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BACTERIAL INFECTIONS

Ectopic Expression of Human Thymosin β4 Confers Resistance to *Legionella pneumophila* during Pulmonary and Systemic Infection in Mice

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ABSTRACT Thymosin beta-4 (Tβ4) is an actin-sequestering peptide that plays important roles in regeneration and remodeling of injured tissues. However, its function in a naturally occurring pathogenic bacterial infection model has remained elusive. We adopted Tβ4-overexpressing transgenic (Tg) mice to investigate the role of Tβ4 in acute pulmonary infection and systemic sepsis caused by *Legionella pneumophila*. Upon infection, Tβ4-Tg mice demonstrated significantly lower bacterial loads in the lung, less hyaline membranes and necrotic abscess, with lower interstitial infiltration of neutrophils, $CD4^+$, and $CD8^+$ T cells. Bronchoalveolar lavage fluid of Tβ4-Tg mice possessed higher bactericidal activity against exogenously added *L. pneumophila*, suggesting that constitutive expression of Tβ4 could efficiently control *L. pneumophila*. Furthermore, qPCR analysis of lung homogenates demonstrated significant reduction of interleukin 1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α), which primarily originate from lung macrophages, in Tβ4-Tg mice after pulmonary infection. Upon *L. pneumophila* challenge of bone marrow-derived macrophages (BMDM) *in vitro*, secretion of IL-1β and TNF-α proteins was also reduced in Tβ4-Tg macrophages, without affecting their survival. The anti-inflammatory effects of BMDM in Tβ4-Tg mice on each cytokine were affected when triggering with tlr2, tlr4, tlr5, or tlr9 ligands, suggesting that anti-inflammatory effects of Tβ4 are likely mediated by the reduced activation of Toll-like receptors (TLR). Finally, Tβ4-Tg mice in a systemic sepsis model were protected from *L. pneumophila*-induced lethality compared to wild-type controls. Therefore, Tβ4 confers effective resistance against *L. pneumophila* via two pathways, a bactericidal and an anti-inflammatory pathway, which can be harnessed to treat acute pneumonia and septic conditions caused by *L. pneumophila* in humans.

KEYWORDS thymosin β4, *Legionella pneumophila*, pulmonary infection, sepsis, bactericidal, anti-inflammatory

Legionella pneumophila, a causative agent of Legionnaires' disease, is one of the intracellular bacterial pathogens that thrive and replicate inside a eukaryotic cell and cause diseases in humans (1, 2). Intratracheal *L. pneumophila* inoculation in A/J mice leads to exponential bacterial growth in the lungs at 24 to 48 h postinfection, followed by a gradual clearance over the next 5 days, leading to the resolution of pulmonary inflammation similarly to the outcome in immunocompetent patients with Legionnaires' disease (3).

During bacterial infection, various immune cells are recruited to the infection sites through chemotactic migration, which is mediated by bacterial factors such as *N*-formylmethionyl-leucyl-phenylalanine (fMLP) (4, 5). fMLP is either a Gram-negative-bacterium-derived or synthetic potent leukocyte chemoattractant interacting with formyl

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peptide receptor (FPR) on the surface of phagocytic leukocytes, such as polymorphonuclear leukocytes (PMNs) (6–9). Neutrophils contribute to the resolution of *L. pneumophila* infection via their own caspase-1 activation and mature interleukin-18 (IL-18) production in *L. pneumophila*-infected C57BL/6 mice (10). Natural killer (NK) cells are recruited to infection sites and activated by interacting with macrophages to manifest cytotoxicity and produce cytokines such as interferon gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) to fight *L. pneumophila* infections in C57BL/6 mice (11). Moreover, depletion of $CD4^+$ or $CD8^+$T cells in A/J mice significantly decreased bacterial clearance and survival against *L. pneumophila* infection (12), demonstrating a critical role of $CD4^+$ or $CD8^+$T cells in the resolution of *L. pneumophila* infection. However, the function of $CD8^+$ and $CD4^+$ T cells is suppressed by *L. pneumophila*-infected macrophages in C57BL/6 mice through the myeloid differentiation factor 88 (MyD88)-dependent pathway (13), and the chemotactic response of human neutrophils may be inhibited by factors like the major secretory protein (Msp), a zinc metalloprotease of *L. pneumophila* (14, 15). Therefore, collective innate and adaptive immune cell activation is required for the successful resolution of *L. pneumophila* infection.

