, were highly expressed in peritoneal Mφ (data not shown). Therefore, MMPs other than these MMPs or GM6001-sensitive ADAM and/or ADAM-TS family members might be involved in the release of sLMIR5 by RAW264.7 cells. Alternatively, it is possible that MMP3 and/or MMP9 may be aberrantly activated in RAW264.7 cells, but not in peritoneal Mφ. Although the involvement of neutrophil-specific proteases in the release of sLMIR5 was suggested, further examination is required to completely understand the relevant mechanism.

Importantly, the pathophysiological role of sLMIR5 in LPS-induced sepsis was clarified by a number of experiments: 1) LMIR5−/− mice were more resistant to LPS- or peritonitis-induced septic death; 2) LMIR5−/− mice exhibited lower levels of proinflammatory cytokine production in response to LPS; 3) systemic and local sLMIR5 quickly increased only in WT mice, but not LMIR5−/− mice, in response to LPS administration or peritonitis induction; 4) in vitro stimulation with LMIR5-Fc, a mimic form of sLMIR5, or recombinant LMIR5 harboring only an extracellular domain of LMIR5 caused cytokine production of peritoneal Mφ; 5) in vivo administration of LMIR5-Fc bound to peritoneal Mφ and induced significant, but not lethal, inflammation; and 6) the resistance to LPS- or peritonitis-induced septic death of LMIR5−/− mice was reduced by in vivo administration of LMIR5-Fc. Although neither LMIR5-Fc nor recombinant soluble LMIR5 is completely equivalent to sLMIR5, these results indicated that sLMIR5 is a positive regulator of LPS-induced lethal inflammation. In contrast, because the mechanism of pathophysiological sepsis is much more complex than that of LPS-induced systemic inflammation, it is possible that sLMIR5 plays a limited role in pathophysiological sepsis. A possible mechanism of LMIR5-mediated, LPS-induced lethal inflammation may be that LPS-activated neutrophils released sLMIR5 that stimulated cytokine production of resident Mφ, resulting in the lethal inflammation. Given that the release of sLMIR5 was enhanced by not only LPS but also other TLR agonists, it is important to examine whether sLMIR5 plays a role in viral or fungal infection models.

Stronger binding of LMIR5-Fc to peritoneal Mφ led us to postulate that the specific ligand for sLMIR5 was highly expressed in peritoneal Mφ. Although we have recently identified TIM1 and TIM4 as endogenous ligands for LMIR5 (15), it was unlikely that these surface molecules were involved in LMIR5-Fc–induced cytokine production in peritoneal Mφ. First, peritoneal Mφ did not express TIM1 at high levels (15, 35). Second, peritoneal Mφ expressed TIM4 but were bound at weak but significant levels by LMIR5 del4-Fc that did not bind to TIM4 (15). Third, LMIR5 del4-Fc as compared with LMIR5-Fc stimulated comparable levels of cytokine production in peritoneal Mφ. It is therefore possible that sLMIR5 binds to and activates an unknown surface molecule other than TIM1/4, resulting in cytokine production of peritoneal Mφ. To identify the unknown ligand, several attempts, including retrovirus-mediated expression cloning, are now under way.

The comparison between sLMIR5 and sTREM-1 is interesting in light of the Ig-like receptor family’s involvement in the pathogenesis of sepsis. Stimulation with TLR agonists augmented the release of both sLMIR5 and sTREM-1, although it increased transcript levels of TREM-1, but not LMIR5 (22–26). The mechanism of TREM-1 generation in neutrophils and monocyes was controversial (22–26), but a recent report has shown that MMPs shed their TREM-1 ectodomain from LPS-stimulated human monocytes (36). Whereas sTREM-1 attenuates excessive inflammatory response by counterregulating TREM-1, an inflammatory amplifier in sepsis (22–26), sLMIR5 amplifies LPS-induced lethal inflammation. Thus, sLMIR5 or sTREM-1 is a positive or negative feedback regulator of LPS-induced inflammation, respectively. Considering that LMIR5 is a DAP12-coupled activating receptor and that DAP12 deficiency downregulates surface expression of LMIR5 (13), the resistance of DAP12−/− mice to LPS-induced lethality might be, in part, because of the lack of sLMIR5 (37). In any case, it is conceivable that cross talk between TLRs and paired immune receptors modulates innate immune responses in sepsis.

