

## INNATE IMMUNITY IS UP-REGULATED BY A VIRULENCE FACTOR p60 DERIVED FROM *LISTERIA MONOCYTOGENES*

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**Abstract** We investigated the effect of p60 that is one of virulence factors of *Listeria monocytogenes* on host immune response in vitro and in vivo. C57BL/6 mice immunized with p60 showed antigen-specific T-helper I type immune response. Mouse macrophage RAW264.7 cells produced tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin-12 and interferon beta (IFN- $\beta$ ) in response to stimulation with recombinant p60. Administration of p60 prior to a sublethal infection with *L. monocytogenes* enhanced innate host resistance in naïve mice. TNF- $\alpha$  and IFN- $\beta$  production from RAW264.7 cells and bone marrow-derived macrophages were Toll-like receptor 4 (TLR4)-dependent. The enhanced clearance of *L. monocytogenes* by p60 administration was not shown in C3H/HeJ mice. Our findings demonstrated that p60 enhances host resistance against *L. monocytogenes* infection through both activation and attenuation of host innate immune response in the TLR4-dependent manner.

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**Key words:** *Listeria monocytogenes*; p60; TLR4; TNF- $\alpha$ ; IFN- $\beta$

*Listeria monocytogenes* is a gram-positive, intracellular-growing bacterium that causes systemic infection in immunocompromised hosts, such as newborns, elderly persons, and pregnant women<sup>1)</sup>. To establish the pathogenicity to host, *L. monocytogenes* attaches, invades and replicates in various types of host cells. A number of listerial virulence factors are involved in the intracellular life of *L. monocytogenes*<sup>2)</sup>. p60 is a 60 kDa extracellular protein produced by *L. monocytogenes*. Regulation of p60 expression is PrfA-independent, but regulated by another secretion system, SecA2<sup>3)</sup>. p60 acts as a murein hydrolase required in the last step of cell division<sup>4)</sup>. p60 has also the potent antigenicity that is next to listeriolysin O<sup>5,6)</sup>. p60-specific CD8<sup>+</sup> T cells induced

by *L. monocytogenes* infection protected host from *L. monocytogenes* infection<sup>7)</sup>. It has been reported that p60 induces strong antigen-specific response to CD4<sup>+</sup> and CD8<sup>+</sup> T cells<sup>8)</sup>, and that p60 activates natural killer cells and induces IFN- $\gamma$ -dependent immune response<sup>9)</sup>. In this study, we investigated the effect of recombinant p60 on host immune response and demonstrated that p60 mediates host innate immune response by activation of nuclear factor kappa B (NF- $\kappa$ B) involved in proinflammatory cytokine production and interferon regulatory factor 3 (IRF3) pathways involved in type I IFN production.

*L. monocytogenes* infection induces Th1 response to the infected mice<sup>10-12)</sup>. Therefore we investigated whether immunization with