Tβ4 is a naturally occurring peptide found in many tissues as a major cellular constituent. Tβ4 is an actin-sequestering peptide and has been shown to affect various cellular functions, including cell proliferation, migration, and differentiation (16). For example, Tβ4 stimulates epithelial healing processes and its protein expression was upregulated in murine corneas during reepithelialization to stimulate corneal and epithelial cell migration (17). Furthermore, Tβ4 was shown to possess antimicrobial cytotoxicity against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Staphylococcus epidermidis* (17, 18). Tβ4 administration significantly reduced mortality rates and lowered inflammatory cytokine levels in the blood of a mouse model of lipopolysaccharides (LPS)-induced sepsis (19, 20). Based on these findings, the protective role of Tβ4 is likely to be associated with the combination of antimicrobial and immune modulation functions.

In light of these studies, we hypothesized that overexpression of Tβ4 could provide a therapeutic benefit during the course of pulmonary and systemic infection. To this end, we adopted Tβ4-overexpressing transgenic (Tg) mice and asked if these mice could be protected from pulmonary *L. pneumophila* infection along with their resistance to systemic sepsis, similar to those in immunocompetent patients with Legionnaires' disease. In this study, we found that Tβ4-Tg mice had approximately 100-fold lower bacterial count (CFU) in the lungs than wild-type controls after 3 days of intranasal *L. pneumophila* infection, and the mice also showed higher survival against intraperitoneal challenge by *L. pneumophila*. Our data demonstrate that the resolution of *L. pneumophila* infection during acute pneumonia and sepsis in Tβ4-Tg mice could be associated with both the anti-inflammatory and bactericidal functions of Tβ4.

RESULTS

Tβ4 transgenic mice showed lower number of bacteria (CFU) in pulmonary infection with *L. pneumophila*. To investigate the role of human Tβ4 in a naturally occurring model of pulmonary infection, human Tβ4-transgenic mice that constitutively express the full-length polypeptide were generated as reported previously (21). mRNA and protein expression of human Tβ4 in Tβ4-Tg lungs was validated using real-time PCR analysis and histology studies, respectively (Fig. 1A and B). Real-time PCR analysis of naive wild-type (WT) and Tβ4-Tg lungs demonstrated that the level of Tβ4 mRNA was approximately 13-fold higher than that in WT lungs (Fig. 1A, right). The immunohistochemistry results using anti-thymosin-β4 antibody show that an increased Tβ4 peptide level was detected in lungs of Tβ4-Tg-C57BL/6 mice compared with those of WT mice, as shown in Fig. 1B. Tβ4, a thrombin-releasable peptide, had earlier demonstrated microbicidal activity against *E. coli* and *S. aureus in vitro* (20). Therefore, ectopic expression of Tβ4 in Tβ4-Tg mice was hypothesized to provide protection to the host against pulmonary *L. pneumophila* infection. To test this hypothesis, WT and Tβ4-Tg mice were intranasally infected with an inoculum of 1×10^9 bacterial CFU and the number of

