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Prevention of Acute Lung Injury by a Novel CD14-Inhibitory Receptor Activator of the NF-κB Ligand Peptide in Mice

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ABSTRACT

Although CD14 has been implicated in the initiation of multiple TLR-mediated inflammatory responses to sepsis and sepsis-related acute lung injury (ALI), an inhibitor of CD14, except for neutralizing Abs, has not been developed. A partial peptide, microglial healing peptide 1 with N-terminal acetylation and C-terminal amidation (MHP1-AcN), derived from the receptor activator of the NF-κB ligand, was recently found to inhibit multiple TLR signaling in the macrophages. Therefore, we hypothesized that the inhibitory effect of MHP1-AcN might be through the inhibition of CD14, a common coreceptor for multiple TLRs. In cultured mouse macrophages, MHP1-AcN was shown to bind to CD14 and compete with LPS for competitive inhibition of CD14, resulting in inhibition of TLR4 signaling, including NF-κB and IFN regulatory factor 3 activation and nuclear translocation. In addition to TLR2, TLR4, and TLR7, MHP1-AcN also inhibited TLR3 signaling and *Escherichia coli* DNA–induced, CD14-dependent TLR9 signals; however, CpG oligodeoxynucleotide–induced, CD14-independent TLR9 signals were not inhibited in the mouse macrophages. In sepsis-induced ALI mouse model, MHP1-AcN treatment showed the reduction in the expression of IL-6 and CCL2 in both the serum and lung tissues. IL-6 levels in the bronchoalveolar lavage fluid and pathological score were also decreased by MHP1-AcN. Thus, MHP1-AcN, a novel CD14 inhibitor, could be a promising agent for treating sepsis-induced ALI. ImmunoHorizons, 2021, 5: 438–447.

INTRODUCTION

Acute lung injury (ALI) or its severe form, acute respiratory distress syndrome (ARDS), is a common syndrome with a high mortality rate, imposing a burden on public health (1, 2). Sepsis is a major cause of ALI (3), and patients with sepsis-related ALI have a higher mortality and more severe multiorgan dysfunction than those with ALI from other causes (4). Endotoxin is involved in ALI, as it has been detected in the plasma samples and bronchoalveolar lavage fluid (BALF) of patients with...
severe ARDS (5). The activation of TLR4, a family of pattern-recognition receptors (PRRs), by endotoxin or LPS initiates a multistep cascade leading to the release of proinflammatory mediators, resulting in the loss of alveolar-capillary barrier integrity and neutrophil recruitment (6, 7). Although the blockade of TLR4 inhibits systemic cytokine responses and improves survival in experimental models (8), the specific TLR4 antagonist failed to improve patient survival in clinical trials (9, 10). Recent studies have shown the importance of suppressing multiple TLR signals for the treatment of ALI, such as TLR2 (11), TLR7 (12), and TLR9 (13). Therefore, inhibition of multiple TLRs has emerged as an important treatment strategy for sepsis-induced ALI.

Previously, we reported that a partial peptide derived from the receptor activator of the NF-κB ligand (RANKL), microglial healing peptide 1 (MHP1), could inhibit TLR2, TLR4, and TLR7 signaling in the macrophages without osteoclast differentiation (14, 15). To increase its stability, we further modified MHP1 with N-terminal acetylation and C-terminal amidation (MHP1-AcN) (16). MHP1-AcN has shown protective effects against ischemic stroke and an imiquimod-induced psoriasis model in mice (15, 17). Although MHP1 was shown to act via RANK, as the knockdown of RANK abolished its inhibitory effect on LPS-induced inflammation (14), it was insufficient to explain its inhibitory effect on TLR2 and TLR7 signaling. Considering the inhibitory effects of MHP1-AcN on multiple TLR signaling, we speculated that MHP1-AcN might interact with a common molecule among TLRs. CD14 is one such molecule and is a GPI-anchored PRR recognizing various pathogen-associated molecular patterns (18, 19). It also is necessary for TLR2, TLR3, TLR4, TLR7, and TLR9 signaling (20–22). For example, CD14 directly binds to LPS and controls the trafficking and signaling functions of TLR4 (23, 24). However, anti-CD14 Abs failed to decrease the inflammatory response consistently in patients experiencing severe sepsis (25, 26), and small molecules targeting CD14 are restricted to TLR4 (27) or TLR2/4 signaling (28).