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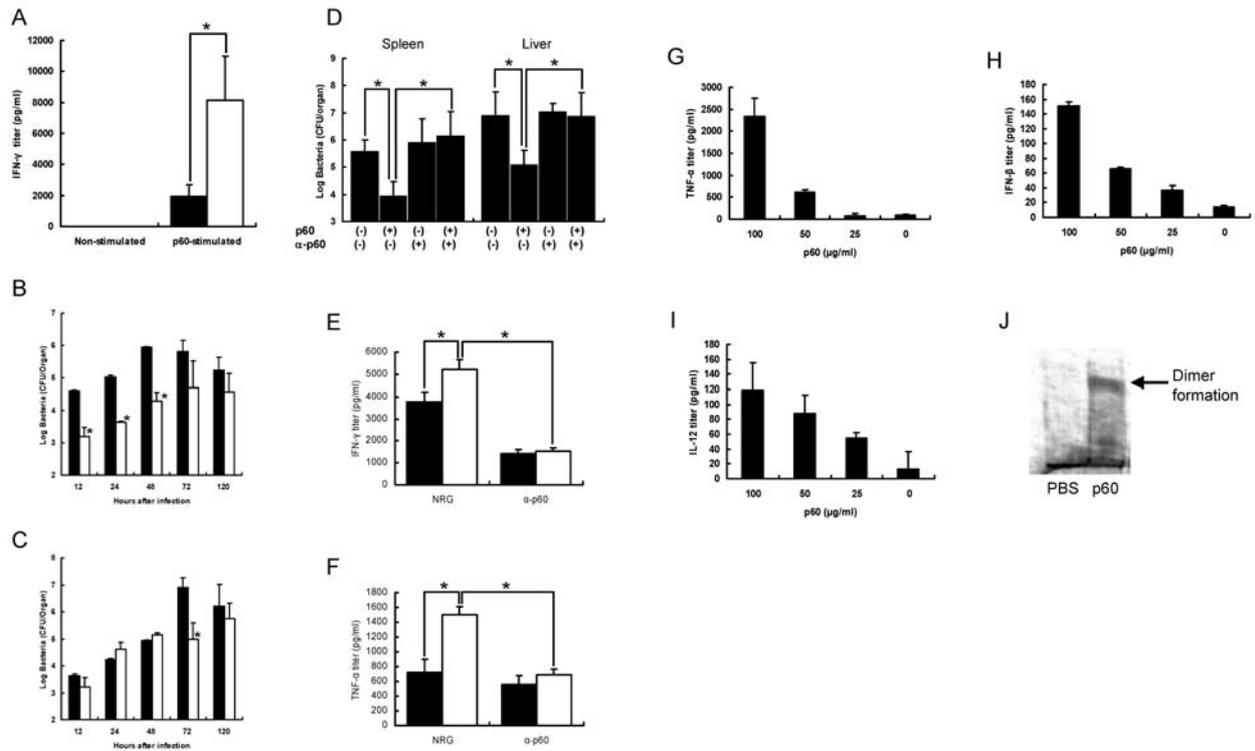
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recombinant p60 induces Th1 response in the immunized mice. Data were expressed as means  $\pm$  standard deviations, and Student's *t* test was used to determine the significance of the differences in the bacterial counts of the specimens by the control and experimental groups. Each experiment was repeated at least twice. C57BL/6 mice were purchased from CLEA Japan, Inc., Tokyo, Japan. Mice were used at 6- to 8-wk-old. Animals 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 article were conducted in accordance with the Animal Research Ethics Committee, Hirosaki University Graduate School of Medicine and followed the Guidelines for Animal Experimentation, Hirosaki University. For preparation of recombinant p60, genomic DNA containing the *iap* gene for p60 was isolated from *L. monocytogenes* by standard procedures<sup>13</sup>. For construction of recombinant p60 expression plasmids, PCR primers were designed to amplify the gene fragment encoding the full length of p60 (forward: CCCGAATTC ATGAAAAAGCAACTATCGCGGC, reverse: CCCCAGCTGTTATACGCGACCGAAGCCAAC, recognized sequence for restriction enzyme was underlined). The *iap* gene was amplified by PCR by using Pyrobest DNA polymerase (Takara, Shiga, Japan). The *iap* fragment was digested with *EcoRI* and *SalI* and subcloned into the pGEX-6p-1 (GE Healthcare Bio-Sciences, Tokyo, Japan) glutathione S-transferase fusion expression vector. Expression, purification of GST-fused recombinant p60, and the cleavage and removal of the GST from recombinant p60 were performed as described previously<sup>13</sup>. Emulsion of recombinant p60 with complete Freund's adjuvant (CFA, Sigma-Aldrich Japan, Tokyo, Japan) was prepared for immunization. Emulsion of PBS and CFA was used for non-immunized control. Mice were subcutaneously

