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Mouse Peptidoglycan Recognition Protein PGLYRP-1 Plays a Role in the Host Innate Immune Response against *Listeria monocytogenes* Infection▽†

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The role of mouse peptidoglycan recognition protein PGLYRP-1 in innate immunity against *Listeria monocytogenes* infection was studied. The recombinant mouse PGLYRP-1 and a polyclonal antibody specific to PGLYRP-1 were prepared. The mouse PGLYRP-1 showed antibacterial activities against *L. monocytogenes* and other Gram-positive bacteria. PGLYRP-1 mRNA expression was induced in the spleens and livers of mice infected with *L. monocytogenes*. The viable bacterial number increased, and the production of cytokines such as gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) was reduced in mice when mice had been injected with anti-PGLYRP-1 antibody before infection. The levels of IFN-γ and TNF-α titers in the organs were higher and the viable bacterial number was reduced in mice injected with recombinant mouse PGLYRP-1 (rmPGLYRP-1) before infection. PGLYRP-1 could directly induce these cytokines in spleen cell cultures. The elimination of intracellular bacteria was upregulated in NMuLi hepatocyte cells overexpressing PGLYRP-1. The enhancement of the elimination of *L. monocytogenes* from the organs was observed in IFN-$\gamma^{-/-}$ mice by rmPGLYRP-1 administration but not in TNF-$\alpha^{-/-}$ mice. These results suggest that PGLYRP-1 plays a role in innate immunity against *L. monocytogenes* infection by inducing TNF-α.

Innate immunity is the frontier of host defense against microbial infections. It is directed to components of microorganisms and recognizes them through a series of pattern recognition receptors, which are conserved through species from insects to mammals (3, 10, 12). Peptidoglycan recognition proteins (PGRPs) also are a family of these pattern recognition proteins and are conserved through species (8, 9, 20, 23, 33, 47).

PGRP was first discovered in the silkworm *Bombyx mori* in 1996 as a 19-kDa protein that could recognize peptidoglycans (PGNs) (61). The genome sequencing of *Drosophila melanogaster* revealed 17 homologues of PGRPs in this fly (8, 56). In these PGRPs, seven short PGRPs have signal peptides and can be secreted. Ten long PGRPs have predicted transmembrane domains and can be transmembrane proteins. *Drosophila* PGRPs are expressed in immunocompetent cells such as hemocytes and are upregulated by PGNs (23). Therefore, it is likely that *Drosophila* PGRPs play a role in insect innate immunity. Recent studies revealed that PGRP-SA, -SD, -LC, and -LE can activate two different pathways, Toll and Imd pathways, that lead to the production of antibacterial peptides (1, 6, 14, 30, 39, 51, 57, 62). In addition, PGRP-LE is reported to be crucial for the induction of autophagy by *L. monocytogenes* in *D. melanogaster* (59). Other proteins, PGRP-SC1b, -LB, and -SA, are known to have PGN-degrading activities (25, 36, 37).

Mammals have four homologues of PGRPs: PGLYRP-1, -2, -3, and -4 (initially named PGRP-S, -L, -Iα, and -Iβ, respectively) in humans (23, 33), mice (24, 32, 35), rats (43), and cattle (53, 54). Mammalian PGLYRP-2 is an *N*-acetyl-muramoyl-L-alanine amidase (9, 13, 34, 55, 62). It can cleave the peptide from the glycan chain of PGNs and is expressed in liver, colon, and skin cells (31, 33, 34, 62), but its function *in vivo* still is unclear (56, 58). Human PGLYRP-1, -3, and -4 show antibacterial activities (34). Molecular and structural mechanisms of mammalian PGRPs for PGN binding and antibacterial activities have been studied (5, 16–19, 26, 48), as have been insect PGRPs (4, 24, 29, 44). Mammalian PGLYRP-1 is a secretory protein (32). This protein constructs dimer formation (32–34), and the metal ions such as calcium and zinc ions enhance the antimicrobial activities (34). Mammalian PGLYRP-1 is considered a pattern recognition receptor (8, 33). Human PGLYRP-1 is localized in neutrophils and is likely to kill phagocytized bacteria (7, 32, 53). Moreover, the immunomodulatory activity of PGLYRP-1 in PGN-induced arthritis has been reported recently for mice (48). However, a mechanism of pattern recognition and ensuing innate immunity in bacterial infection still is unclear.

Listeria monocytogenes is a Gram-positive intracellular bacterium that is important as an opportunistic pathogen in humans such as immunocompromised hosts, pregnant women, and their fetuses. Macrophages contribute to innate immunity against *L. monocytogenes* infection, although recent reports showed that granulocytes, including neutrophils, play a role in host resistance against the early stage of *L. monocytogenes* infection as well as macrophages (15, 60). Tumor necrosis factor-alpha (TNF-α) and gamma interferon (IFN-γ) are known to be important in host resistance against *L. monocytogenes* infection (2, 11, 21, 22, 40–42, 44). The production of these cytokines is induced by *L. monocytogenes* infection via several pattern recognition receptors and their downstream components (3). However, the role of PGLYRP-1 in the protection against *L. monocytogenes* infection is not clear.

In this study, we investigated the immunomodulatory activ-

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ities of mouse PGLYRP-1 in innate immune systems as well as antibacterial activities. We demonstrated that mouse PGLYRP-1 plays an important role in host resistance against *L. monocytogenes* infection.

MATERIALS AND METHODS

Mice. C57BL/6 mice were purchased from Clea Japan Inc., Tokyo, Japan. IFN-γ-deficient (IFN-$\gamma^{-/-}$) and TNF-α-deficient (TNF-$\alpha^{-/-}$) mice (C57BL/6 background) were developed as previously reported (50, 52). Mice were cared for under specific-pathogen-free conditions in the Institute for Animal Experimentation, Hirosaki University Graduate School of Medicine. All animal experiments in this study were performed by following the guidelines for animal experimentation of Hirosaki University.

Infection. *L. monocytogenes* strain 1b 1684 (41) was used in this study. Bacteria grown in tryptic soy broth (BD Bioscience, Sparks, MD) were dispersed and stored at −80°C until use. C57BL/6, IFN-$\gamma^{-/-}$, and TNF-$\alpha^{-/-}$ mice were infected intravenously with sublethal doses of 5×10^5 CFU (for C57BL/6 mice) and 1×10^5 CFU (for IFN-$\gamma^{-/-}$ and TNF-$\alpha^{-/-}$ mice) of *L. monocytogenes* in phosphate-buffered saline (PBS). When the effect of PGLYRP-1 administration was tested, 5×10^6 CFU of *L. monocytogenes* in PBS was infected.