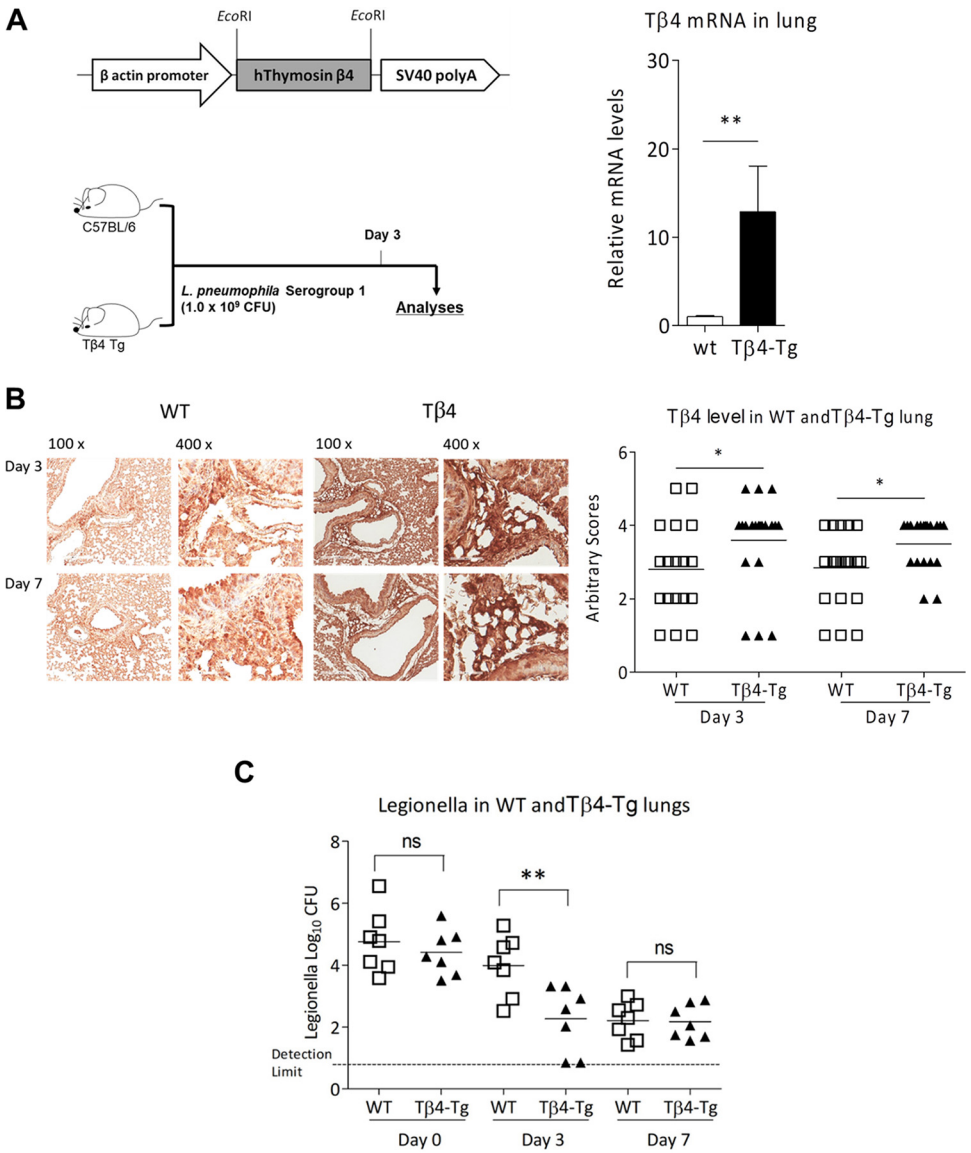

FIG 1 Thymosin $\beta 4$ transgenic (T$\beta 4$-Tg) mice cleared more *L. pneumophila* serogroup 1 (Lpn) during pulmonary infection. (A, upper left) Generation of human thymosin $\beta 4$ transgenic mice. The 1.5-kb human thymosin $\beta 4$ fragment was subcloned into an EcoRI/EcoRI site of ATB vector in pCAGGS. Injectable DNA, including the β actin promoter, human T$\beta 4$ cDNA, and SV40 poly A sequence was microinjected into fertilized mouse eggs of C57BL/6. Injected eggs were transferred into pseudopregnant recipients. Potential T$\beta 4$-Tg founders were analyzed by PCR of mouse tail DNA. (A, lower left) Schematic representation of pulmonary infection by *L. pneumophila* serogroup 1. Wild-type and thymosin $\beta 4$ Tg mice were infected with 1.0×10^9 *L. pneumophila*, then bacterial CFU and immune cell types in individual mice were examined. (A, right) Real-time PCR analysis of T$\beta 4$ mRNA in naive WT and T$\beta 4$-Tg lungs was performed in a LightCycler System (Applied Biosystem). The relative quantity of T$\beta 4$ mRNA was calculated using the $\Delta\Delta C_T$ method and GAPDH RNA level for normalization. The experiment was repeated three times ($n = 4$ mice/group). (B) Immunohistochemistry results of lungs from WT and T$\beta 4$-Tg mice at 3 and 7 days after nasal inoculation of 1×10^9 *L. pneumophila* ($n = 4$ mice/group). Lung slices were stained with rabbit polyclonal anti-thymosin $\beta 4$ antibody. Thymosin $\beta 4$ was visualized using diaminobenzidine (DAB) (brown) and counterstained with hematoxylin. The histology of DAB-stained lungs was examined with under 100× and 400× magnification, and 4 fields were photographed for each lung and scored for levels of T$\beta 4$ (scores from 1 [lowest] to 5 [highest]), then the populations were plotted. (C) WT and T$\beta 4$-Tg mice were infected with 1.0×10^9 *L. pneumophila*. On days 0, 3, and 7 following infection, bacterial CFU counts were determined in the lungs of these WT and T$\beta 4$-Tg mice. The deposition counts of *L. pneumophila* at $t = 0$ were measured from the lung samples harvested at 2 h following intranasal inoculation. The CFU counts of surviving *L. pneumophila* in the WT and T$\beta 4$-Tg mice were compared by pooling the results from two different sets of infection experiments ($n = 4$ mice/group 1, $n = 3$ mice/group 2).