injected with emulsion containing 50  $\mu$ g/mouse of recombinant p60 in 100  $\mu$ l/mouse emulsion twice at the 2 weeks interval. Two weeks after the second immunization, spleens and sera were obtained for further analyses or immunized mice were prepared for lethal challenge of bacterial infection. *L. monocytogenes* 1b 1684 cells were prepared as described previously<sup>14</sup>. Mice were infected with viable *L. monocytogenes* intravenously at the indicated dose, and spleens and livers were obtained at various time points. Single cell suspensions were prepared and production of IFN- $\gamma$  in response to ex vivo stimulation with p60 was determined by enzyme-linked immunosorbent assay (ELISA). Determination of IFN- $\gamma$  was carried out as described previously<sup>15,16</sup>. For determination of viable bacteria, organs were homogenized in phosphate-buffered saline (PBS) containing 1% 3-[(cholamidopropyl) dimethylammonio] 1-propanesulfate (CHAPS, Wako Pure Chemical Industries, Osaka, Japan). The numbers of viable bacteria in the organs of infected animals were counted by plating serial 10-fold dilutions of organ homogenates on tryptic soy agar (BD Diagnosis Systems, Sparks, MD, USA). Colonies were routinely counted 24 h later. Splenic cells from the p60-immunized mice showed higher production of IFN- $\gamma$  in response to stimulation with p60 compared with that in the non-immunized mice (Fig. 1A). This result indicated that immunization with recombinant p60 induced p60-specific Th1-skewed immunity. Then we investigated whether immunization with p60 is able to protect the immunized mice from a lethal infection with *L. monocytogenes*. The result showed that immunization with p60 did not protect from lethal *L. monocytogenes* infection (data not shown), suggesting that immunization with p60 was not sufficient to protect the immunized mice from lethal *L. monocytogenes* infection.

Next we assessed the effect of administration



**Figure 1** (A) Mice were immunized with 50  $\mu\text{g}$  of recombinant p60 as described in Materials and Methods. Spleens were obtained 2 weeks after second immunization, and single cell suspensions of splenocytes were prepared. One million/ml of splenocytes in 24-well plate were re-stimulated with 100  $\mu\text{g}/\text{ml}$  of recombinant p60 for 48 h. Then the culture supernatants from non-immunized (filled) and p60-immunized (open) were collected and titers of IFN- $\gamma$  were determined by ELISA. (B, C) Mice were administered with 100  $\mu\text{g}/\text{mouse}$  of recombinant p60 (open) or PBS as control (filled) intravenously 6 h prior infection, then they were infected with  $5 \times 10^5$  CFU/mouse of *L. monocytogenes* intravenously. The numbers of viable *L. monocytogenes* in the spleens (B) and livers (C) at various time points were determined. (D-F) Anti-p60 antibody canceled the effect of p60 on host resistance against *L. monocytogenes* infection in vivo. Mice were administered with 1 mg/mouse of rabbit anti-p60 antibody intravenously, and then they were administered with 100  $\mu\text{g}/\text{mouse}$  of recombinant p60 or PBS as control intravenously 6 h prior infection. Mice were infected with  $5 \times 10^5$  CFU/mouse of *L. monocytogenes* intravenously. (D) The numbers of viable bacteria in the organs were determined on day 3 after infection. The titers of (E) IFN- $\gamma$  and (F) TNF- $\alpha$  in the spleens from p60-administered (open) or control (filled) were determined by ELISA 24 h after infection. An asterisk indicates that the value is significantly different from the control group ( $P < 0.01$ ). (G-K) RAW264.7 cells were prepared to  $1 \times 10^6$  /ml in 24-well plate and stimulated with various doses of recombinant p60 for 24 h. Then cells and culture supernatants were collected and titers of TNF- $\alpha$  (G), IL-12 (H) and IFN- $\beta$  (I) in culture supernatants were determined by ELISA. (J) IRF3 dimerization of protein extract from p60-stimulated cells. RAW264.7 cells were prepared to  $1 \times 10^6$  /ml in 24-well plate and stimulated with 100  $\mu\text{g}/\text{well}$  of recombinant p60 for 12 h. The stimulated cells were lysed and protein was extracted. Native PAGE was carried out and IRF3 dimerization was detected by western blotting.

of p60 on innate immunity. Recombinant p60 was administered intravenously 6 h prior infection, and the numbers of viable *L. monocytogenes* were determined at various time points. p60-injected mice showed the lower bacterial numbers in the spleens (Fig. 1C) and the livers (Fig. 1D) compared with the PBS-injected controls, indicating that administration of p60 enhanced host resistance against *L. monocytogenes* infection in the early phase. Then we assessed whether

antibody against p60 could neutralize the effect of p60 in vivo (Figs. 1D-F). To generate anti-p60 antibody, rabbits were hyperimmunized with recombinant p60 as previously described<sup>13</sup>. Sera were pooled and the immunoglobulin fraction was obtained by precipitation with a saturated ammonium sulfate solution, followed by extensive dialysis against PBS. The concentration of immunoglobulin was determined by Bradford assay. Anti-p60 antibody was administered 24