In this study, we examined whether MHP1-AcN interacted with CD14 and its inhibitory effect on the downstream signaling of CD14/TLR4 upon LPS stimulation. Furthermore, we examined its efficacy in TLR3 and TLR9 signaling associated with CD14. Finally, to clarify whether MHP1-AcN could treat sepsis-induced ALI as a novel CD14 inhibitor, we also evaluated its protective effects in vivo using a mouse model of sepsis-induced ALI by LPS challenge.

MATERIALS AND METHODS

Peptide design and synthesis

Synthetic MHP1-AcN (Ac-LMVYYVKTSIKIPSSHNLKGGST KNWNSG-NH₂) was purchased from ILS (Ibaragi, Tsukuba, Japan), dissolved in double-distilled water (H₂O) to make a 2-mg/ml solution, and stored at 4°C until use. Biotinylated MHP1-AcN at C-terminal [Ac- LMVYYVKTSIKIPSSHNLKGGST KNWNSG-NH₂] and biotinylated MHP1 at N-terminal (biotinyl-LMVYYVKTSIKIPSSHNLKGGST KNWNSG-NH₂) trifluoroacetate salt was purchased from Bachem Japan K. K (Shinagawa, Japan), dissolved in double-distilled H₂O to make a 2-mg/ml solution, and stored at 4°C until use.

In vivo LPS challenge

In this study, we examined whether MHP1-AcN interacted with CD14 and its inhibitory effect on the downstream signaling of CD14/TLR4 upon LPS stimulation. Furthermore, we examined its efficacy in TLR3 and TLR9 signaling associated with CD14. Finally, to clarify whether MHP1-AcN could treat sepsis-induced ALI as a novel CD14 inhibitor, we also evaluated its protective effects in vivo using a mouse model of sepsis-induced ALI by LPS challenge.

Histological analysis

For histological examination, the left lung was dissected and fixed in 4% paraformaldehyde overnight before embedding in paraffin blocks. Samples were cut into 4-μm-thick sections using a rotary microtome and stained with H&E (Muto Pure Chemicals, Tokyo, Japan). Stained sections were observed using a digital microscope (FSX-100; Olympus, Tokyo, Japan). The histological scoring parameters included alveolar congestion, alveolar hemorrhage, infiltration, or aggregation of neutrophils in the airspace or vessel wall and thickness of the alveolar wall or hyaline membrane formation. The score of each item was recorded as follows: 0, normal; 1, mild; 2, moderate; 3, severe; and 4, very severe.

ELISA

The concentrations of IL-6 and CCL2 were measured using the following commercially available ELISA kits: IL-6, Mouse IL-6 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN) and CCL2, Mouse CCL2/JE/MCP-1 Quantikine ELISA Kit (R&D Systems). The serum concentration of each cytokine was determined in duplicate for each mouse sample. IL-6 concentration
in the macrophage culture medium was also analyzed in duplicate for each experimental condition.

**Real-time reverse transcription PCR (quantitative RT-PCR)**

Lung tissues were collected 6 h after LPS application. RAW 264.7 cells (1 × 10^5 cells) or bone marrow-derived macrophages (BMDMs) (2 × 10^5 cells) were plated in 12-well plastic culture dishes. After overnight culture, the medium was replaced with DMEM supplemented with 4% FBS. LPS (no. L4391, *Escherichia coli* O111:B4; Sigma-Aldrich) and MHP1-AcN (100 μg/ml) were added simultaneously to the medium for 3 or 24 h. The final concentration of LPS was 100 ng/ml, following product information. mRNA was isolated using an RNeasy Mini Kit (QIAGEN), according to the manufacturer’s instructions. cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer’s instructions. The oligonucleotide primers were purchased according to the following identification: Il6, Mm00446190; Il1b, Mm00434228; Nos2, Mm00440502; Ccl2, Mm99999056; Tnfa, Mm00443258; CD14, Mm00438094; Tlr4, Mm00445273; and Gapdh, Mm99999915 (Applied Biosystems). The 5′ nuclease assay was performed on a MicroAmp Optical 384-well reaction plate using an ABI PRISM 7900 Sequence Detection System (Applied Biosystems), according to the manufacturer’s instructions. The expression levels of the target genes were quantified with triplicated repeated 3′84-well plates in an ABI PRISM7900 Sequence Detection System (Applied Biosystems). The expression levels were normalized to the level of Gapdh expression in each individual sample.

