The Agonists of Formyl Peptide Receptors Prevent Development of Severe Sepsis after Microbial Infection

Sang Doo Kim, Yoon-Keun Kim, Ha Young Lee, You-Sun Kim, Seong Gyu Jeon, Suk-Hwan Baek, Dong-Keun Song, Sung Ho Ryu and Yoe-Sik Bae

_J Immunol_ 2010; 185:4302-4310; Prepublished online 3 September 2010;
doi: 10.4049/jimmunol.1001310
http://www.jimmunol.org/content/185/7/4302

Supplementary Material

http://www.jimmunol.org/content/suppl/2010/09/03/jimmunol.1001310.DC1

References

This article cites 53 articles, 20 of which you can access for free at:
http://www.jimmunol.org/content/185/7/4302.full#ref-list-1

Subscription

Information about subscribing to _The Journal of Immunology_ is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
The Agonists of Formyl Peptide Receptors Prevent Development of Severe Sepsis after Microbial Infection

Sang Doo Kim,*† Yoon-Keun Kim,‡ Ha Young Lee,*† You-Sun Kim,‡ Seung Hyun Jeon,‡ Suk-Hwan Baek,§ Dong-Keun Song,¶ Sung Ho Ryu,‡ and Yoe-Sik Bae*†

Severe sepsis, a principal cause of death in intensive care units, occurs when host immune defenses fail to combat invading microbes. In this paper, we report that the administration of peptide agonists of formyl peptide receptors, including Trp-Lys-Tyr-Met-Val-D-Met (WKYMVm), protected against death by enhanced bactericidal activity and inhibition of vital organ inflammation and immune cell apoptosis in a cecal ligation and puncture (CLP) sepsis mouse model. The administration of WKYMVm also enhanced the production of type 1 (IFN-γ and IL-12) and type 17 (IL-17 and TGF-β) cytokines in CLP mice. In contrast, the administration of WKYMVm inhibited the production of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) in the CLP mice. The therapeutic and bactericidal effects of WKYMVm were partly reversed in IFN-γ−/− mice, whereas target organ inflammation was not. Meanwhile, the therapeutic and anti-inflammatory effects of WKYMVm were partly reversed in IL-17−/− mice. In addition, the administration of WKYMVm also enhanced type 1 and type 17 Th cell responses in mice sensitized with LPS plus Ags. These results suggest that the agonists of formyl peptide receptors effectively prevent development of severe sepsis following microbial infection partly via augmentation of type 1 and type 17 immune responses. The Journal of Immunology, 2010, 185: 4302–4310.

Sepsis is a complex clinical syndrome that results from a harmful or damaging host response to infection (1). The septic response is a contributing factor in >200,000 deaths annually in the United States (2). The annual incidence of severe sepsis and septic shock has increased to >750,000 during the past 20 years (3). Studies indicate that 50–70% of patients with nosocomial severe sepsis or septic shock experience a less severe stage of the septic response (i.e., systemic inflammatory response syndrome, or sepsis) lasting at least 1 d during their hospital stay (4, 5). Research is needed to develop adjunctive agents that can dampen the septic response before severe sepsis or septic shock occurs.

Recent studies indicate that sepsis-induced mortality is accompanied by an inability to regulate the inflammatory response because of substantial impairment of the innate immune system during early sepsis (i.e., during the first 6 h) (6–8). In addition, excessive lymphocyte apoptosis occurs during sepsis, resulting in the clinical signs of multiorgan failure (9, 10). Moreover, studies indicate that cytokine levels are markedly altered during sepsis; in particular, the levels of such proinflammatory cytokines as TNF-α and IL-1β are greatly increased (11–13). These findings indicate that the effective prevention of mortality by severe sepsis or septic shock requires drugs that enhance the bactericidal activity of phagocytes, inhibit the production of proinflammatory mediators, and prolong Ag-specific adaptive immune responses.

Formyl peptide receptors (FPRs) are pattern recognition receptors for formylated peptides found in Gram-negative bacteria (14); they are G-protein–coupled classical chemoattractant receptors present in phagocytic cells, such as neutrophils, monocytes, macrophages, and dendritic cells (15). Three FPRs (FPR1, FPR2, and FPR3) have been identified in humans, and two mouse FPRs (mFPR1 and mFPR2) have been identified as human counterparts in mice (15). Past studies indicated that the in vitro activation of FPR family members induces leukocyte chemotactic migration and bactericidal activity via superoxide anion generation in neutrophils and monocytes (16–18). Trp-Lys-Tyr-Met-Val-D-Met (WKYMVm), a synthetic peptide capable of stimulating chemotactic migration in phagocytes (19), binds to members of the FPR family (mFPR1 and mFPR2 in mice, and FPR1, FPR2, and FPR3 in humans) in vitro (17, 20, 21), and enhances the bactericidal activity of monocytes and neutrophils via the production of superoxide anions (18, 19). WKYMVm has also been found to enhance monocyte survival by blocking apoptosis (22). Thus, we investigated the therapeutic effects and functional mechanisms of the FPR agonist WKYMVm in the development of severe sepsis after microbial infection, based on the notion that the major cause of sepsis-induced mortality is deficiency of host innate and adaptive immune responses and subsequent uncontrolled infections and that FPR activation enhances host defense mechanisms.