surviving bacterial CFU was measured at 0, 3, and 7 days after infection, and infiltrated immune cells were determined at 3 days postinfection. Compared to WT mice, Tβ4-Tg mice showed a decrease in bacterial count (CFU) at 3 days postinfection, suggesting that Tβ4 exerted antimicrobial activity on pulmonary *L. pneumophila in vivo* (Fig. 1C). Notably, we did not observe a significant difference in the CFU counts in the WT and Tβ4-Tg C57BL/6 mice at 7 days after infection, suggesting that the antibacterial effect of Tβ4 occurs immediately following infection and reaches a maximal level within 3 days. By day 7, *L. pneumophila* clearance was comparable between the WT and Tβ4-Tg mice (Fig. 1C). Therefore, ectopic expression of Tβ4 in the mouse protects hosts from *L. pneumophila*-induced pulmonary infection.

Tβ4-Tg mice show less inflammatory features in the lung. We examined the levels of infiltrated immune cells and structures of lung tissues on days 3 and 7 after intranasal inoculation of *L. pneumophila* (Fig. 2). From hematoxylin and eosin (H&E) staining results, it was apparent that hyaline membranes, which are composed of fibrinous exudate, cellular debris, and red blood cells, had less overflow into Tβ4-Tg lungs compared to that in WT at 3 days after infection (Fig. 2A). Fewer hyaline membranes, composed of proteins and dead cells of the alveoli (the tiny air sacs in the lung), were attached to blood vessels of Tβ4-Tg lungs, and lower interstitial infiltration of inflammatory cells was seen (Fig. 2A). Consistent with low infiltration of immune cells, there were fewer dead cells in Tβ4-Tg lungs on day 7 after infection, thereby leading to lower levels of necrotic abscess (Fig. 2A). Combining all these indicators, relative severity of inflammation was lower in Tβ4-Tg lungs than in WT lungs on days 3 and 7, while overall inflammatory responses faded away after day 7 (Fig. 2A and B). These histological observations were supported by flow cytometry analysis of immune cell types and numbers infiltrated into both WT and Tβ4-Tg lungs (Fig. 2C and D).

Upon fluorescence-activated cell sorting (FACS) analysis of WT and Tβ4-Tg lungs, PMNs, $CD4^+$ T cells, and $CD8^+$ T cells, which are known to be essential to control pulmonary *L. pneumophila* infection in C57BL/6 and A/J mice (10, 12), were found to be relatively less recruited to Tβ4-Tg lungs. This might be associated with the lower pulmonary *L. pneumophila* counts (CFU) and anti-inflammatory function of Tβ4 polypeptide (Fig. 1C and Fig. 2). Reduction in the number of PMNs and natural killer cells was apparent even at 6 h of intranasal *L. pneumophila* infection (Fig. S1 in the supplemental material), indicating that Tβ4 controls inflammatory responses early in the immune response. Therefore, both bactericidal and anti-inflammatory activities of thymosin β4 are correlated with reduced immune cell recruitment to the Tβ4-Tg lungs during pulmonary *L. pneumophila* infection.