**Isolation and culture of primary macrophages**

BMDMs were prepared by culturing bone marrow from the femurs of 6-wk-old male C57BL/6J mice in DMEM/F-12 containing 10% FBS, 1% penicillin-streptomycin, and 10 ng/ml M-CSF (PeproTech). Nonadherent cells were removed after 24 h and cultured for 7 d.

**Cell culture and transfection**

RAW 264.7 and HEK 293 cells were obtained from the RIKEN Gene Bank (Tsukuba, Japan) and maintained in 5% carbon dioxide (CO2) at 37°C in DMEM (Nacalai Tesque) or the RPMI 1640 medium (Nacalai Tesque) supplemented with 10% FBS (Thermo Fisher Scientific) and 1% penicillin-streptomycin mixed solution (Nacalai Tesque). NF-κB luciferase stable RAW 264.7 cells were obtained from AnGen (Ibaraki, Japan) and maintained in 10% FBS RPMI 1640 containing 100 ng/ml hygromycin B (Nacalai Tesque). RAW 264.7 cells (5 × 10^4 cells) or BMDMs (4 × 10^4 cells) were plated on a 96-well plastic plate. After overnight culture, the medium was replaced with DMEM supplemented with 4% FBS. Poly(I:C) HMW (InvivoGen), CpG oligodeoxynucleotide (ODN) 1826 (InvivoGen), R837 (InvivoGen), or *E. coli* DNA (InvivoGen) was added to the medium with MHP1-AcN at the same time. After 24 h of incubation, the medium was collected for ELISA. The final concentration of poly(I:C) was 1 μg/ml (RAW 264.7) or 5 μg/ml (BMDMs), CpG ODN 1826 was 1 μg/ml, R837 was 1 μg/ml, and *E. coli* DNA was 2 μg/ml following product information. HEK 293 cells were transfected with pCMV3-CD14-HA (Sino Biological) using Lipofectamine 2000 (Invitrogen) for 48 h.

**Immunoprecipitation, Western blotting, and immunostaining**

For immunoprecipitation, cells were washed twice with chilled PBS and lysed with lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 0.5% NP40, 10% glycerol, and protease inhibitor mixture [Roche Diagnostics]). Samples were centrifuged at 16,000 × g for 10 min. The supernatant was collected, and the protein concentration was determined. Immunoprecipitation from samples containing equal amounts of protein was with High-Capacity Streptavidin Agarose (Thermo Fisher Scientific) or with Red Anti-HA Affinity Gel (Sigma-Aldrich) overnight at 4°C, washed twice with lysis buffer, and eluted with 2× Laemmli Sample Buffer at 37°C for 30 min prior to Western blotting. Extraction of separate cytoplasmic and nuclear protein fractions was performed using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific), following product information. Blotted membranes were incubated overnight at 4°C with primary Abs and washed with TBS containing 0.1% Tween 20 before incubation with HRP-conjugated secondary Ab for 1 h at room temperature, followed by Chemi-Lumi One L (Nacalai Tesque). For immunostaining, cells on glass-bottom dishes were fixed in 4% paraformaldehyde for 15 min and permeabilized with 0.2% Triton X-100 for 3 min. The samples were blocked in 5% skim milk, followed by incubation with primary Abs overnight at 4°C. The corresponding secondary Abs were labeled with Alexa Fluor 488 (Invitrogen). Nuclear staining was performed with DAPI. Images were collected using confocal microscopy (FX10i FluoView; Olympus). Abs and reagents employed in immunoprecipitation, Western blotting, and immunostaining included the following: anti-CD14 (EPR21847; Abcam), anti-RANK (AF692; R&D Systems), anti-phospho-IRF3 (Ser395) (4D4G) (Cell Signaling Technology), anti-NF-κB p65 (D14E12) (Cell Signaling Technology), anti-HA (clone 16B12; BioLegend), anti-Spl (no. 07-645; Merck Millipore), anti-GAPDH (MAB374; Sigma-Aldrich), anti-mouse IgG-HRP (NA931V; GE Healthcare), anti-rabbit IgG-HRP (NA934V; GE Healthcare), anti-goat IgG-HRP (sc-2354; Santa Cruz Biotechnology), and HRP-Streptavidin (Sigma-Aldrich).