Spleen cells and macrophages. Spleens from C57BL/6 mice were minced and filtrated through stainless mesh (size, 100). Erythrocytes were lysed with 0.83% (wt/vol) ammonium chloride and then washed three times with Dulbecco's modified Eagle medium (DMEM; Nissui Pharmaceutical Co., Tokyo, Japan). Splenic macrophages were prepared as follows: spleen cells resuspended in DMEM supplemented with 10% (vol/vol) fetal calf serum (FCS; Nichirei Biosciences Inc., Tokyo, Japan) and 0.03% L-glutamine (Wako Pure Chemical Co., Osaka, Japan) were plated on 25-cm^2 culture flasks (Asahi Glass Co., LTD., Tokyo, Japan) for 2 h in a 5% CO_2 incubator. The adherent cells were isolated by a cell scraper and counted. Spleen cells and splenic macrophages were cultured in the media in a 5% CO_2 incubator. Mouse hepatocyte cell line NMuLi cells (DS Pharma Biomedical, Osaka, Japan) were maintained in DMEM with 10% (vol/vol) FCS.

Expression of mouse PGLYRP-1. RNA was isolated from the spleens of C57BL/6 mice infected with *L. monocytogenes* 0, 0.5, 1, 2, 4, 6, and 12 h after infection using TRIzol reagents (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA from three mice was pooled and used for real-time reverse transcription-PCR (RT-PCR). Primers for the amplification of *tag7* genes, coding for PGLYRP-1 (25), were p-132 (5′-CAT ATG TGC AGT TTC ATC GTG CCC CGC AG-3′) and p-133 (GGA TCC TCA CTC TCG GTA GTG TTC CCA GC). Primers for the amplification of mouse glyceraldehyde-3-phosphate dehydrogenase (mGAPDH) genes were mGAPDH-F (5′-TGA AGG TCG GTG TGA ACG GAT TTG G-3′) and mGAPDH-R 5′-ACG ACA TAC TCA GCA CCG GCC TCA C-3′). PCR conditions consisted of preheating at 94°C for 10 min, 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s, and a final elongation at 72°C for 10 min. PCR products were observed in the agarose gel after electrophoresis. The quantitative analysis of the expression of PGLYRP-1 was performed by an i-Cycler system (Bio-Rad Japan, Tokyo, Japan) using iQ SYBR green Supermix (Bio-Rad Japan) as the chromogen. The expression was analyzed as the relative expression compared to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

rmPGLYRP-1. RNA samples were obtained from spleens of C57BL/6 mice 2 h after *L. monocytogenes* infection. Primers for cloning were the same as those for amplification (described above) and were designed for subcloning to the pET15b expression vector (Merck Chemical KgaA, Darmstadt, Germany). After PCR, PCR products were purified and ligated to pCR II TA cloning vector. After the transformation and sequencing of the insert, it was subcloned into pET15b by the NdeI-BamHI restriction site. The expression host, Rosetta (DE3) pLysS, was transformed by this plasmid. After the cultivation of these bacteria in 500 ml LB broth (BD Biosciences) with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol for 2 h, recombinant protein production was induced by the addition of 1 mM isopropylthiogalactopyranoside, and the bacteria were cultured for 2 h. The cultivated bacteria were disrupted by sonication, and recombinant proteins were precipitated in inclusion bodies. Purified inclusion bodies were solubilized by 6 M guanidine-HCl in the washing buffer (50 mM sodium phosphate buffer, pH 7.0, 300 mM NaCl) and immediately reprecipitated by diluting the guanidine concentration to 0.6 M. After centrifugation, the resulting pellets were resolubilized by 6 M guanidine in the washing buffer supplemented with 0.3% *N*-laurolyl sarcosinate and 1 mM dithiothreitol, and the concentration of guanidine was diluted to 2 M by slowly adding the washing buffer with supplements. Solubilized recombinant mouse PGLYRP-1 (rmPGLYRP-1) then was charged to a Talon 6× histidine affinity column (Takara Bio Inc., Ohtsu, Japan). The column was washed with the washing buffer containing a slowly lowered concentration of guanidine from 2 to 0 M using an Econo gradient pump system (Bio-Rad Japan). The charged rmPGLYRP-1 then was eluted by 150 mM imidazole in the washing buffer. The eluted proteins were concentrated and exchanged with the washing buffer using Vivaspin filter units (Sartorius AG, Goettingen, Germany). Digestion by thrombin and the subsequent removal of thrombin were performed using a thrombin cleavage capture kit (Novagen, Madison, WI), and histidine tag-cleaved rmPGLYRP-1 was stored at −20°C before use. When used for *L. monocytogenes* infection, 100 μg of rmPGLYRP-1 was administered to mice 6 h before infection.

Tests for endotoxin contamination. To assess the contamination of lipopolysaccharide (LPS) derived from host *Escherichia coli*, *Limulus* amebocyte lysate (LAL) tests were performed using PYROTELL kits (Seikagaku Corp., Tokyo, Japan). Endotoxin was below 3 pg/100 μg rmPGLYRP-1. In some experiments, rmPGLYRP-1 was inactivated by heating the sample for 30 min at 95°C. LPS from *E. coli* was purchased from Sigma-Aldrich Japan, Tokyo, Japan.

Rabbit antibodies against rmPGLYRP-1. The rabbits were immunized with 1 mg of rmPGLYRP-1 plus 1 ml of Imject alum (Pierce Biotechnology Inc., Rockford, IL) per rabbit three times at 2-week intervals. The sera were obtained from the rabbits 1 week after final immunization. Immunoglobulin was purified using DEAE-sephacel resin (GE Healthcare, Piscataway, NJ) according to the standard protocol and stored at −80°C before use. The titer of antibodies was 1:102,400, as determined by enzyme-linked immunosorbent assay (ELISA). Mice were administered 1 mg of the antibody 24 h before *L. monocytogenes* infection. Normal rabbit globulin (NRG) was injected as a negative control. NRG was prepared from normal rabbit sera using the same protocol as that for the antibody.