Tβ4-Tg mice show lower levels of cytokine gene expression in the lung. When lungs are infected with *L. pneumophila*, both pro- and anti-inflammatory cytokines increase dramatically during the early phase of infection in A/J mice and C57BL/6 mice (22, 23). Since alveolar macrophages are the primary cell type that is infected by *L. pneumophila* in C57BL/6 mice (24), we examined the expression of established cytokines of activated macrophages (IL-1β, IFN-γ, TNF-α, IL-6 [proinflammatory cytokines in activated A/J mice and human macrophages], and IL-10 [anti-inflammatory cytokine in activated A/J mice and human macrophages]) (22, 25). To explore whether these cytokine levels were affected by Tβ4 expression, we examined the mRNA levels of IFN-γ, TNF-α, IL-1β, IL-6, and IL-10, using real-time PCR at 6 h after intranasal inoculation with *L. pneumophila*. As seen in Fig. 3A, Tβ4-Tg lungs showed significantly less IL-1β and TNF-α upon *L. pneumophila* infection. IL-6 and IL-10 also had a tendency toward lower expression in Tβ4-Tg lungs infected with *L. pneumophila*, although the differences were not statistically significant. The level of IFN-γ in Tβ4-Tg lungs was comparable to that of WT at 6 h after pulmonary infection (Fig. 3A). These data suggest that Tβ4 might suppress the mRNA expression of proinflammatory IL-1β, TNF-α, and IL-6, as well as anti-inflammatory IL-10, at an early time point. Nonetheless, Tβ4-Tg mice did not show any difference in the death rates of PMN, alveolar macrophages, and monocytes in their lungs (Fig. 3B). Together, these data demonstrate that reduction in pro-