h prior to p60 administration. *L. monocytogenes* infection was carried out 6 h after p60 injection and bacterial numbers in the organs of mice on day 3 after infection (Fig. 1D), IFN- $\gamma$  titer (Fig. 1E) and TNF- $\alpha$  titer (Fig. 1F) 24 h after infection were determined by ELISA. The enhancement of bacterial elimination from the organs was inhibited by anti-p60 antibody (Fig. 1D). p60 treatment augmented IFN- $\gamma$  and TNF- $\alpha$  production from the spleens of mice infected with *L. monocytogenes*, and anti-p60 suppressed the enhanced production of both cytokines (Figs. 1E, F).

We assessed the effect of recombinant p60 on macrophage responses *in vitro*. Mouse macrophage cell line RAW264.7 was purchased from Dainippon Sumitomo Pharmaceutical Co. Ltd., Osaka, Japan. Cells were cultured in Dulbecco's modified Eagle medium (DMEM, Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% of fetal calf serum (FCS, JRH Biosciences, Lenexa, KS, USA), 3% of L-glutamine (Wako). RAW264.7 cells were stimulated with recombinant p60 and titers of TNF- $\alpha$ , IL-12 and IFN- $\beta$  in the culture supernatants were determined. Production of IL-12 was determined by ELISA described previously<sup>15,16</sup>. Titers of IFN- $\beta$  in samples were determined by mouse IFN- $\beta$  ELISA kit (PBL Biomedical Laboratories, Piscataway, NJ, USA). The significant production of TNF- $\alpha$  (Fig. 1G), IL-12 (Fig. 1H) and IFN- $\beta$  (Fig. 1I) was induced by the treatment with recombinant p60. We confirmed an activation of nuclear factor  $\kappa$ B (NF $\kappa$ B) in p60-stimulated RAW264.7 cells by electrophoretic mobility shift assay (data not shown). For assessment of IRF3 pathway activation, IRF3 dimerization was assessed by native polyacrylamide gel electrophoresis using 10% polyacrylamide gel and transferred to Immobilon<sup>TM</sup>-P transfer membrane (Millipore Corp. Bedford, CA, USA). After blocking, the membrane was incubated with a primary

antibody that is specific to IRF3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Then the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG. Immunoreactive bands were visualized using ECL<sup>TM</sup> Western Blotting Analysis System (GE Healthcare). Stimulation with p60 induced IRF3 dimerization (Fig. 1J). These results showed that recombinant p60 induced TNF- $\alpha$ , IL-12 and IFN- $\beta$  production in mouse macrophages depending on NF- $\kappa$ B and IRF3 pathways. Infection with *L. monocytogenes* induces a host Th1 response, which is a critical in acquired resistance against *L. monocytogenes*<sup>17,18</sup>. TNF- $\alpha$  is a critical factor for host resistance against infection with *L. monocytogenes*, and IL-12 is key cytokine that drives Th1 response<sup>19-22</sup>. And mice lacking receptor of type I IFN exhibited enhanced resistance against *L. monocytogenes* infection<sup>23-25</sup>. These results suggested that p60 induced the expression of cytokines that were involved in both augmentation and attenuation of host resistance against infection with *L. monocytogenes*.