Materials and Methods

Animals and sepsis models

Male wild-type (WT) Institute of Cancer Research (ICR) mice were used for evaluating therapeutic effects of FPR activation; IL-12Rβ2−/−, IFN-γ−/−, and IL-17−/− deficient mice (kindly donated by Y.C. Sung, Pohang University of Science and Technology, Pohang, Republic of Korea) and their WT...
controls (B57BL/6 and BALB/c background) were used for mechanism studies. All experiments involving animals adhered to the guidelines and received the approval of the Institutional Review Committee for Animal Care and Use at Dong-A University, Busan, Korea. For cell ligation and puncture (CLP), mice were anesthetized with pentothal sodium (50 mg/kg, i.p.), and a small abdominal midline incision was made to expose the cecum. The cecum was then ligated below the ileocecal valve, punctured twice through both surfaces (or once for the measurement of cytokine production), using a 22-gauge needle, and the abdomen was closed. Sham CLP mice were subjected to the same procedure, but without puncture of the cecum. For the LPS or Escherichia coli model, E. coli (1 × 10⁹ cells/mouse) or 60 mg/kg LPS was injected i.p., respectively. Survival was monitored once daily for 10 d.

**Measurement of bactericidal activity in vivo**

At 24 h after CLP, peritoneal lavage fluids were collected and cultured overnight on blood-agar base plates (TRYPTICASE SOY AGAR DEEPS; BD Biosciences, San Jose, CA) at 37°C. The numbers of CFUs were counted as described previously (23).

**Quantification of pulmonary edema**

The extent of pulmonary edema was quantified by evaluating the wet/dry (W/D) weight ratio of the lung, as described previously (24). Whole harvested wet lungs were weighed and then placed in an oven for 48 h at 60°C. The dry weight was measured, and the W/D weight ratio was calculated.

**Tissue histology**

Mice were subjected to CLP surgery and given PBS or WKYMVm (Anygen, Gwangju, Republic of Korea) at a dose of 4 mg/kg 2 h later. The mice were euthanized 24 h after surgery, and their lungs were fixed, sectioned, and stained with H&E for morphological analysis. The images of H&E-stained lung tissue samples were taken using a virtual microscope system (Aperio Technologies, San Diego, CA).

**Immunohistochemistry for apoptosis evaluation**

A TUNEL assay was performed in paraffin-embedded tissue sections that were first deparaffinized using a standard histological protocol. The sections were then permeabilized with Triton X-100 at 4°C for 2 min and flooded with TdT enzyme and digoxigenin-deoxyuridine triphosphate reaction buffer (TUNEL) reagent for 60 min at 37°C. The percentage of apoptotic cells (TUNEL-positive cells) was determined by counting 500 splenocytes under a light microscope. We also performed immunofluorescence staining for cleaved caspase-3 in paraffin-embedded tissue sections that were first deparaffinized using a standard histological protocol, as previously described (25).

**Isolation of mouse neutrophils and measurement of H₂O₂**

Mouse neutrophils were isolated from peripheral blood using a Histopaque-1077 solution (Sigma-Aldrich, St. Louis, MO), as described previously (26). Neutrophils isolated from sham, CLP⁺, or CLP⁺ WKYMVm-treated mice were stimulated with 100 nM PMA for 1 h. Freshly isolated neutrophils from normal mice were incubated with cytochalasin B (5 μM) for 5 min, and then the cells were stimulated with various concentrations of WKYMVm for 10 min. To investigate the role of mFPR1 and mFPR2, neutrophils were preincubated with N-β-butoxycarbonyl-Phe-Leu-Phe-Leu-Phe (Boc-PLLP, 10 μM), Trp-Arg-Trp-Trp-Arg-Trp-Arg-Trp-Arg-Trp-Arg-Val (Boc-PLLP, 10 μM), or vehicle (DMSO) for 30 min prior to adding WKYMVm (100 nM) for 10 min. H₂O₂ in the supernatant was measured using an H₂O₂ assay kit (Molecular Probes, Eugene, OR) according to the manufacturer’s protocol.