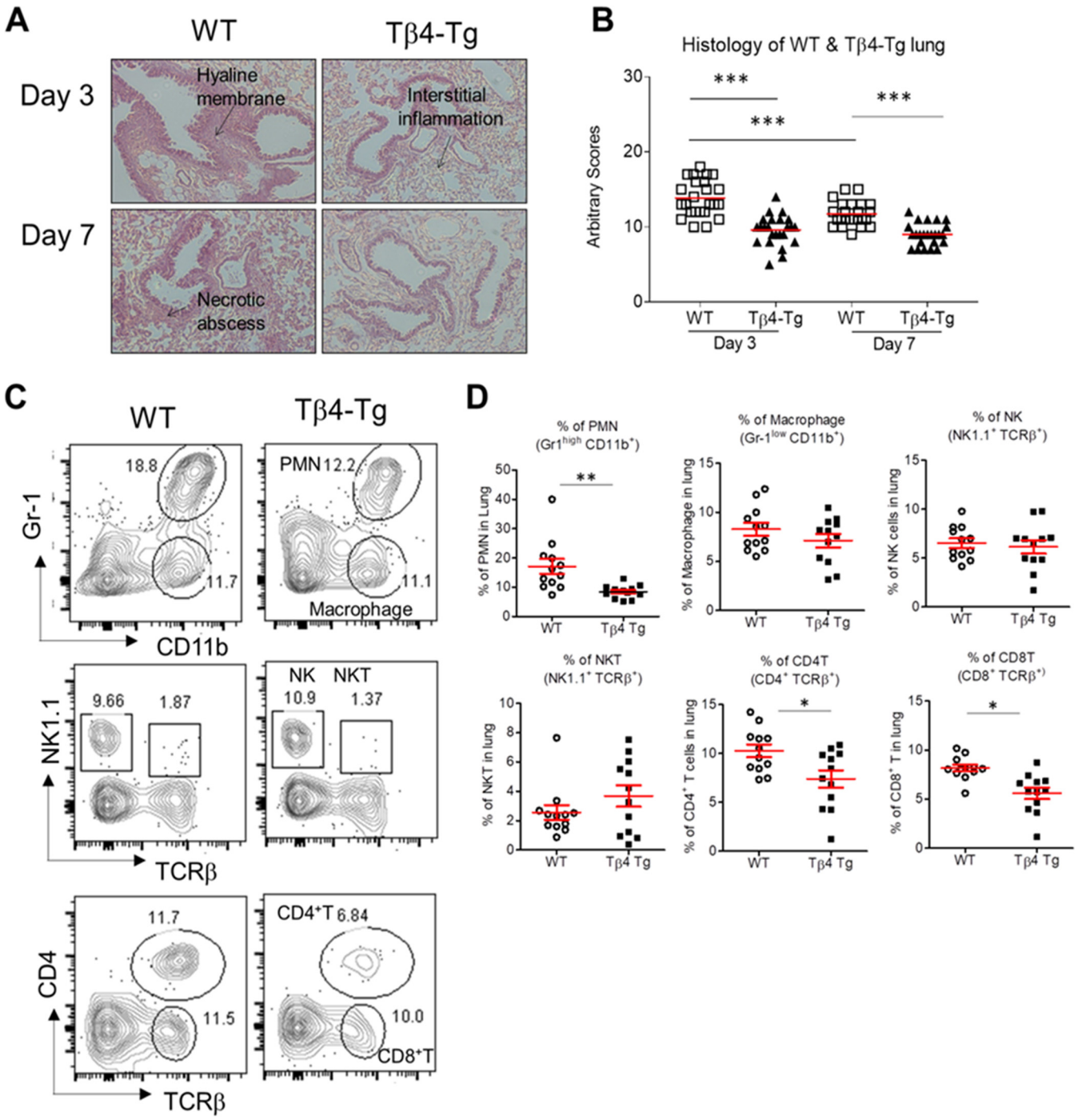

FIG 2 Thymosin β4 Tg mice showed reduced immune cell infiltration in the lung. (A) Histopathology was examined on day 3 and day 7 after *L. pneumophila* lung challenge. WT and Tβ4-Tg mice ($n = 4$ mice/group) were sacrificed, their lungs were formalin fixed and stained with hematoxylin and eosin. Representative figures are shown from each group. (B) H&E-stained lungs were examined by microscope and 6 fields were photographed for each lung and scored for necrotic abscess, hyaline membrane disease, and interstitial inflammation (scores from 1 [lowest] to 6 [highest]). Total scores were between 3 and 18, after which the populations were plotted. (C) Representative flow cytometric analysis of individual cell types in the WT and Tβ4-Tg lungs at 3 days after pulmonary *L. pneumophila* infection. (D) Frequency of each type of immune cell in the lung was calculated and compared between two groups. The data were produced by pooling the three different sets of *L. pneumophila* infection experiments ($n = 12$ mice/group). *, $P < 0.05$ and ***, $P < 0.001$ were considered significant (one-way ANOVA followed by pairwise Tukey test).