Assay of antibacterial activities. *Salmonella enterica* serovar Typhimurium χ3306, *Escherichia coli* strain IFO3806, and clinical isolates of *Staphylococcus aureus*, *S. epidermidis*, *Pseudomonas aeruginosa*, and *Serratia marcescens* were used for assays of antibacterial activities in addition to *L. monocytogenes*. Bacteria were precultured overnight and subcultured for 4 h in tryptic soy broth. Assay mixtures for antibacterial activities contained 1×10^6 CFU of bacteria tested and rmPGLYRP-1 or bovine serum albumin (fraction V; Sigma-Aldrich, Japan) in 1 ml of assay buffer (5 mM Tris-HCl, pH 7.6, 150 mM NaCl, 2.5 mM $CaCl_2$, and 5% glycerol supplemented with 1% LB broth). Where indicated, 1 mM zinc chloride (Wako) or 1 mM EDTA (EDTA, Wako) were added to the assay mix. After 0.5, 1, 3, and 6 h, 100 μl of mixture was recovered, and serially diluted specimens were plated on tryptic soy agar (BD Sciences). The colony number was counted 24 h later. Neutralization by anti-mPGLYRP-1 antibody was accomplished by adding 1 mg of the antibody to the mixture.

***In vitro* stimulation of spleen cells and macrophages.** Spleen cells and splenic macrophages were plated on 24-well culture plates at a concentration of 1×10^6 cells/ml in DMEM supplemented with 10% (vol/vol) FCS, and 0.4, 2, or 10 μg of rmPGLYRP-1 was added. The culture supernatant fluids were collected 48 h later and used for cytokine assays. In some experiments, the cells were plated on 24-well culture plates (Asahi Glass, Tokyo, Japan) at a concentration of 1×10^6 cells/ml in DMEM supplemented with 10% (vol/vol) FCS with 1×10^7 CFU of *L. monocytogenes*. After 30 min, the medium was changed to DMEM supplemented with 10% (vol/vol) FCS and 5 μg/ml gentamicin and cultured for 12 h. Culture supernatant fluids were collected and used for cytokine assays.

Construction of PGLYRP-1 with enhanced green fluorescent protein (EGFP) expression plasmid. pEGFP-C2 vector was purchased from Takara Bio Inc. The gene encoding PGLYRP-1 was amplified using cDNA from spleens of *L. monocytogenes*-infected mice (described above) by the primers p505 (5′-GAA TTC ATG TTG TTT GCC TGT GCT CTC CTT G-3′) and 507 (5′-GGA TCC TCA CTC TCG GTA GTG TTC CCA GC-3′) with EcoRI and BamHI restriction sites, respectively. This fragment was ligated into pEGFP-C2 by restriction sites, and the plasmid with PGLYRP-1 was designated pEGFP-C2/PGLYRP-1.

Overexpression of PGLYRP-1 in hepatocyte NMuLi cells and observation of *L. monocytogenes* in the cells. NMuLi cells were seeded on 24-well culture plates at a concentration of 2×10^5 cells/ml in DMEM supplemented with 10% (vol/vol) FCS for 12 h. pEGFP-C2/PGLYRP-1 and control pEGFP-C2 vectors were transfected with the support of Lipofectamine 2000 (Invitrogen) and incubated for 24 h. Cells were infected with *L. monocytogenes* at a multiplicity of infection (MOI) of 100. After 30 min of incubation, the extracellular bacteria were eliminated with 5 μg/ml gentamicin and additionally incubated for 6 h. Bacteria were sequentially labeled with rabbit anti-*Listeria* spp. (ViroStat, Portland, ME) and rhodamine-conjugated goat anti-rabbit immunoglobulin G (MP Bio Japan, Tokyo, Japan) and observed by fluorescent microscopy (Olympus IX71; Olympus, Tokyo, Japan). GFP- or GFP-PGLYRP-1-overexpressing cells

and intracellular rhodamine-labeled *L. monocytogenes* inside the cells were counted for 10 fields and analyzed statistically.

Preparation of organ and cell samples for determination of bacterial numbers and for cytokine assays. Spleens and livers from infected mice were homogenized in PBS containing 1% (wt/vol) 3-[(cholamidopropyl)-dimethyl-ammonio]-1-propanesulfate (CHAPS; Wako) to prepare 10% (wt/vol) homogenates. NMuli cells seeded on 24-well culture plates at a concentration of 2×10^5 cells/well also were homogenized in 1 ml of PBS containing 1% (wt/vol) CHAPS. The numbers of viable *L. monocytogenes* cells were established by plating 10-fold serial dilutions of a bacterial solution in PBS on tryptic soy agar plates. Colonies were counted 24 h later.

Cytokine assays. TNF-α levels were determined by ELISA using a cyto-set Elisa kit (Invitrogen) according to the manufacturer's instrumentation. IFN-γ ELISA was performed as previously described (40).

SDS-PAGE and Western blotting. SDS-PAGE was performed as previously reported (27). For the confirmation of the purity of rmPGLYRP-1, 2 μg of recombinant protein was applied to SDS-PAGE. Endogenous PGLYRP-1 in sera of mice were detected at various time points after *L. monocytogenes* infection. Exogenous rmPGLYRP-1 was detected in sera, spleens, and livers of mice at various time points after the injection of 100 μg of rmPGLYRP-1. Protein concentration was determined using a Bio-Rad protein assay (Bio-Rad Japan) according to the manufacturer's instructions and normalized to 20 μg/well. Separated proteins were transferred to Immobillon-P membranes (Millipore Japan, Tokyo, Japan) by electrophoresis. The polyclonal antibody prepared in this study was used to detect mPGLYRP-1. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (MP Bio, Japan) was used as a second antibody, and color reaction was performed with 3,3′-diaminobenzidine, tetrahydrochloride (Wako) as a substrate.

Statistical analysis. Data were expressed as means ± standard deviations (SD), and the Mann-Whitney U test was used to determine the significance of the differences.

RESULTS

PGLYRP-1 was induced by *L. monocytogenes* infection in mice. Mouse PGLYRP-1 is known to be highly produced in neutrophils (32). To estimate whether *tag7* gene expression and the production of PGLYRP-1 are regulated by bacterial infections, we investigated the expression of PGLYRP-1 in spleens and livers of mice infected with *L. monocytogenes*. After infection, mice were sacrificed and RNA was obtained from the spleens and livers for RT-PCR. The expression of PGLYRP-1 mRNA was upregulated 30 min to 6 h after infection (Fig. 1A). Quantitative real-time RT-PCR also was carried out, and a consistent expression pattern was observed (see Fig. S2 in the supplemental material). Moreover, to investigate that PGLYRP-1 is secretory, sera were taken from mice infected with *L. monocytogenes* and PGLYRP-1 was detected by anti-mPGLYRP-1 antibody. PGLYRP-1 protein was detected 3 h after infection in the sera and continued to 12 h (Fig. 1B). These results indicated that PGLYRP-1 was induced in the early stages of *L. monocytogenes* infection in mice.