**Reporter assay**

The NF-κB luciferase stable RAW 264.7 cells were plated on 96-well plastic plates and incubated with LPS (10 ng/ml) or LPS with MHP1-AcN (100 μg/ml) for 24 h. After incubation, luciferase activity was analyzed using the Luciferase Assay System (Promega, Madison, WI) and a microplate luminometer (Centro XS3 LB960; Berthold Technologies, Wildbad, Germany).
Germany) according to the manufacturer’s instructions. The protein concentration of each sample was measured using the Takara Bradford Protein Assay Kit (Takara Bio). The luciferase activity results were normalized to the protein concentration in each sample.

Silver staining
Gels were stained by using Pierce Silver Stain (Thermo Fisher Scientific) according to the manufacturer’s instructions.

Statistical analysis
All values are expressed as mean ± SD. Multiple comparisons were evaluated by ANOVA, followed by the Dunnett multiple comparison test. The two groups were compared using an unpaired two-tailed Student t test. Differences were considered significant at p < 0.05. Statistical analyses were performed using Prism 6.0 (GraphPad, San Diego, CA).

RESULTS

MHP1-AcN as a competitive inhibitor of CD14 competing with LPS for CD14 binding
We first examined whether MHP1-AcN interacts with CD14. RAW 264.7 cells, and BMDMs were incubated with the same amount of double-distilled H2O, unlabeled MHP1-AcN, or N- or C-terminal biotinylated MHP1 for 30 min, followed by streptavidin pulldown and Western blot assay. As expected, MHP1-AcN bound to CD14 and RANK (Fig. 1A, Supplemental Fig. 1). Because CD14 recognizes LPS and initiates TLR4 signaling (24), we next examined whether MHP1-AcN competitively binds to CD14 in the presence of LPS. HEK 293 cells were transfected with HA-CD14, incubated with biotinylated LPS, and different doses of MHP1-AcN (1, 10, and 100 μg/ml) for 10 min. We observed that MHP1-AcN competed with LPS for CD14 binding (Fig. 1B). Interestingly, the amount of LPS bound to CD14 at different doses of MHP1-AcN was consistent with the results of LPS-induced IL-6 levels in our previous report (16). At 10 μg/ml of MHP1-AcN treatment, the amount of LPS that bound to CD14 or LPS-induced IL-6 was reduced by ~50%; however, ~90% reduction was observed at 100 μg/ml of MHP1-AcN. These results indicate that MHP1-AcN is a competitive inhibitor of CD14, competing with LPS for CD14 binding, although MHP1-AcN could also bind to RANK.

MHP1-AcN inhibited LPS-induced NF-κB and IRF3 activation and nuclear translocation
Because CD14 controls the trafficking and signaling function of TLR4 (24), resulting in the activation of NF-κB and IRF3 mediated by MyD88 and TRIF, respectively (29, 30), we next checked whether MHP1-AcN could inhibit LPS-induced NF-κB and IRF3 activation and nuclear translocation of TLR4 intracellular signaling. By examining total protein distribution, NF-κB p65 was observed in the nuclear fraction after 1 h of LPS stimulation in both RAW 264.7 cells and BMDMs; however, the translocation was diminished by MHP1-AcN treatment (Fig. 2A). To further confirm the activation of NF-κB, we stimulated NF-κB luciferase stable RAW 264.7 cells with LPS (10 ng/ml) or LPS with MHP1-AcN (100 μg/ml) for 24 h. NF-κB luciferase activity was significantly decreased by MHP1-AcN treatment (Fig. 2C). We also examined the subcellular distribution of NF-κB p65 using confocal microscopy. After LPS stimulation (10 ng/ml) for 1 h, cytosolic NF-κB p65 was localized to the nucleus in BMDMs. However, NF-κB p65 remained diffusely distributed in the cytosol following MHP1-AcN treatment (Fig. 2D). Similar results were also observed in RAW