**Neutrophil bactericidal activity**

Neutrophil bactericidal activity was measured according to the method of Yan et al. (23). Neutrophils were incubated at 37°C on 13-mm plastic cover slips in 60-mm plastic culture dishes (1 × 10⁶ neutrophils/cover slip) for 1 h. Nonadherent cells were removed with PBS. Adherent neutrophils were incubated with 10⁹ opsonized E. coli for 1 h. After washing away the unengulfed E. coli, E. coli-engulfed neutrophils were stimulated with several concentrations of WKYMVm for 1 h. Then, adherent neutrophils were lysed by adding cold distilled water for 5 min. The supernatants were taken and cultured overnight on Luria-Bertani plates. The number of viable bacteria in the neutrophils was determined by colony counting of live bacteria. The percentage of bacteria killed was calculated as 100 × (1 — number of CFUs after WKYMVm stimulation/number of CFUs before WKYMVm stimulation). To investigate the role of mFPR1 and mFPR2, neutrophils were preincubated with Boc-PLLP (10 μM), WRW4 (10 μM), or vehicle (DMSO) for 30 min prior to adding WKYMVm (1 μM) for 1 h.

**Cytokine measurement after CLP or LPS injection**

To measure cytokines in the peritoneal lavage fluids or serum, the fluids or serum was collected at various times between 4 h and 72 h after CLP or i.p.

**FIGURE 1.** The therapeutic effects of WKYMVm in sepsis models. A, Various doses of WKYMVm were injected s.c. four times into CLP mice at 2, 14, 26, and 38 h after CLP. B, Various doses of WKYMVm were injected s.c. four times into CLP mice at 10, 22, 34, and 48 h after CLP. C, CLP mice were given zero, one, two, three, four, or five injections of WKYMVm (4 mg/kg, s.c.). D, FPR family agonists or scrambled peptide (4 mg/kg, respectively) were injected s.c. four times into CLP mice at 2, 14, 26, and 38 h post-CLP. E, Boc-PLLP (4 mg/kg), WRW4 (4 mg/kg), or Boc-PLLP (4 mg/kg) + WRW4 (4 mg/kg) was injected s.c. 2 h before CLP. After CLP, Boc-PLLP (4 mg/kg) or WRW4 (4 mg/kg) was injected s.c. four times at 12-h intervals 2 h before WKYMVm treatment (4 mg/kg, s.c.). F, WKYMVm (4 mg/kg) was injected s.c. four times into mice 2, 14, 26, and 38 h after peritoneal injection with E. coli (1 × 10⁹ cells/mouse). G, PBS or WKYMVm (4 mg/kg) was injected s.c. four times into mice 2, 14, 26, and 38 h after i.p. injection of 60 mg/kg LPS. n = 16–24 (A–E) or n = 15 (F, G) mice per group. *p < 0.05; **p < 0.01; ***p < 0.001 compared with the vehicle control (A–G).
application of LPS; cytokines present in the peritoneal fluids or serum were measured by ELISA (eBioscience, San Diego, CA).

Cytokine release from inflammatory cells in vitro

Mouse neutrophils (3 \times 10^6 cells/0.3 ml) were placed in RPMI 1640 medium containing 5% FBS in 24-well plates and kept in a 5% CO₂ incubator at 37°C. The neutrophils were then incubated with LPS (100 ng/ml) for 3 and 6 h, respectively, in the presence or absence of WKYMVm (0.1 and 1 μM). LPS (100 ng/ml) was added to the cells 30 min later, and cell-free supernatants were collected, centrifuged, and measured for IL-1β or TNF-α by ELISA (BD Biosciences Pharmingen, San Diego, CA) according to the manufacturer’s instruction. As for the effects of IL-17 on the production of proinflammatory cytokines by LPS, 5 \times 10^5 cells/ml mouse macrophage cell lines (RAW264.7) were stimulated with 0.1 and 10 ng/ml LPS with or without 10 ng/ml IL-17 (R&D Systems, Minneapolis, MN). TNF-α levels in the supernatants were measured by ELISA (BD Biosciences Pharmingen) 20 h after the incubation.

Cytokine measurement from regional lymph nodes

C57BL/6 WT mice were intranasally sensitized with 10 μg OVA (Sigma-Aldrich) with and without i.p. injection of WKYMVm (4 mg/kg) on days 0, 1, and 2. Regional lymph node (LN) cells were isolated 24 h after the last sensitization. The isolated cells (4 \times 10^6 cells/ml) were incubated with nonspecific stimuli with anti-CD3 (1 μg/ml) + anti-CD28 (1 μg/ml) (BD Biosciences Pharmingen) or with 100 μg/ml OVA. IFN-γ, IL-17, IL-4, and IL-10 in the supernatants were measured 6 and 16 h after the nonspecific stimuli and 24, 48, and 72 h after OVA-specific stimuli, respectively.

Statistical analysis

Survival data were analyzed using the log-rank test. All other data were evaluated using ANOVA. The Bonferroni test was used for post hoc comparisons, and statistical significance was set a priori at \( p < 0.05 \).