Anti-PGLYRP-1 antibody increased susceptibility to *L. monocytogenes* infection and reduced TNF-α and IFN-γ production. We assessed whether endogenous PGLYRP-1 is involved in host resistance against *L. monocytogenes* infection. Recombinant protein was prepared and purified as a dimer (see Fig. S1), consistently with the previous report (34). Polyclonal antibodies against mPGLYRP-1 were prepared by the immunization of rabbits with this protein, and the specificity was confirmed by Western blotting, although minor nonspecific bands were detected (Fig. 1B; also see Fig. S3 in the supplemental material). Mice were injected with anti-PGLYRP-1 antibody or NRG 24 h before infection with a sublethal dose of *L. monocytogenes*. The viable bacterial numbers in the spleens and livers were measured on days 1, 2, 3, and 5 after infection. The numbers in both organs increased in anti-PGLYRP-1 antibody-injected mice on days 2 and 3 after infection compared to that in NRG-injected animals (Fig. 2A and B). However, the effect of anti-PGLYRP1 antibody was not observed on day 5 of infection. The levels of IFN-γ and TNF-α in the sera and spleens were determined next, because TNF-α and IFN-γ are critical in host resistance against *L. monocytogenes* infection (2, 11, 21, 22, 41, 42, 46). The titers of both cytokines in the organs tested were significantly decreased in the anti-PGLYRP-1 antibody-treated group compared to that of the NRG-treated group 24 h after infection (Fig. 2C and D). These results indicated that endogenous PGLYRP-1 is involved in host resistance against *L. monocytogenes* infection and the production of IFN-γ and TNF-α.

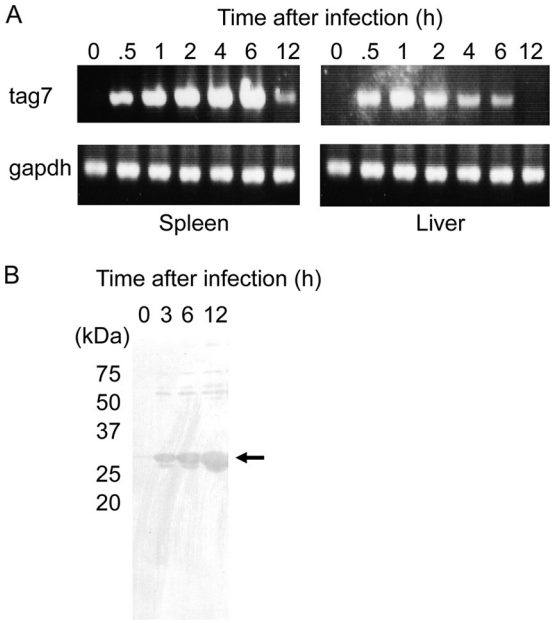

FIG. 1. Expression of PGLYRP-1-coding *tag7* gene and PGLYRP-1 protein after *L. monocytogenes* infection. (A) Mice were infected with 5×10^5 CFU of *L. monocytogenes*, the spleens and livers were obtained 0, 0.5, 1, 2, 4, 6, and 12 h after infection, and RNA was extracted from the organs. The expression of *tag7* and *gapdh* was estimated by RT-PCR. Representative data are shown for three independent experiments. (B) Mice were infected with 5×10^5 CFU of *L. monocytogenes*, and the sera were obtained 0, 3, 6, and 12 h after infection. The protein concentration was adjusted to 20 μg/well and applied to Western blotting. Representative data are shown for three independent experiments.

rmPGLYRP-1 enhanced the elimination of *L. monocytogenes* from the organs of mice. We investigated the effect of the administration of rmPGLYRP-1 on antilisterial resistance. We first assessed whether rmPGLYRP-1 is able to induce cytokines *in vivo*. Naive mice were administered rmPGLYRP-1, and the levels of IFN-γ and TNF-α in the sera and spleens were measured 24 h later. The concentration of IFN-γ and TNF-α in sera increased in rmPGLYRP-1-treated mice compared to that of the PBS-treated group (Fig. 3A and B). Mice then were injected with rmPGLYRP-1 followed by *L. monocytogenes* infection 6 h later, and bacterial numbers were determined in the spleens and livers on days 1, 2, 3, and 5 of

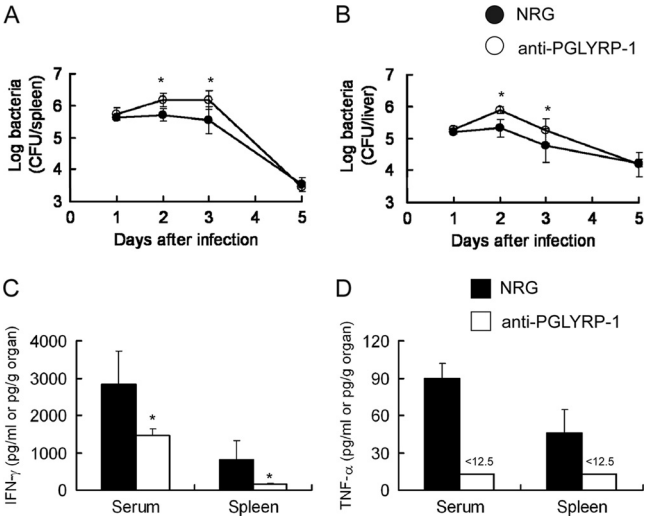

FIG. 2. Inhibition of elimination of *L. monocytogenes* and endogenous cytokine production by administration of anti-mPGLYRP-1 antibody in mice. C57BL/6 mice were injected with 1 mg of anti-PGLYRP-1 antibody or normal rabbit globulin (NRG) 24 h before infection with 5×10^5 CFU of *L. monocytogenes*, and viable bacterial numbers in the spleens (A) and livers (B) from mice were determined 1, 2, 3, and 5 days after infection. Similarly, titers of IFN-γ (C) and TNF-α (D) in the sera and spleens 24 h after infection were determined in an anti-PGLYRP-1 antibody-treated group and NRG-treated group. The threshold of detection is indicated by <12.5. Each group consists of six mice. Bars show SD. An asterisk indicates a significant difference from the NRG-treated group at $P < 0.05$.