![FIGURE 1. MHP1-AcN was a competitive inhibitor of CD14 competing with LPS for CD14 binding.](https://doi.org/10.4049/immunohorizons.2000112)
264.7 cells (Supplemental Fig. 2). In addition, phosphorylation of IRF3 S396 (in mice, S388), crucial for nuclear translocation and induction of type I IFNs (31), was significantly inhibited by MHP1-AcN treatment (Fig. 2B). Overall, these complementary approaches suggested that MHP1-AcN inhibited LPS-induced intracellular TLR4 signaling, including NF-κB and IRF3 activation and nuclear translocation.

MHP1-AcN regulated LPS-induced proinflammatory cytokine and CD14 expression in macrophages

Aberrant activation of NF-κB results in the transcriptional induction of inflammatory cytokines and chemokines involved in inflammation development and progression. In this study, we examined whether MHP1-AcN regulated LPS-induced proinflammatory gene expression, including IL-6, IL-1β, NOS2, and TNF-α in the macrophages. After LPS treatment (100 ng/ml), the upregulation of IL-6, IL-1β, NOS2, and TNF-α was strikingly reduced by MHP1-AcN (100 μg/ml) at both 3 (Fig. 3A) and 24 h (Fig. 3C). Because CD14 expression is regulated by NF-κB (24), we further examined CD14 expression. LPS-induced a rapid increase in CD14 mRNA level at 3 h, which decreased almost to normal levels at 24 h. MHP1-AcN inhibited the rapid increase in CD14 mRNA levels (Fig. 3B, 3D). These results indicated that MHP1-AcN regulated LPS-induced proinflammatory cytokine expression as well as CD14 expression in the macrophages.

MHP1-AcN inhibited TLR3 and TLR9 ligand–induced IL-6 production in macrophages

Next, we examined whether MHP1-AcN could inhibit CD14-related TLR signaling in addition to TLR2, -4, and -7, reported in our previous studies. TLR3 signaling is involved in the pathogenesis of ARDS (32), in which CD14 mediates the uptake of dsRNA into TLR3-containing endosomes, thereby promoting TLR3 activation (22). Another study suggested that CD14 is necessary for TLR9-dependent induction of proinflammatory cytokines.

FIGURE 2. LPS-induced nuclear translocation of NF-κB and IRF3 was inhibited by MHP1-AcN.

(A and B) Immunoblot analysis of subcellular distribution of NF-κB p65 and phosphorylated IRF3 in RAW 264.7 cells and BMDMs after treatment with LPS (10 ng/ml) or LPS with MHP1-AcN (100 μg/ml) for 1 h (A) or 2 h (B). These are representatives of three independent experiments. GAPDH (cytoplasm) and Sp1 (nucleus) served as fractionation and loading controls. (C) NF-κB luciferase stable RAW 264.7 cells incubated with LPS (10 ng/ml, n = 4) or LPS with MHP1-AcN (100 μg/ml, n = 4) for 24 h. Luciferase activity measured and normalized with protein concentration. (D and E) Confocal immunofluorescence microscopy images of fixed BMDMs treated with PBS, LPS (10 ng/ml), and LPS with MHP1-AcN (100 μg/ml) for 1 h and stained with anti–NF-κB p65 Ab and DAPI nuclear stain. Scale bars, 20 μm (D); 5 μm (E). Error bars represent SD. Multiple comparisons were evaluated by one-way ANOVA with Dunnett correction. The experiments were repeated three times for each group. *p < 0.05, **p < 0.01 versus the LPS group.
cytokines (21). Thus, we reasoned that MHP1-AcN could inhibit TLR3 and TLR9 ligand–induced inflammation by targeting CD14. Poly(I:C) and CpG ODNs are the most commonly used synthetic TLR3 and TLR9 agonists, respectively. As expected, we observed that MHP1-AcN dose-dependently decreased poly(I:C)-induced IL-6 production in RAW 264.7 cells and