**Results**

Administration of FPR agonists protects against sepsis-induced mortality

To investigate whether WKYMVm has a therapeutic effect on experimental sepsis, CLP was performed on albino ICR mice, and their survival was monitored for up to 10 d. Within 2 d after CLP, mouse mortality dramatically increased (Fig. 1A). To examine the effect of WKYMVm treatment, mice were injected s.c. with various doses of the peptide or with PBS as a negative control beginning 2 h after CLP. Treatment with 4 or 8 mg/kg WKYMVm dramatically increased mouse survival, compared with the PBS-injected controls (Fig. 1A). When WKYMVm was injected 10 h post-CLP, the therapeutic effect was similar to that observed following injection of the peptide 2 h post-CLP (Fig. 1B). In terms of injection frequency, survival was greatly improved when 4 mg/kg WKYMVm was injected 2 h post-CLP and at 12-h intervals three or four additional times (Fig. 1C). Given these results, our subsequent experiments were performed in CLP mice, using 4 mg/kg WKYMVm beginning 2 h after CLP and at 12-h intervals three additional times.

Because WKYMVm is reported to bind FPR family receptors (17, 20, 21), the therapeutic effect of other FPR ligands was also evaluated. The therapeutic effect of Trp-Lys-Tyr-Met-Val-Met (27), N-formyl-Met-Leu-Phe, and MMK-1 peptide (LESIFRSLLFVRM) (28) was lower than that of WKYMVm (Fig. 1D). Moreover, the inactive scrambled peptide Val-Trp-Met-Tyr-Met-Val-Met had no therapeutic effect (Fig. 1D). To evaluate the roles of mFPR1 and mFPR2, an FPR1 antagonist (Boc-PLPLP) (29) and an FPR2 antagonist (WRW4) (30) were administered prior to WKYMVm treatment, mice were injected s.c. with various doses of the peptide or with PBS as a negative control beginning 2 h after CLP. Administration of FPR agonists protects against sepsis-induced mortality.

**Figure 2**

Effect of WKYMVm on CLP-induced bacterial colony count, lung inflammation, and immune cell apoptosis. A, WKYMVm (4 mg/kg) was injected s.c. two times into CLP mice 2 and 14 h after CLP. Peritoneal lavage fluids collected 24 h after sham, CLP, or CLP + WKYMVm administration were cultured overnight on blood-agar base plates at 37°C; the number of CFUs was then counted. B, WKYMVm (4 mg/kg, s.c.) was administered 2 and 14 h after CLP, and the lungs were used to measure the W/D weight ratio 24 h after CLP in ICR mice. PBS or WKYMVm (4 mg/kg, s.c.) was administered 2 h and 14 h after CLP. The mice were sacrificed 24 h after surgery. The lungs were stained with H&E (original magnification \times 400). The data are representative of eight mice per group. C, WKYMVm (4 mg/kg) was injected s.c. two times into CLP mice 2 and 14 h post-CLP. The spleen, which was collected 24 h after sham, CLP + PBS, or CLP + WKYMVm administration, was used for a TUNEL assay (right) (original magnification \times 400). TUNEL-positive cells were counted (left). D, The spleen from the mice described in C was used for immunofluorescent staining with FITC-conjugated cleaved-caspase-3 Ab (original magnification \times 100). Data are expressed as the mean \pm SE. \( n = 16 \) for A; \( n = 5 \) for B; \( n = 8 \) for C. \(* p < 0.05; **p < 0.01; ***p < 0.001.\) The data are representative of eight mice per group (C, D).
treatment in CLP mice. The therapeutic effects of WKYMVm were completely reversed by pretreatment with WRW 4, but only partly reversed by Boc-PLPLP (Fig. 1E).

We also evaluated the therapeutic effects of WKYMVm in other sepsis mouse models. Lethality was reduced in mice inoculated with \textit{E. coli} (1 $\times$ 10^9 cells/mouse) and treated s.c. with 4 mg/kg WKYMVm four times at 12-h intervals beginning 2 h after inoculation, compared with \textit{E. coli}-inoculated mice treated with PBS (Fig. 1F). Moreover, WKYMVm also reduced the mortality of mice injected i.p. with 60 mg/kg LPS (Fig. 1G).

FIGURE 3. WKYMVm enhances bactericidal activity in vitro via H$_2$O$_2$ production. \(A\), Adherent neutrophils were incubated with 10^6 opsonized \textit{E. coli} for 1 h, and stimulated with vehicle (PBS) or WKYMVm (0.1–1000 nM) for 1 h. The number of viable bacteria in the neutrophils was then determined. \(B\), Adherent neutrophils were incubated with 10^6 opsonized \textit{E. coli} for 1 h, and Boc-PLPLP (10 $\mu$M), WRW 4 (10 $\mu$M), or Boc-PLPLP (10 $\mu$M) + WRW 4 (10 $\mu$M) was added 30 min prior to the addition of WKYMVm (100 nM) for 1 h. The number of viable bacteria in the neutrophils was then determined. \(C\), Mouse neutrophils were stimulated with vehicle (PBS) or WKYMVm (1–1000 nM) for 15 min. Boc-PLPLP (10 $\mu$M), WRW 4 (10 $\mu$M), or Boc-PLPLP (10 $\mu$M) + WRW 4 (10 $\mu$M) was added 5 min prior to the addition of WKYMVm (100 nM). \(E, PBS\) or WKYMVm (4 mg/kg) was injected s.c. two times into CLP mice 2 and 14 h post-CLP. At 24 h after CLP, peritoneal neutrophils were isolated. Neutrophils isolated from sham, CLP, or CLP + WKYMVm mice were stimulated with PMA (100 nM) for 30 min. Data are expressed as the mean ± SE. \(n = 16\) for \(A; n = 4\) for \(B; n = 8\) for \(C–E. \) *\(p < 0.05; \)**\(p < 0.01; \)***\(p < 0.001.