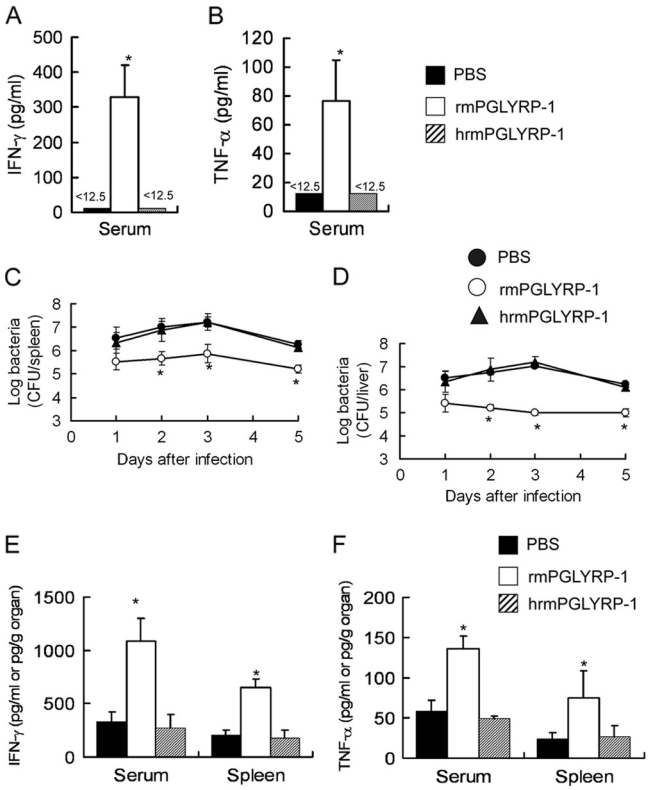

FIG. 3. Enhancement of host resistance against *L. monocytogenes* infection and cytokine production by administration of rmPGLYRP-1. C57BL/6 mice were injected with 100 μg of rmPGLYRP-1, heat-inactivated rmPGLYRP-1, or PBS, and titers of IFN-γ (A) and TNF-α (B) in the sera were determined 24 h after administration. Similarly, C57BL/6 mice were injected with 100 μg of rmPGLYRP-1, heat-inactivated rmPGLYRP-1, or PBS 6 h before infection with 5×10^6 CFU of *L. monocytogenes*, and viable bacterial numbers in the spleens (C) and livers (D) from mice were determined 1, 2, 3, and 5 days after infection. Titers of IFN-γ (E) and TNF-α (F) in the sera and spleens were determined for the rmPGLYRP-1-treated group, heat-inactivated rmPGLYRP-1-treated group, and PBS-treated group 24 h after infection. The threshold of detection is indicated by <12.5. Each group consists of six mice. Bars show SD. An asterisk indicates a significant difference from the PBS-treated group and heat-inactivated rmPGLYRP-1-treated group at $P < 0.05$.

infection. The numbers of *L. monocytogenes* cells in the organs significantly decreased when rmPGLYRP-1 had been administered on days 1, 2, 3, and 5 after infection (Fig. 3C and D). We next carried out the detection of exogenous PGLYRP-1 in the bloodstream and organs of mice. The thick band of PGLYRP-1 was detected at 12 and 24 h in sera and rapidly disappeared (see Fig. S3 in the supplemental material). In contrast, PGLYRP-1 still could be detected in livers and spleens thereafter (see Fig. S3). The titers of TNF-α and IFN-γ in the sera and spleens also were measured 24 h after infection. IFN-γ and TNF-α production in *L. monocytogenes*-infected mice increased significantly at 24 h in the rmPGLYRP-1-injected groups compared to that of the PBS-injected group (Fig. 3E and F).

rmPGLYRP-1 showed antibacterial activities. Mammalian PGLYRP-1 is known to show bactericidal activities against Gram-positive bacteria (31, 32). In this study, we investigated the antibacterial activities of rmPGLYRP-1 against some species of Gram-positive and Gram-negative bacteria. Two hundred μg of rmPGLYRP-1 was added to 1 ml of the bacterial suspension, and viable bacterial numbers were determined during 6 h of incubation. rmPGLYRP-1 showed no antibacterial activity against the Gram-negative bacteria examined (data not shown). In contrast, rmPGLYRP-1 revealed antibacterial activities against Gram-positive *S. aureus*, *S. epidermidis*, and *L. monocytogenes* (Fig. 4A, B, and C). We investigated whether anti-mPGLYRP-1 antibody could neutralize the antibacterial activities of rmPGLYRP-1. No antibacterial activity was observed against *L. monocytogenes* when rmPGLYRP-1 had been treated with anti-PGLYRP-1 antibody (Fig. 4C). The antibacterial activity was not significantly enhanced by the addition of zinc chloride (Fig. 4D and E). However, the addition of EDTA inhibited the antibacterial activity of PGLYRP-1 (Fig. 4D and E).

rmPGLYRP-1 induced cytokine production in spleen cells. We next investigated the involvement of PGLYRP-1 in IFN-γ and TNF-α production *in vitro*. Naïve spleen cells and splenic macrophages were stimulated with 0.4, 2, or 10 μg of rmPGLYRP-1 for 48 h. Cytokine titers in the culture supernatant fluids were measured. Spleen cells produced IFN-γ and TNF-α by stimulation with rmPGLYRP-1 in a dose-dependent manner (Fig. 5). To investigate whether PGLYRP-1 could activate spleen cells *in vivo*, mice then were injected with 100 μg of rmPGLYRP-1, spleen cells and splenic macrophages were obtained 6 h later, and these cells were stimulated with 1×10^6 CFU of viable *L. monocytogenes in vitro*. After 12 h of incubation in the presence of gentamicin, TNF-α and IFN-γ