FIGURE 3. MHP1-AcN regulated LPS-induced proinflammatory cytokine and CD14 expression in macrophages. (A and B) Quantitative PCR (qPCR) analysis of IL-6, IL-1β, NOS2, TNF-α, and CD14 mRNA expression in RAW 264.7 cells and BMDMs after treatment with PBS (control), LPS (100 ng/ml), and LPS with MHP1-AcN (100 µg/ml) for 3 h (n = 3 or 4). Error bars represent SD. Multiple comparisons were evaluated by one-way ANOVA with Dunnett correction. Similar results were obtained from three independent experiments. *p < 0.05, **p < 0.01 versus the LPS group. n.s., not significant.

FIGURE 4. MHP1-AcN inhibited TLR3 and TLR9 ligand–induced IL-6 production in macrophages. RAW 264.7 cells and BMDMs incubated with (A) poly(I:C) (1 or 5 µg/ml), (B) CpG ODN 1826 (1 µg/ml), or (C) E. coli DNA (2 µg/ml) in the presence or absence of MHP1-AcN (10, 30, or 100 µg/ml) for 24 h (n = 3 or 4). Cultured medium collected and analyzed for IL-6 production by ELISA. Error bars represent the SD. Multiple comparisons were evaluated by one-way ANOVA with Dunnett correction. Similar results were obtained from three independent experiments. **p < 0.01 versus the group treated with poly(I:C) or CpG ODN or E. coli DNA without MHP1-AcN.
BMDMs (Fig. 4A). Interestingly, MHP1-AcN had no effect on CpG ODN–induced inflammation (Fig. 4B). A recent study suggested that CD14 was not required for the binding or responses to A-, B-, and C-class CpG ODNs (33). In addition, another report found that the macrophage activation differed by bacteri al DNA and synthetic CpG ODNs (34). Thus, we next examined the effect of MHP1-AcN on E. coli DNA–activated TLR9 signaling. In contrast to CD14-independent CpG ODNs, we observed that MHP1-AcN inhibited E. coli DNA–induced IL-6 production in macrophages (Fig. 4C). We previously showed MHP1-AcN inhibited LPS (TLR4 ligand) and R837 (TLR7 ligand) induced IL-6 in MG6 and RAW 264.7 cells (15, 16); in this study, we observed IL-6 was also inhibited in primary macrophages (Supplemental Fig. 3). These results

**FIGURE 5.** MHP1-AcN protected mice from sepsis-induced ALI.

Mice i.p. treated with PBS (control, n = 4), LPS (1 mg/kg, n = 6), LPS with low-dose MHP1-AcN (4 mg/kg, n = 6), and LPS with high-dose MHP1-AcN (10 mg/kg, n = 6) for 6 h. (A) Serum IL-6 and CCL2 levels are measured by ELISA. (B) IL-6 levels in BALF are measured by ELISA. (C) IL-6 and CCL2 mRNA levels in the lungs analyzed by quantitative PCR (qPCR) and normalized to the PBS group. (D) Histological score measured and calculated on a four-point scale: 0, normal; 1, mild; 2, moderate; 3, severe; and 4, very severe, evaluating each item, including alveolar congestion, alveolar hemorrhage, infiltration or aggregation of neutrophils in the airspace or vessel wall, and thickness of the alveolar wall or hyaline membrane formation. (E) Representative H&E-stained sections of the left lung. Scale bar, 100 μm. Error bars represent the SD. Multiple comparisons are evaluated by one-way ANOVA with Dunnett correction. Similar results were obtained from two independent experiments.*p < 0.05, **p < 0.01 versus the LPS group. n.d., not detected; n.s., not significant.
indicate that MHP1-AcN inhibits CD14-related TLR3 and TLR9 ligand–induced inflammation. Importantly, because MHP1-AcN had no effect on CD14-independent, CpG ODN–mediated inflammation, it further implied that the inhibitory effect on multiple TLR signaling of MHP1-AcN was through CD14.