FIGURE 4. Role of IL-12 and IFN-$\gamma$ in WKYMVm-induced protection against severe sepsis. \(A\), WKYMVm (4 mg/kg) was injected s.c. four times into CLP mice, at 2, 14, 26, and 38 h after CLP. Separate groups of animals were given sham, CLP + PBS, or CLP + WKYMVm treatment. Left panel, IL-12; right panel, IFN-$\gamma$. Data are expressed as the mean ± SE. \(n = 8\) for \(A. \) *\(p < 0.05; \)**\(p < 0.01\), compared with CLP + PBS. \(B\), WT C57BL/6 or IL-12R$\beta$2-deficient mice were s.c. injected with WKYMVm (4 mg/kg) or PBS four times, at 2, 14, 26, and 38 h after CLP. \(C\), WT C57BL/6 or IFN-$\gamma$-deficient mice were s.c. injected with WKYMVm (4 mg/kg) or PBS four times, at 2, 14, 26, and 38 h after CLP. \(D, \) WKYMVm (4 mg/kg) was injected s.c. two times into CLP mice 2 and 14 h after CLP. Peritoneal lavage fluids collected 24 h after CLP or CLP + WKYMVm administration were cultured overnight on blood-agar plates at 37°C, and the CFUs were counted. \(E, Lungs\) were used to measure the W/D weight ratio 24 h after CLP in IFN-$\gamma$-deficient and WT C57BL/6 mice. Data are expressed as the mean ± SE. **\(p < 0.001. \) \(n = 8\) for \(D\) and \(E. \)
Enhanced survival by WKYMVm is associated with enhanced bactericidal effect and with inhibition of vital organ inflammation and immune cell apoptosis

Because CLP-induced lethality was significantly associated with bacterial colony counts in the peritoneal fluid (1), we investigated whether FPR agonist WKYMVm affected bacterial clearance from peritoneal fluid. We found that WKYMVm treatment dramatically reduced the i.p. bacterial colony count by 99.8% 24 h after CLP (Fig. 2A).

Mortality after sepsis is known to be significantly associated with vital organ dysfunction. We found that an indicator of acute lung inflammation, the lung W/D weight ratio, was significantly increased in CLP-induced mice treated with PBS, and that this indicator was completely reversed by treatment with WKYMVm (Fig. 2B). Moreover, histological analyses revealed that CLP-induced lung inflammation was completely reversed by WKYMVm (Fig. 2B).

CLP-induced sepsis caused immune cell apoptosis in the spleen; however, this effect was dramatically inhibited by WKYMVm treatment (Fig. 2C). Previous reports demonstrated that lymphocyte apoptosis is mediated by the activation of several critical caspases, including caspase-3 (31). Similarly, CLP-induced sepsis enhanced the activation of caspase-3, whereas WKYMVm dramatically inhibited it (Fig. 2D).

WKYMVm enhances bacterial clearance and H2O2 generation in phagocytes

To determine whether WKYMVm treatment increases bactericidal activity in vitro, mouse neutrophils were allowed to ingest E. coli for 1 h and were then stimulated with 0.1–1000 nM WKYMVm for 1 h. Such treatment markedly enhanced the bactericidal activity of the neutrophils in a dose-dependent manner (Fig. 3A). Because mFPR1 and mFPR2 are expressed in neutrophils (data not shown), we investigated the roles of mFPR1- and mFPR2-mediated pathways in WKYMVm-induced bactericidal activity. Neutrophils pretreated with Boc-PLPLP or WRW4 before WKYMVm treatment had significantly inhibited bactericidal activity (Fig. 3B).

Because the bacterial killing effect within phagocytes was related to their H2O2 generation (32), we evaluated whether H2O2 generation in mouse neutrophils was enhanced by in vitro WKYMVm treatment. The present study showed that WKYMVm treatment elicited an increase in neutrophil H2O2 generation, with maximal activity at 100–1000 nM of WKYMVm (Fig. 3C). In addition, WKYMVm-induced H2O2 generation within neutrophils was partially reversed by pretreatment of the FPR1 antagonist (Boc-PLPLP) or the FPR2 antagonist (WRW4); however, the WKYMVm-induced H2O2 generation was completely reversed by pretreatment of the FPR1 + FPR2 antagonists (Fig. 3D). Moreover, the current study also showed that neutrophils derived from CLP-induced mice failed to produce H2O2 with the stimulus of PMA; however, H2O2 generation was significantly enhanced in neutrophils derived from CLP-induced mice treated with WKYMVm, compared with neutrophils from mice not having treatment (Fig. 3E).