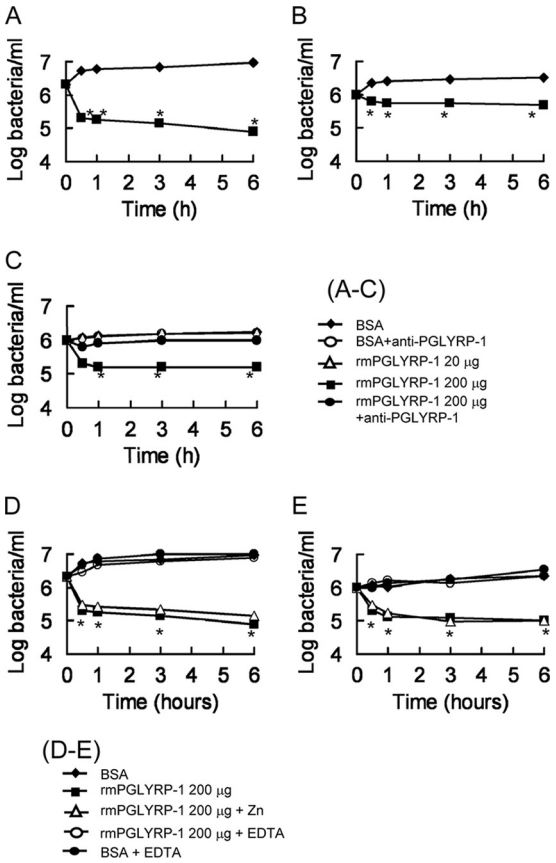

FIG. 4. Antibacterial activities of rmPGLYRP-1 against Gram-positive and Gram-negative bacteria. (A to C) An assay mixture for antibacterial activity contained 1×10^6 CFU of *S. aureus* (A), *S. epidermidis* (B), and *L. monocytogenes* (C) rmPGLYRP-1 or bovine serum albumin (BSA) as a control in 1 ml of the assay buffer with or without anti-mPGLYRP-1 antibody (1 mg/ml). After 0.5, 1, 3, and 6 h of incubation, 100 μl of the mixture was taken and plated on tryptic soy agar plates after serial dilution. Each result was obtained from six independent assays. An asterisk indicates that a value is significantly different from the value obtained from BSA controls at $P < 0.01$. (D, E) An assay mixture for antibacterial activity contained 1×10^6 CFU of *S. aureus* (D) and *L. monocytogenes* (E) rmPGLYRP-1 or BSA as a control in 1 ml of the assay buffer with or without 1 mM zinc chloride or 1 mM EDTA. Each result was obtained from six independent assays. An asterisk indicates that a value is significantly different from the value obtained from BSA controls at $P < 0.01$.

levels in culture supernatant fluids were measured. TNF-α and IFN-γ production was significantly enhanced in rmPGLYRP-1-injected mice compared to that of the PBS-treated group (Fig. 6). These results indicated that PGLYRP-1 could induce TNF-α and IFN-γ production *in vitro* and *in vivo*.

NMuLi hepatocyte cells overexpressing PGLYRP-1 showed increased resistance to *L. monocytogenes* infection. We showed that mPGLYRP-1 induces TNF-α and IFN-γ production *in vitro* (Fig. 5 and 6). To investigate further whether PGLYRP-1 enhances the elimination of *L. monocytogenes*, we constructed NMuLi hepatocyte cells overexpressing PGLYRP-1 with GFP as a marker of successful transfection. Transfected hepatocytes were infected with *L. monocytogenes*. Rhodamine-labeled *L. monocytogenes* cells were observed in green-labeled cells under microscopy 6 h after infection, and the intracellular bacterial

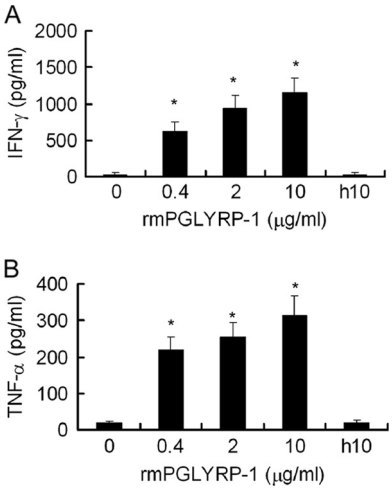

FIG. 5. Cytokine production by stimulation with rmPGLYRP-1 *in vitro*. (A) Naïve mouse spleen cells were stimulated with 0.4, 2, or 10 μg of rmPGLYRP-1 or 10 μg of heat-inactivated rmPGLYRP-1 for 48 h, and IFN-γ in culture supernatant fluids was assayed. (B) Splenic macrophages were stimulated with 0.4, 2, or 10 μg of rmPGLYRP-1 or 10 μg of heat-inactivated rmPGLYRP-1 for 48 h, and TNF-α in culture supernatant fluids was assayed. h10 means 10 μg of heat-inactivated rmPGLYRP-1. Each group consists of six wells of the plates. Bars show SD. An asterisk indicates that the value is significantly different from that of the unstimulated culture and heat-inactivated rmPGLYRP-1 at $P < 0.01$.

number in GFP- or GFP-PGLYRP-1-overexpressing cells was counted. The number of *L. monocytogenes* cells inside PGLYRP-1-overexpressing cells decreased compared to that of GFP-overexpressing control cells (Fig. 7).

rmPGLYRP-1 failed to enhance host resistance against *L. monocytogenes* infection in TNF-α$^{-/-}$ mice. To confirm the role of enhanced IFN-γ and TNF-α production induced by PGLYRP-1, we investigated the effect of rmPGLYRP-1 administration on host resistance against *L. monocytogenes* infection in IFN-γ$^{-/-}$ and TNF-α$^{-/-}$ mice. These mice were in-

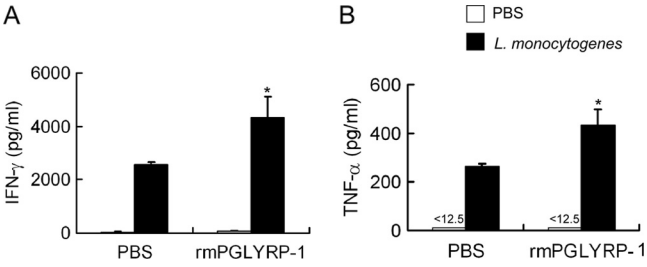

FIG. 6. Enhancement of cytokine production in spleen cells and macrophages by systemic administration of rmPGLYRP-1. (A) Spleen cells were obtained from C57BL/6 mice 6 h after the injection of 100 μg of rmPGLYRP-1 or PBS *in vivo* and then were stimulated with PBS or 1×10^7 CFU of viable *L. monocytogenes* cells for 12 h *in vitro*. IFN-γ titers in culture supernatant fluids were assayed. (B) Splenic macrophages from C57BL/6 mice pretreated with rmPGLYRP-1 or PBS were stimulated with PBS or viable *L. monocytogenes* cells for 12 h *in vitro*. TNF-α titers in culture supernatant fluids were assayed. The threshold of detection is indicated by <12.5. Each group consists of six wells of the plates. Bars show SD. An asterisk indicates that the value is significantly different from that obtained from PBS-pretreated groups at $P < 0.01$.

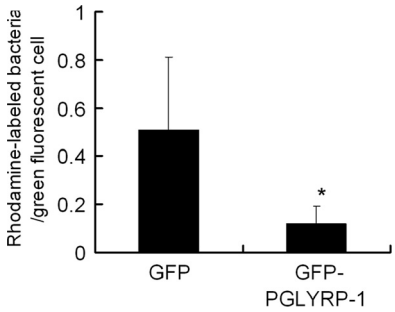

FIG. 7. Increased elimination of *L. monocytogenes* in PGLYRP-1-overexpressing hepatocytes. NMuLi hepatocyte cells seeded at a concentration of 2×10^5 cells/well were transfected with pEGFP-C2/PGLYRP-1 or pEGFP-C2 control vector. Both transfected cells were infected with *L. monocytogenes* for 30 min, and cell cultivation was continued with the elimination of extracellular bacteria by gentamicin. After 6 h of cultivation, *L. monocytogenes* cells were labeled with rhodamine, and rhodamine-labeled bacterial numbers in GFP-labeled cells were counted and the ratio of the bacterial number to the cell number was calculated. An asterisk indicates a significant difference from the pEGFP-C2-transfected group at $P < 0.05$.