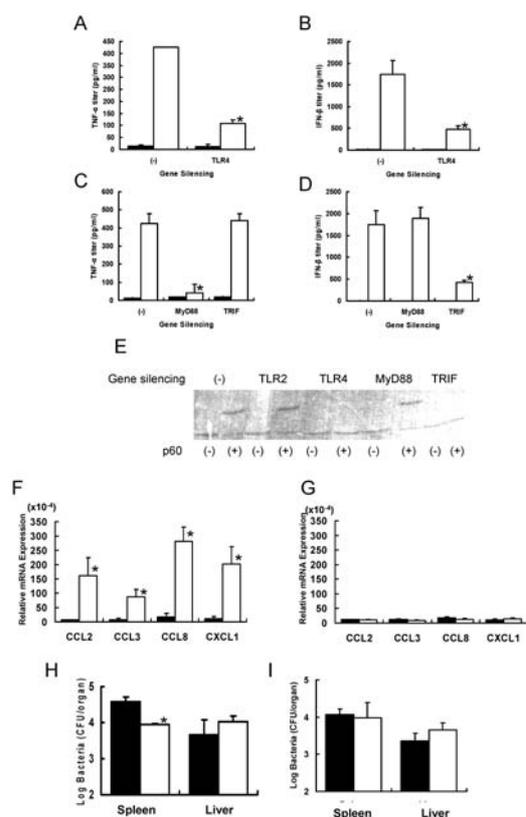
Host cells recognize diverse microbial pathogens by Toll like receptor (TLR)<sup>26</sup>. Next we investigated whether TLR family might be involved in p60-activated signal transduction in mouse macrophages. Pam<sub>3</sub>CSK<sub>4</sub> (EMC microcollections GmbH, Germany) for TLR2, poly I:C (Sigma) for TLR3, Lipid A (Peptide Institute, Inc., Osaka, Japan) for TLR4, and Imiquimod (BIOMOL International, Inc., Plymouth Meeting, PA, USA) for TLR7 were used as TLR agonists. Expression of TLR2, TLR3, TLR4, TLR7, MyD88 and TRIF in RAW264.7 cells was suppressed using small interference RNA (siRNA). siRNAs were purchased from Qiagen, Tokyo, Japan, and gene silencing was carried out using HiPerFect reagent (Qiagen) according to manufacturer's instruction. For confirmation of effect of siRNA, expression of TLRs, MyD88 and TRIF was assessed by reverse transcriptase (RT)-PCR.

Gene silencing of TLR2, TLR3, TLR4, and TLR7 with siRNA successfully suppressed production of TNF- $\alpha$  and IFN- $\beta$  induced by each TLR agonist from RAW264.7 (data not shown). Therefore, we investigated the effect of gene silencing of TLRs on p60-induced cytokine production in mouse macrophages. TNF- $\alpha$  and IFN- $\beta$  production in p60-stimulated cells was suppressed when the expression of TLR4 was silenced (Figs. 2A, B). Knock-down of TLR2, TLR3, or TLR7 showed no change of the production of TNF- $\alpha$  or IFN- $\beta$  (data not shown). Then we investigated whether the TLR4-dependent signal transduction pathway might be involved in p60-stimulated cytokine production. Signals transduction by TLR4 stimulation is mediated by myeloid differentiation factor 88 (MyD88) or Toll/IL-1 receptor domain-containing adaptor-inducing IFN- $\beta$  (TRIF)<sup>26</sup>. Our results showed that suppression of MyD88 expression inhibited TNF- $\alpha$  production in p60-stimulated macrophages, whereas the silencing of TRIF gene expression did not alter (Fig. 2C). Conversely, IFN- $\beta$  production in p60-stimulated macrophages decreased by suppression of TRIF expression but not by suppression of MyD88 (Fig. 2D). Dimerization of IRF3 was inhibited by suppression of the TLR4 expression (Fig. 2E). These results suggested that TLR4 might be involved in cytokine production in mouse macrophages by stimulation with p60.

To confirm that the inducible potential of recombinant p60 for TNF- $\alpha$  and IFN- $\beta$  production might be TLR4-dependent, we investigated cytokine production by stimulation with recombinant p60 in bone marrow-derived macrophage (BMDM) derived from TLR4-deficient C3H/HeJ mice. TLR4-lacking C3H/HeJ and C3H/HeN as the control strain of C3H/HeJ strain were purchased from CLEA Japan. Bone marrow cells were prepared and differentiated to BMDM in RPMI 1640 containing 10% FCS supplemented with 5  $\mu$ g/ml of macrophage