Bactericidal effects by WKYMVm are partly dependent on a type 1 cytokine (IFN-γ)-mediated pathway

We found that WKYMVm enhanced IL-12 levels in the peritoneal fluids 24 h after CLP (Fig. 4A, left panel). Moreover, IFN-γ levels in the peritoneal fluids were augmented 8 and 24 h after CLP (Fig. 4A, right panel). To investigate the role of type 1 cytokines (IL-12 and IFN-γ) in the WKYMVm-induced survival effect after sepsis, the CLP model was applied to IL-12Rβ2–deficient, IFN-γ–deficient, and WT control mice (C57BL/6 background). CLP-induced lethality was significantly decreased in WT mice treated with WKYMVm (Fig. 4B). Because mortality in IL-12Rβ2–deficient and WT control mice was similar, the increase in IL-12 levels mediated by WKYMVm plays an important role in the survival effect after sepsis.

FIGURE 5. Role of WKYMVm in the production of proinflammatory cytokines. A, WKYMVm (4 mg/kg) was injected s.c. four times into CLP mice and then cytokine levels in the peritoneal fluids were measured at different time points after CLP. Separate groups of animals were subjected to sham, CLP + PBS, or CLP + WKYMVm treatment. Left panel, TNF-α; middle panel, IL-1β; right panel, IL-6. Data are expressed as the mean ± SE. n = 8. *p < 0.05 compared with CLP + PBS. B, WKYMVm (4 mg/kg) was injected s.c. into mice 2 h and 14 h after i.p. injection of 60 mg/kg LPS; plasma was collected 24 h later and then the levels of IL-1β and TNF-α were measured. C, Mouse neutrophils were preincubated with PBS or WKYMVm (0.1 and 1 μM) for 30 min and then stimulated with PBS or LPS (100 ng/ml) for 3 h. The levels of TNF-α (left panel) and IL-1β (right panel) were measured by ELISA. Data are presented as the mean ± SE. n = 16 for B and C. *p < 0.05; ***p < 0.001.
with WKYMVm; the enhanced survival by WKYMVm was partly reversed in IL-12Rβ2-deficient mice (Fig. 4B), but almost completely reversed in IFN-γ-deficient mice (Fig. 4C).

On the basis of the finding that enhanced survival by WKYMVm treatment was partly dependent on a type 1 cytokine-mediated pathway, we also evaluated whether the enhanced bactericidal activity by WKYMVm was type 1 cytokine dependent. The bactericidal effects augmented by WKYMVm treatment were partly reversed in IFN-γ-deficient mice (Fig. 4D). In terms of an anti-inflammatory effect of WKYMVm, lung inflammation (W/D weight ratio) enhanced by CLP was not reversed in IFN-γ-deficient mice (Fig. 4E). In addition, CLP-induced immune cell apoptosis in the spleen was not observed in IFN-γ-deficient mice, regardless of WKYMVm treatment (Supplemental Fig. 1).

**WKYMVm inhibits the production of proinflammatory cytokines**

The levels of the proinflammatory cytokines (TNF-α, IL-1β, and IL-6) after CLP were significantly decreased in the peritoneal fluids of mice treated with WKYMVm, compared with mice treated with PBS (Fig. 5A). To evaluate the effects of WKYMVm on the proinflammatory cytokine production by LPS, the in vivo production of proinflammatory cytokines was measured following i.p. application of LPS (60 mg/kg). The plasma levels of TNF-α and IL-1β 4 h after LPS administration were significantly decreased in mice treated with WKYMVm (4 mg/kg), compared with mice treated with PBS (Fig. 5B). Moreover, the direct release of TNF-α and IL-1β from mouse neutrophils by LPS was inhibited by in vitro WKYMVm treatment in a dose-dependent manner (Fig. 5C).