**MHP1-AcN protected mice from sepsis-induced ALI**

Finally, we examined the efficacy of MHP1-AcN in a sepsis-induced ALI model. Mice were treated with 1 mg/kg of LPS, together with MHP1-AcN (4 mg/kg for low dose and 10 mg/kg for high dose) or PBS into peritoneal for 6 h. After the LPS challenge, the serum, BALF, and lung were collected. Because previous studies showed that plasma IL-6 is an efficient predictor of poor prognosis (35) and aCLL2 is an important chemokine in the pathogenesis of ARDS (36), we measured IL-6 and CCL2 production in the serum. Administration of LPS elicited a robust increase in IL-6 and CCL2 in the serum, whereas these inflammatory mediators were significantly decreased in mice treated with MHP1-AcN (Fig. 5A). To examine inflammation in the lungs, we measured IL-6 in BALF and expression of IL-6 and CCL2 mRNA in the lung. IL-6 levels in BALF were significantly decreased by high-dose MHP1-AcN treatment. Low-dose MHP1-AcN treatment showed a tendency to reduce BALF IL-6, but no significant difference was observed (Fig. 5B). Expression of Il6 and CCL2 in the lungs was also reduced by MHP1-AcN treatment (Fig. 5C). In addition, pathological changes in the lungs were detected using H&E staining (Fig. 5E), and their pathological scores were calculated (Fig. 5D). High-dose MHP1-AcN treatment attenuated infiltration or aggregation of neutrophils in the airspace or vessel walls and reduced the thickness of the alveolar wall. Similar to the BALF IL-6 result, low-dose MHP1-AcN treatment showed a tendency to attenuate the lung injury without a significant difference. These results suggest the potential effects of MHP1-AcN in protecting mice from sepsis-induced ALI by LPS challenge.

**DISCUSSION**

In this study, we demonstrated that the partial RANKL peptide, MHP1-AcN, is a (to our knowledge) novel CD14 inhibitor that competes with LPS for CD14 binding, thereby inhibiting TLR4 signaling. In addition to TLR2, TLR4, and TLR7, MHP1-AcN inhibited TLR3- and CD14-dependent TLR9 signaling. MHP1-AcN also protected mice from sepsis-induced ALI by LPS challenge through inhibition of neutrophil infiltration with reduced IL-6 and CCL2 expression.

Sepsis-induced ALI can be of pulmonary (direct) or extrapulmonary (indirect) origin. In direct sepsis-induced ARDS, lung dysfunction begins following bacterial/viral pulmonary infection, in which innate immune cells function via PRRs, including TLRs. Among the TLRs, TLR4-mediated recognition of LPS is an important trigger of the inflammatory response. Therefore, great efforts have been made to develop new drugs targeting TLR4 to compete with sepsis and sepsis-associated multiorgan failure, including ALI/ARDS. Among all TLR4 antagonists developed so far, only TAK-242 and eritoran reached the clinical trials. Unfortunately, neither TAK-242 (resatorvid), a small-molecule compound selectively binding to an intracellular TLR4 domain, nor eritoran, a competitive inhibitor of MD-2 competing with LPS, was beneficial for improving patient survival (9, 10). One possible explanation is the limited inhibition of TLR4 signaling. For example, a recent study suggested that multiple TLRs, including TLR2, TLR9, and, especially, TLR4, simultaneously contribute to the pathogenesis of sepsis-induced ALI (37). Additionally, the combined administration of anti-TLR2 and anti-TLR4 mAbs increased the survival more than twice when compared with anti-TLR4 Ab alone (38). TLR3 and TLR9 signaling are also necessary for sepsis-induced cardiac dysfunction (39). Considering these recent findings, anti-CD14 Ab might be an attractive strategy because of its broad coverage of TLR signals; however, it failed to decrease the inflammatory response consistently in patients experiencing severe sepsis despite promising anti-inflammatory effects in human low-grade endotoxemia (25, 26, 40). Also, IgG-mediated reaction, which was associated with anaphylaxis (41), is one of major problems for its clinical use. Alternatively, small molecules targeting CD14, such as IAXO-101 and VB-201, might be other possible alternatives, but their actions are restricted to TLR2 and TLR4 (27, 28). From these viewpoints, the inhibitory effects on multiple TLRs of MHP1-AcN are unique and might be more suitable for the treatment of ALI than the currently used agents.