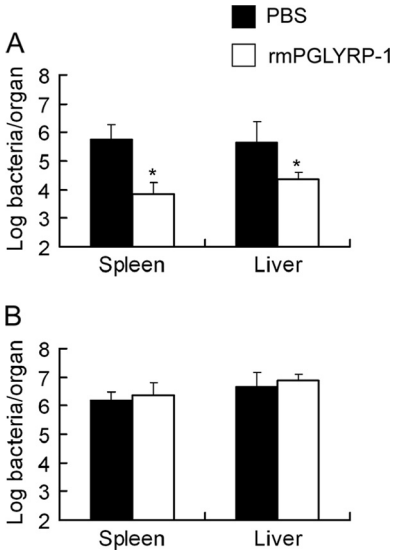

FIG. 8. Effect of rmPGLYRP-1 on host resistance against *L. monocytogenes* infection in IFN-$\gamma^{-/-}$ mice and TNF-$\alpha^{-/-}$ mice. IFN-$\gamma^{-/-}$ mice and TNF-$\alpha^{-/-}$ mice were injected with 100 μg of rmPGLYRP-1 or PBS 6 h before infection with 1×10^5 CFU of *L. monocytogenes*. The number of viable *L. monocytogenes* cells in organs from IFN-$\gamma^{-/-}$ mice (A) and TNF-$\alpha^{-/-}$ mice (B) was determined on day 3 after infection. Each group consists of six mice. An asterisk indicates the significant difference from the PBS-treated group at $P < 0.01$.

jected with 100 μg of rmPGLYRP-1 prior to infection with *L. monocytogenes*, and the viable bacterial number was determined on day 3 after infection. The bacterial number in the organs of IFN-$\gamma^{-/-}$ mice decreased significantly in the rmPGLYRP-1-injected group to a level comparable to that of wild-type mice (Fig. 8A). Conversely, the bacterial numbers were not significantly different between the rmPGLYRP-1-injected group and PBS control group in TNF-$\alpha^{-/-}$ mice (Fig. 8B). These results indicated that TNF-α, but not IFN-γ, is involved in the enhancement of antilisterial resistance by PGLYRP-1.

Possibility of endotoxin contamination in rmPGLYRP-1 was excluded. To exclude the possibility of endotoxin contamination of the rmPGLYRP-1 preparation, LAL tests were carried out. The endotoxin level was below 3 pg/100 μg rmPGLYRP-1. We investigated whether the low dose of LPS could modulate antilisterial resistance. Mice were injected with 3 pg of LPS 6 h before *L. monocytogenes* infection. This amount of LPS failed to affect bacterial proliferation in the organs on day 2 of infection (data not shown). LPS is known to be heat stable. Therefore, we assessed whether heat-inactivated rmPGLYRP-1 could modulate cytokine production and antilisterial resistance. Naive mice were injected with heat-inactivated rmPGLYRP-1, and serum IFN-γ and TNF-α levels were determined. The titers of both cytokines were under the detectable level at 24 h (Fig. 3A and B). IFN-γ and TNF-α in the sera also were determined 4 h after injection with heat-inactivated rmPGLYRP-1, and both cytokines were under the detectable level (data not shown). Moreover, mice were injected with heat-inactivated rmPGLYRP-1 6 h before *L. monocytogenes* infection, and the bacterial number in the spleens and livers was determined on days 1, 2, 3, and 5 after infection. The viable bacterial numbers were similar between heat-inactivated rmPGLYRP-1-treated mice and PBS-treated animals (Fig. 3C and D). The induction of IFN-γ and TNF-α production by heat-inactivated rmPGLYRP treatment was estimated in *L. monocytogenes*-infected mice. The titers of both cytokines in heat-inactivated rmPGLYRP-1-treated mice were at the same level as that for the PBS-treated group 24 h after infection (Fig. 3E and F). We also assessed the involvement of endotoxin contamination in stimulation with rmPGLYRP-1 *in vitro*. The production of IFN-γ and TNF-α did not occur when heat-inactivated rmPGLYRP-1 was added (Fig. 5). The enhanced elimination of *L. monocytogenes* in pEGFP-C2/PGLYRP-1-transfected hepatocytes also excluded the possibility of LPS, because PGLYRP-1 expressed in NMuLi hepatocyte cells caused reduced numbers of *L. monocytogenes* (Fig. 7). These results suggested that the activation of antilisterial resistance and cytokine induction by rmPRLYRP-1 is not affected under the detectable dose of endotoxin in the recombinant preparation.

DISCUSSION

Enzymatic characteristics of mammalian PGRPs, including structure biological studies, have been investigated (5, 16–19, 26, 49). However, the actual physiological functions of PGRPs remain unclear (8). In this report, we studied the functions of mouse PGLYRP-1 from the point of innate immunity during *L. monocytogenes* infection.

The expression of the *tag7* gene was upregulated in spleens and livers soon after *L. monocytogenes* infection (Fig. 1A), and PGLYRP-1 was released into the bloodstream beginning 3 h after infection (Fig. 1B). Endogenous PGLYRP-1 reduced bacterial numbers until day 3 of infection (Fig. 2A and B), and the administration of rmPGLYRP-1 before infection enhanced the elimination of *L. monocytogenes* from the organs until day 5 of infection (Fig. 3C and D). However, the effect of anti-PGLYRP-1 antibody or rmPGLYRP-1 was not observed on day 5 of infection (Fig. 2A and B). $CD8^+$ T cells reportedly

are critical in adaptive host defense against *L. monocytogenes* infection (38). Previous studies of adaptive immune responses to *L. monocytogenes* infection demonstrated that H2-M3-restricted $CD8^+$ T cells reach high frequencies in the spleen on days 5 to 6, and the number of major histocompatibility complex class I-restricted $CD8^+$ T cells peaks on days 7 to 9 of sublethal infection (38). In this study, the effects of anti-PGLYRP-1 antibody or rmPGLYRP-1 were restricted in the early stage of infection. Neutrophils as well as macrophages play a critical role in host resistance against early-stage *L. monocytogenes* infection (45). Previous studies reported that the expression of mammalian PGLYRP-1 is restricted to neutrophils (32, 53, 54). Although the accurate cellular source of PGLYRP-1 is not identified in this study, it is possible that macrophages are able to produce PGLYRP-1 as well as neutrophils (unpublished observation). Moreover, we showed that PGLYRP-1 without a secretion signal sequence autonomously activated NMuLi hepatocyte cells (Fig. 7). These results suggest that PGLYRP-1 plays a role in innate host defense against *L. monocytogenes* infection in the early stage.