**The anti-inflammatory effects of WKYMVm are dependent on an IL-17–mediated pathway**

The administration of WKYMVm increased IL-17 levels in the peritoneal fluids as early as 4 h after CLP in mice, compared with mice treated with PBS; the administration of WKYMVm also enhanced the levels of anti-inflammatory cytokines (IL-10 and TGF-β) in the peritoneal fluids from 8 to 12 h after CLP (Fig. 6A). To investigate the role of IL-17 in the survival effect enhanced by the administration of WKYMVm, the CLP model was applied to IL-17–deficient and WT control mice (BALB/c background). This study showed that survival after CLP without WKYMVm treatment was increased in IL-17–deficient mice compared with WT mice (Fig. 6B); in addition, IFN-γ production in serum was enhanced in IL-17–deficient mice compared with WT mice 6 h after i.p. administration of PBS or LPS (10 and 100 μg) (Supplemental Fig. 2). However, the enhanced survival after WKYMVm treatment was partly reversed in IL-17–deficient mice (Fig. 6B). In terms of the role of IL-17 in the anti-inflammatory effects of the FPR agonist, lung inflammation (W/D weight ratio) inhibited by WKYMVm in WT mice was not observed in IL-17–deficient mice (Fig. 6C). Thus, we evaluated the effect of IL-17 on the production of proinflammatory cytokines by LPS. The in vitro experiment showed that the production of TNF-α from macrophages 20 h after in vitro stimulation of LPS (0.1 and 10 ng/ml) was significantly inhibited by coincubation of IL-17 (10 ng/ml) (Fig. 6D).
Administration of FPR agonist WKYMVm enhances type 1 and type 17 adaptive immune responses

Finally, we evaluated the effects of the administration of FPR agonists on Ag-specific T cell responses based on the finding that WKYMVm treatment induces the upregulation of IFN-γ and IL-17 production 24 h after CLP. Our previous studies indicated that airway application of an Ag with 10 μg of LPS induced a type 1 adaptive immune response (T cell priming) rather than T cell tolerance to inhaled Ags (33); this model was applied to WT C57BL/6 mice and then T cell cytokine production in the regional LNs was evaluated 24 h after 3 d of airway sensitization with an Ag (OVA) (Fig. 7A). This study showed that IFN-γ production after nonspecific (anti-CD3 + anti-CD28) or OVA-specific stimuli was markedly enhanced in mice treated with WKYMVm, compared with mice treated with PBS (Fig. 7B). Similarly, IL-17 production of T cells in the regional LNs after nonspecific and Ag-specific stimuli was also markedly enhanced by the administration of WKYMVm (Fig. 7C). In addition, IL-10 production after anti-CD3/CD28 or OVA stimuli was mildly enhanced by treatment with WKYMVm (Fig. 7D); however, IL-4 production was detected in no group (data not shown).

Discussion

Many patients with severe sepsis or septic shock die despite aggressive management (2, 3). One means of preventing severe sepsis is to neutralize endotoxin; however, the potential of such a target for therapeutic intervention is controversial (34). In placebo-controlled clinical trials, mAbs of endotoxins did not prevent the death of patients with severe Gram-negative bacterial sepsis (35). Other adjunctive therapies that are intended to control the inflammation regardless of the microbial stimuli—such as agents that directly or indirectly interfere with the activity of inflammatory mediators [e.g., platelet-activating factor antagonist (36), rIL-1 receptor antagonist (37, 38), genetically engineered soluble receptors for TNF-α, and mAbs to TNF-α (39)]—have not prevented the death of patients with severe sepsis or septic shock (34). In this paper, we demonstrated that the administration of FPR family agonist WKYMVm after the induction of sepsis by CLP effectively prevented CLP-induced lethality in mice via multiple therapeutic pathways: 1) bactericidal activity that is partly mediated by IFN-γ; 2) an anti-inflammatory effect via the downregulation of proinflammatory mediators, which is partly mediated by IL-17; and 3) an antiapoptotic effect on immune cells. Recently, Park and colleagues (40) demonstrated that a classical chemoattractant, platelet-activating factor, shows protective activity against LPS-induced endotoxic shock in an experimental animal model. On the basis of all these findings, we suggest that the functional role of classical chemoattractants and their receptors should be reconsidered as important target molecules for the development of therapeutic agents against infectious diseases.

Following the initial microbial signals and host interaction, there is widespread activation of the innate immune system, which coordinates host defense against microbes. One of the key defense mechanisms involving phagocytes is the elaboration of intracellular toxic mediators, such as reactive oxygen species and NO. Our data clearly show that the mFPR activation by WKYMVm enhanced H2O2 production, which was associated with enhanced bacterial clearance; mFPR1 or mFPR2 blockades significantly inhibited WKYMVm-induced H2O2 production in neutrophils. These data suggest that the bactericidal effect enhanced by mFPR activation is related to augmentation of the production of bactericidal mediators within phagocytes.