In conclusion, sLMIR5 is released by neutrophils in response to LPS, and amplifies lethal inflammation in LPS-induced sepsis. Our results provide a mechanistic insight into septic death and will help develop the novel therapies to treat sepsis.

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References


Supplemental Figure 1. Establishment of an ELISA system for detecting sLMIR5. (A) Different concentrations of recombinant LMIR5 (left panel) or 10 ng/ml of LMIR5-Fc, LMIR3-Fc, or human IgG1 dissolved in PBS (right panel) were incubated on plates coated with anti-LMIR5 mAb (2C12). The captured samples were then incubated with biotinylated anti-LMIR5 Ab and HRP-conjugated avidin. After adding TMB substrate, the absorbance was calculated according to the manufacturer’s protocol. (B and C) The concentrations of sLMIR5 in the culture supernatants of Ba/F3, BW5147, or RAW264.7 cells (upper panel) (B) or LMIR5- or mock-transduced RAW264.7 cells (C) were measured by ELISA. Recombinant LMIR5 was used as a protein standard. Ba/F3, BW5147, or RAW264.7 cells were stained with anti-LMIR5 Ab (bold line histograms) or with control Ab (shaded histogram) (lower panel) (B). Data are presented as mean ± SD and represent three independent experiments.
**Supplemental Figure 2.** Stimulation with recombinant LMIR5 or LMIR5-Fc induces IL-6 production of peritoneal Mφ through its interaction with an unknown ligand. (A) Peritoneal Mφ were stimulated for 24 h with 10, 20, or 100 μg/ml of recombinant LMIR5 harboring only an extracellular domain of LMIR5. The concentrations of IL-6 in the culture supernatants were measured by ELISA. (B) Ba/F3 cells transduced with Flag-TIM1, Flag-TIM4, or mock, or peritoneal Mφ were stained with LMIR5-Fc (bold line histogram) or control Fc (shaded histogram) (*upper panel*), with LMIR5 del4-Fc (bold line histogram) or control Fc (shaded histogram) (*middle panel*), with anti-Flag mAb or anti-TIM4 mAb (bold line histogram) or control Ab (shaded histogram) (*lower panel*). Data are representative of three independent experiments. (C) Peritoneal Mφ were stimulated with 20 μg/ml LMIR5-Fc, LMIR5 del4-Fc, control Fc, or human IgG1 for 24 h. The concentrations of IL-6 in the culture supernatants were measured by ELISA. (A and C) Data are presented as mean ± SD (*P < .05) (**P < .01) and represent three independent experiments.
**Supplemental Figure 3.** sLMIR5 is not detected in serum or PLF of LMIR5−/− mice. (A) The cell lysates or culture supernatants of WT or LMIR5-deficient neutrophils were immunoprecipitated with anti-LMIR5 Ab or control Ab, and then immunoblotted with anti-LMIR5 Ab (Left panel). The cell lysates of WT or LMIR5-deficient neutrophils were immunoblotted with anti-α-tubulin mAb as control (right panel). (B) WT or LMIR5-deficient neutrophils were incubated with or without 100 ng/ml LPS for 24 h. The concentrations of sLMIR5 in the culture supernatants were measured by ELISA. (C) The concentrations of sLMIR5 in serum (left panel) or PLF (right panel) of WT or LMIR5−/− mice at 1.5 h after intraperitoneal injection of LPS (4 mg/kg) were measured by ELISA. Data are presented as mean ± SD (*P < .01) and represent four independent experiments.
**Supplemental Figure 4.** LMIR5 deficiency did not influence LPS-induced cytokine production in peritoneal Mφ or neutrophils. Peritoneal Mφ or BM neutrophils derived from WT or LMIR5<sup>−/−</sup> mice were incubated with or without 1 or 100 ng/ml LPS for 24 h. The concentrations of IL-6 in the culture supernatants were measured by ELISA. Data are presented as mean ± SD and represent three independent experiments.