colony stimulating factor (Sigma) for 3 days. Then adherent cells were washed and cultured for 7 days in fresh medium. Cells were detached with 5 mM EDTA in PBS, and used for further assays. We assessed the expression of chemokines including CCL2, CCL3, CCL8 and CXCL1 in the spleens from p60-stimulated mice. Total RNA from samples were prepared using TRIzol reagent (Invitrogen Co., Carlsbad, CA, USA) according to manufacturer's instruction. First-strand cDNAs were synthesized by reverse transcription of 1  $\mu$ g total RNA using random primers (Takara) and reverse transcriptase Moloney murine leukemia virus (Invitrogen). Primers used in this study and protocol for quantitative real time RT-PCR were described previously<sup>27</sup>. All experiments were run in duplicate and non-template controls and dissociation curves were used to detect primer-dimer conformation and nonspecific amplification. The threshold cycle ( $C_T$ ) of each target product was determined and set in relation to the amplification plot of glyceraldehyde-3-phosphate (GAPDH). The detection threshold is set to the log linear range of the amplification curve and kept constant (0.05) for all data analysis. Difference in  $C_T$  values ( $\Delta C_T$ ) of two genes was used to calculate the relative expression [relative expression =  $2^{-(C_T \text{ of target genes} - C_T \text{ of GAPDH})} = 2^{-\Delta C_T}$ ]. The expression of chemokines was enhanced by administration of p60 in C3H/HeN mice (Fig. 2F), but not in TLR4-deficient C3H/HeJ mice (Fig. 2G). These chemokines are involved in chemotaxis or activation of macrophages and neutrophils<sup>28-30</sup>. CCL2 is involved in host resistance against *L. monocytogenes* infection<sup>30</sup>, and CCL3 is also important for Th1 response against *L. monocytogenes* infection<sup>29</sup>. Host resistance against *L. monocytogenes* infection is augmented in mice lacking CXCR3, a receptor for CCL8 and CXCL1<sup>28</sup>. These results suggested that p60 induced the expression of these chemokines in vivo, depending on TLR4.

To investigate whether recombinant



**Figure 2** (A-E) RAW264.7 cells were prepared to  $1 \times 10^6$  / ml in 24-well plates and the expression of TLR4 (A, B), MyD88 or TRIF (C, D) was suppressed by siRNA treatment. Then the cells were stimulated with 100  $\mu$ g/well of recombinant p60 for 24 h. The culture supernatants were collected and the titers of TNF- $\alpha$  (A, C) and IFN- $\beta$  (B, D) in culture supernatants from the PBS-treated (filled) and p60-treated (open) cells were determined by ELISA. (E) IRF3 dimerization by treatment with recombinant p60 in gene-silenced RAW264.7 cells. RAW264.7 cells were prepared to  $1 \times 10^6$  /ml in 24-well plates and the expression of TLRs, MyD88 or TRIF was suppressed by siRNA treatment. Then cells were stimulated with 100  $\mu$ g/well of recombinant p60 for 12 h. The stimulated cells were lysed and extracted proteins were developed by native PAGE. IRF3 was visualized by Western blotting. (F, G) In vivo expression of chemokines in the spleen from p60-treated mice. C3H/HeN (F) or C3H/HeJ (G) mice were treated with 100  $\mu$ g of recombinant p60 intravenously and the spleens were obtained 6 h after p60 administration. Then RNAs of the spleens from PBS-treated control (filled) and p60-treated (open) mice were prepared and the expression of CCL2, CCL3, CCL8, and CXCL1 was determined by quantitative real-time RT-PCR. (H, I) Host resistance against *L. monocytogenes* infection by pre-treatment of p60 in TLR4-deficient mice. C3H/HeN (H) or C3H/HeJ (I) mice were injected with 100  $\mu$ g of recombinant p60 intravenously and  $5 \times 10^5$  CFU/mouse of *L. monocytogenes* was infected 6 h after p60 administration. Spleens and livers from infected mice were obtained 12 h and bacterial numbers in the organs from PBS-treated control (filled) and p60-treated (open) mice were determined. An asterisk indicates that the value is significantly different from the control group ( $P < 0.01$ ).

p60 affects host immune response or not, we assessed the effect of administration of recombinant p60 on host resistance against *L. monocytogenes* infection to TLR4-deficient mice. Bacterial growth in the spleens of C3H/HeN mice administered with recombinant p60 was suppressed compared with the PBS-treated control mice at 12 h (Fig. 2H) after infection. The numbers of *L. monocytogenes* in the organs of TLR4-deficient C3H/HeJ mice showed no significant difference between the p60-treated and PBS-treated groups (Fig. 2I). These results suggested that recombinant p60 enhanced host innate resistance against infection with *L. monocytogenes*, depending on TLR4.

In this study, we demonstrated that recombinant p60 induces both proinflammatory cytokines and type I IFN and that listerial virulence factor p60 modulates host immune responses by up-regulation of both proinflammatory cytokines and chemokines as positive factors and type I IFN as negative factors during *L. monocytogenes* infection.

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