FIGURE 7. Effect of WKYMVm on type 1 and type 17 adaptive immune responses. A. Study protocol for the generation of Ag-specific memory T cells. The production of IFN-γ (B), IL-17 (C), and IL-10 (D) from memory T cells in the regional LNs. Immune cells isolated from the regional LNs were pooled as the four groups and then the pooled cells in each group were stimulated with nonspecific (anti-CD3 plus anti-CD28) and OVA-specific stimuli.
After severe sepsis, there is an increase in the level of IL-4 and a decrease in the level of IFN-γ that may result in impaired cellular immunity (41). Therapies designed to augment production of IFN-γ may thus be beneficial in the treatment of sepsis. Properties of IFN-γ for defense against microbes include stimulation of phagocyte bactericidal activity, stimulation of Ag presentation through class I and class II MHC molecules, and orchestration of leukocyte–endothelium interactions (42). Severe sepsis down-regulates IFN-γ production (41). A clinical study demonstrated that rIFN-γ–treated patients with sepsis showed an improved clinical course (43). Our data revealed that the administration of WKYMVM enhanced IFN-γ production as early as 8 hrs after CLP; in addition, the administration of WKYMVM augmented IFN-γ production 24 hrs after CLP. Moreover, the administration of WKYMVM was found to enhance Ag-specific type 1 adaptive immune responses. In terms of the role played by IFN-γ in the therapeutic effects of WKYMVM, the survival enhanced by administration of WKYMVM was reversed in IFN-γ–deficient mice. This therapeutic effect was positively associated with the bactericidal effects of IFN-γ, although target organ inflammation was not related to IFN-γ. These findings suggest that the therapeutic effect of WKYMVM is partly mediated by the bactericidal effects of IFN-γ.

The downregulation of immunity that accompanies sepsis is related to the development of lymphocyte apoptosis; thus, inhibition of sepsis-induced lymphocyte apoptosis is a good therapeutic target. Indeed, the administration of WKYMVM inhibited apoptosis of immune cells in the spleen. Recent evidence suggests that IFN-γ induces apoptosis in Ag-specific Th1 cells in the spleen (44). Our data also indicate that immune cell apoptosis was not observed in IFN-γ–deficient mice after CLP. These findings suggest that the treatment of sepsis with rIFN-γ induces adverse effects that compromise the therapeutic effect of IFN-γ, and that FPR activation by WKYMVM may be a superior therapeutic approach.

Innate immune responses to infection can cause vital organ inflammation, which leads to multiple organ failure (the clinical hallmark of severe sepsis) (1). The recognition of microbial signals by blood neutrophils and monocytes in sepsis progression triggers the production or release of proinflammatory mediators that increase blood flow to infected tissues, enhance the permeability of local blood vessels, and recruit inflammatory cells to the site of infection (45). Our results indicate that mFPR activation by WKYMVM inhibits the production of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, following CLP. Moreover, our in vitro and in vivo experiments demonstrate that mFPR activation inhibits the production of proinflammatory cytokines induced by LPS (a prototype of microbial signals). These findings suggest that the survival effect enhanced by FPR activation is also related to downregulation of proinflammatory cytokine production; this indicates that FPR activation, compared with blockade of individual proinflammatory mediators, may be a superior therapeutic target for sepsis. Very recently it was shown that blocking the mFPRs reduces tissue damage in sepsis-like syndromes induced by the administration of mitochondria in a rat experimental model (46). Wang and colleagues (47) also demonstrated that mFPR2−/− mice showed markedly reduced severity in OVA/alum-induced allergic airway inflammation, suggesting an essential role for mFPR2 in the progression of allergic inflammation. At this point, it is not clear what causes these different roles for mFPRs. It is possible to assume that mFPRs have dual roles for regulating inflammatory cytokine production, depending on environmental status.

Cellular sources for IL-17 have recently been specifically linked to the Th17 lineage (48); however, IL-17 production does not seem to be limited to Th17 cells (49–51). IL-17 has potent actions in mobilizing, recruiting, and activating neutrophils, and has been associated with many inflammatory diseases, including autoimmune diseases (52). However, the role of IL-17 in the development of severe sepsis has been investigated little; neutralization of IL-17 with Abs improved survival after CLP (53). Our in vivo data also showed that survival after CLP without the administration of WKYMVM was higher in IL-17–deficient mice than in WT control mice; this finding may be explained by the fact that IFN-γ production was enhanced in the absence of IL-17. Interestingly, our in vivo data showed that survival enhanced by the administration of the FPR agonist WKYMVM was partly reversed in IL-17–deficient mice; moreover, target organ inflammation inhibited by the administration of WKYMVM did not occur in IL-17–deficient mice. In addition, our data indicate that the administration of WKYMVM enhanced IL-17 production after CLP, and subsequently inhibited the production of proinflammatory cytokines (TNF-α, IL-1β, and IL-6), which is accompanied by up-regulation of IL-10 and TGF-β production. Moreover, our data show that proinflammatory cytokine production from inflammatory cells by in vitro stimulation of low or high doses of LPS was significantly inhibited by coincubation of IL-17. These findings suggest that the anti-inflammatory effect of mFPR agonists in the sepsis model can be partly explained by the downregulation of proinflammatory cytokine production by IL-17 augmented by mFPR activation.

In conclusion, FPR agonists, including WKYMVM, effectively prevent development of severe sepsis after microbial infection via multiple pathways. Thus, FPRs and their specific agonists might prove useful in the development of efficient therapeutic agents for sepsis.

Disclosures
S.D.K., Y.-K.K., S.H.R., and Y.-S.B. have pending patent applications. All other authors have no financial conflicts of interest.

References