PGLYRP-1 is a secretory protein (8, 9, 33), and mammalian PGLYRP-1 has antibacterial activities against Gram-positive bacteria (31–33), suggesting the contribution to protection from bacterial infections by its antibacterial activity (32). In this study, rmPGLYRP-1 showed antibacterial activities against Gram-positive bacteria such as *S. aureus*, *S. epidermidis*, and *L. monocytogenes* but not against Gram-negative bacteria (Fig. 4). These results were almost comparable to those of the previous report (34), although the antibacterial activity of rmPGLYRP-1 against *S. aureus* was lower than that of human PGLYRP-1 (34) and *L. monocytogenes* is sensitive to PGLYRP-1 (34). This contradiction may be due to the difference of species or preparation of proteins. The fact that N-glycosylation is required for the bactericidal activity of human PGLYRP-1 (34) might explain the weak activity of rmPGLYRP-1, because the rmPGLYRP-1 used herein was prepared by an *E. coli* overexpression system and was not N-glycosylated.

In *D. melanogaster*, PGRP-SA, the homologue of PGLYRP-1, recognizes PGNs through the Toll signaling pathway and induces antibacterial peptide production (6, 39), thus PGLYRP-1 is involved in insect innate immunity. We investigated the possibility that mPGLYRP-1 is involved in cytokine-mediated innate immunity against *L. monocytogenes* infection. TNF-α and IFN-γ are known to be crucial cytokines in innate immunity against *L. monocytogenes* infection and following macrophage activation in mice (2, 11, 21, 22, 40–42, 46). TNF-α and IFN-γ production in the spleens and sera was suppressed when endogenous PGLYRP-1 had been neutralized by anti-PGLYRP-1 antibody (Fig. 2C and D), suggesting that PGLYRP-1 is involved in the induction of the production of both cytokines. The inhibition of IFN-γ production was incomplete by anti-PGLYRP-1 antibody treatment (Fig. 2C). This effect might lead to the result that the increase of bacterial numbers in spleens and livers by anti-PGLYRP-1 antibodies was marginal (Fig. 2A and B). Therefore, to ensure that PGLYRP-1 enhanced IFN-γ and TNF-α production, IFN-γ and TNF-α responses were examined after the administration of rmPGLYRP-1. Indeed, TNF-α and IFN-γ production was induced by the administration of rmPGLYRP-1 (Fig. 3A and B), and the production of both cytokines was enhanced by *L. monocytogenes* infection (Fig. 3E and F). The induction of TNF-α and IFN-γ production by rmPGLYRP-1 also was shown by experiments with spleen cell cultures (Fig. 5 and 6). These results support that PGLYRP-1 is involved in protection from *L. monocytogenes* infection by inducing TNF-α and IFN-γ production.

We next addressed whether both TNF-α and IFN-γ are critically involved in the enhancement of host resistance against *L. monocytogenes* infection by PGLYRP-1. The bacterial numbers in spleens and livers 3 days after infection were significantly reduced by the administration of PGLYRP-1 before infection in IFN-$\gamma^{-/-}$ mice but not in TNF-$\alpha^{-/-}$ mice (Fig. 8), suggesting that TNF-α, but not IFN-γ, induced by PGLYRP-1 mainly contributes to the protection against *L. monocytogenes* infection. This result is consistent with our previous study that TNF-α is more important in the protection of *L. monocytogenes* than IFN-γ (40).

It is unclear whether the rmPGLYRP-1 preparation used in this study was correctly refolded and has the same specific activity as native proteins. The heat-treated rmPGLYRP-1 lost the inducing ability of TNF-α and IFN-γ production (Fig. 3E and F and 5C and D), suggesting that rmPGLYRP-1 is active in the nondenatured condition, although the antibacterial activities of rmPGLYRP-1 were lower than those of human PGLYRP-1 because of the possibility of the incomplete refolding and the absence of N-glycosylation (34). We also examined the effect of zinc ions on the antibacterial activities. Zinc is important for antibacterial activities of human PGRPs (34). However, the addition of zinc ions did not cause increased antibacterial activities (Fig. 4D and E), suggesting that rmPGLYRP-1 already contains metal cationic ions such as a zinc ion (34). This suggestion was strongly supported by the addition of EDTA, a metal cation chelator, which suppressed antibacterial activities (Fig. 4D and E). We also showed that rmPGLYRP-1 as prepared herein was a dimer (see Fig. S1 and S3 in the supplemental material). This result supports the idea that the recombinant proteins were recovered in the native form.

The administration of 100 μg of rmPGLYRP-1 may not reflect physiological conditions (Fig. 1B; also see Fig. S3 in the supplemental material). Indeed, the administration of 10 μg of rmPGLYRP-1 failed to affect the bacterial number and cytokine responses in *L. monocytogenes* infection in mice (data not shown). The administration of antibodies against PGLYRP-1 incompletely inhibited cytokine responses (Fig. 2C and D). These results suggest that an additional mechanism that is involved in protection by PGLYRP-1 from *L. monocytogenes* infection should be considered (3, 10, 12).

We also noticed endotoxin contamination, because we prepared rmPGLYRP-1 by using an *E. coli* overexpression system. LPS induces inflammatory cytokine production via the Toll-like receptor 4-NF-κB signaling pathway (3). In addition to LAL tests, which proved no contamination of LPS in rmPGLYRP-1, we showed that heat-inactivated rmPGLYRP-1 lost the ability to induce cytokine production and to modulate antilisterial resistance (Fig. 3 and 5). The enhanced elimination of *L. monocytogenes* from pEGFP-C2/PGLYRP-1-transfected hepatocytes supports the evidence, because the contamination of LPS can be eliminated in this system (Fig. 7).

Recent studies demonstrated that the expression of

PGLYRP-1 was upregulated by transcription factor NF-κB in brain ischemia (28) and that PGLYRP-1 may regulate the inflammatory effect of PGLYRP-2 (46). The next subject of investigation should be to elucidate the precise role of PGLYRP-1 in *L. monocytogenes* infection. In summary, for the first time we demonstrated that the expression of PGLYRP-1 is induced by *L. monocytogenes* infection and that PGLYRP-1 plays a role in the protection from *L. monocytogenes* infection through the induction of TNF-α and IFN-γ, especially TNF-α.

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