In indirect origin, sepsis-induced ALI is proposed to be mediated by accumulated neutrophils in the lungs, closely related to proinflammatory mediators such as IL-6 and CCL2. In MHP1-AcN–treated mice, the neutrophil activation was prevented. As early control of neutrophil activation in patients with sepsis-induced ALI is associated with better outcomes (42) despite overlapping pathogenetic mechanisms between direct and indirect origin (43), the inhibitory effects of neutrophil infiltration of MHP1-AcN are also promising for the treatment of ALI.

Interestingly, MHP1-AcN did not reduce soluble CD14 (sCD14) concentration in the serum (Supplemental Fig. 4). CD14 exists as a soluble form as well as a membrane-bound form. In cells lacking membrane-bound CD14, such as the endothelial and epithelial cells, complexes of LPS and sCD14 can stimulate these cells, leading to their activation and cytokine production, playing a pivotal role in the inflammatory reaction of sepsis (44). Elevated concentrations of sCD14 are seen in patients with sepsis and may be a biomarker for the severity of disease (45). Consistent with these reports, we found elevated serum CD14 concentration after LPS challenge.

One possible explanation why sCD14 was not decreased in the current study might be that the ELISA for CD14 could not discriminate between sCD14–MHP1 complexes and monomeric sCD14 because similar results were observed in anti-CD14 therapy for human sepsis (46). Although the function of sCD14
remains controversial, further studies are necessary to determine whether the sCD14–MHP1 complexes are biologically active.

We focused on CD14 in the current study, but MHP1-AcN was also shown to bind to RANK. In addition, we previously showed that the anti-TLR4 signal effects of MHP1-AcN were decreased in RANK knockdown in RAW 264.7 cells (14). RANKL/RANK signaling was demonstrated to attenuate proinflammatory cytokine production in mice exposed to LPS (47) and ischemic stroke (48). Similar results were observed in BV2 microglia lacking RANK, as RANK knockout abolished most effects of RANKL pretreatment (49). Although the precise mechanism by which RANKL/RANK signaling affects TLR signaling is still unclear, all the effects of recombinant RANKL require their pretreatment prior to inflammatory insults, as it could reshape host responses by regulating the expression of TLR adapter protein (49). In contrast, MHP1-AcN does not require pretreatment for its anti-TLR signal effects. This suggests that the anti-TLR signal effects of MHP1-AcN might be mainly dependent on the inhibition of the CD14/TLR4 signal, although the RANKL/RANK signal might be partly responsible for its anti-TLR4 signal effects.

In summary, we found that MHP1-AcN is a novel CD14 inhibitor suppressing multiple TLR signaling. Although further studies are necessary for its clinical use, this peptide will provide an opportunity to inhibit inflammatory responses at a very proximal point, making it a rational therapeutic approach to treat polymicrobial sepsis and sepsis-related organ dysfunction.

DISCLOSURES

A patent application has been filed for MHPs (application no. 2015-102502 and PCT/JP2016/064446; M.S., H.N., R.M.). R.M. is a founder and shareholder of AnGes and a former board member. R.M. is also a shareholder of Funpep. M.S. and H.N. are shareholders of the MH Peptide. This company has a contract for priority rights of negotiation regarding the MHP-1 patient with the Osaka University. There are no conflicts of interest between any authors and the MH Peptide, as adjudicated by the Conflict-of-Interest Committee at Osaka University. The other authors have no financial conflicts of interest.

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