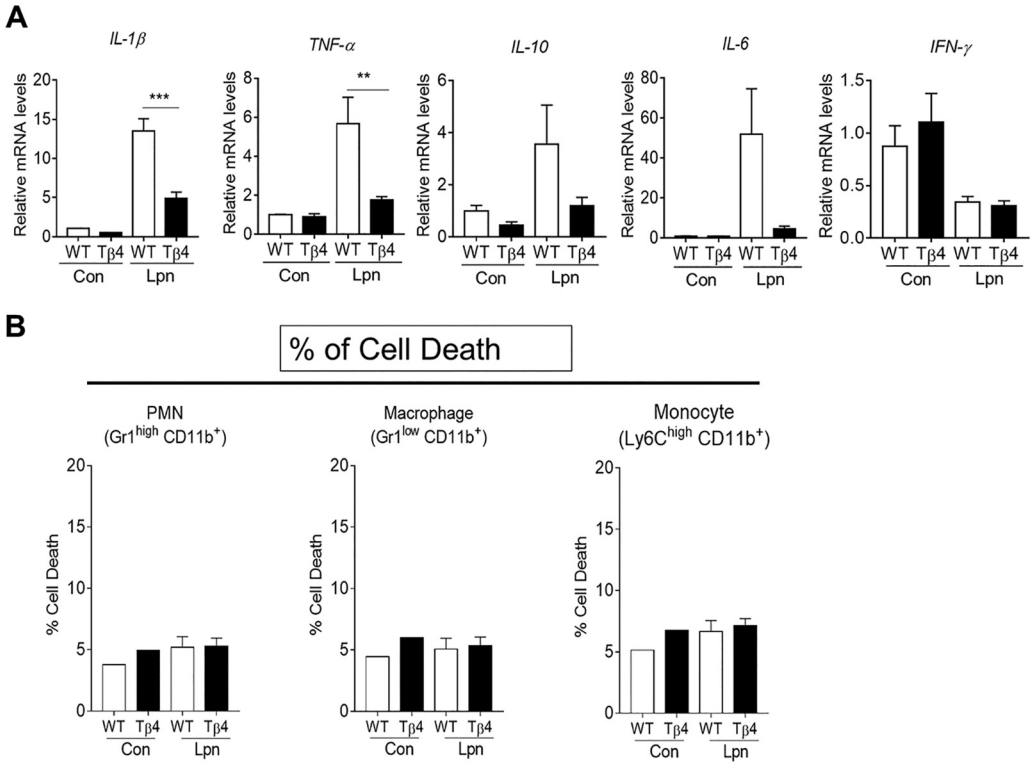

FIG 3 Levels of cytokine mRNAs and cell viability in WT and Tβ4-Tg lungs at 6 h after pulmonary *L. pneumophila* infection. (A) Real-time PCR analyses were performed in triplicate in a LightCycler System (Applied Biosystem). Total RNAs of WT and Tβ4-Tg lungs were extracted using TRIzol reagent at 6 h after pulmonary *L. pneumophila* infection. The relative quantity of each cytokine mRNA was calculated using the $\Delta\Delta C_T$ method and GAPDH RNA level for normalization. The means $\pm$ standard deviations were calculated by pooling the numbers from two different sets of infection experiment ($n = 9$ mice/group). (B) Cell death percentages of immune cells were determined using flow cytometry following incubation of lung cell homogenates with 7-AAD. The data show mean values and standard deviations (SD) obtained from five mice ($n = 5$ mice/group). **, $P < 0.01$ and ***, $P < 0.001$ were considered significant (one-way ANOVA followed by pairwise Tukey test).

and anti-inflammatory cytokines by Tβ4 might contribute to the protection against inflammatory tissue damages by pulmonary *L. pneumophila* infection.

Tβ4-Tg macrophages produced lower levels of cytokines following stimulation with *L. pneumophila* or various Toll-like receptor ligands *in vitro*. We next examined whether thymosin β4 possesses bactericidal function against *L. pneumophila* using naive bronchoalveolar lavage (BAL) fluids harvested from WT or Tβ4-Tg mice in *in vitro* killing assays. An inoculum of *L. pneumophila* was added to both WT and Tβ4-Tg BAL specimens and the remaining CFU was measured under each condition following incubation at 37°C for 1 h and 4 h. As shown in Fig. 4A, Tβ4-Tg BAL fluid killed approximately 30% more *L. pneumophila in vitro* compared to the corresponding WT fluid within 4 h, suggesting that human Tβ4 is actively secreted into the airway lumen of transgenic mice and is likely to mediate *in vitro* killing of *L. pneumophila* by Tβ4-Tg BAL fluid.

Although macrophages from C57BL/6 mice are normally nonpermissive to *L. pneumophila* (26), Tβ4 overexpression might confer an additional bactericidal effect against *L. pneumophila*. To test this, we examined the survival rate of *L. pneumophila* in WT and Tβ4-Tg bone marrow-derived macrophages (BMDM). In a macrophage survival assay, there was no significant difference in the number of surviving bacteria between WT and Tβ4-Tg BMDM, indicating that Tβ4 macrophages did not have enhanced bactericidal activity against *L. pneumophila* (Fig. 4B). During bacterial infection, bacterial components are sensed by inflammasomes that induce caspase-1 activation in macrophages, resulting in cytokine production and cell death (27). In studies using chicken

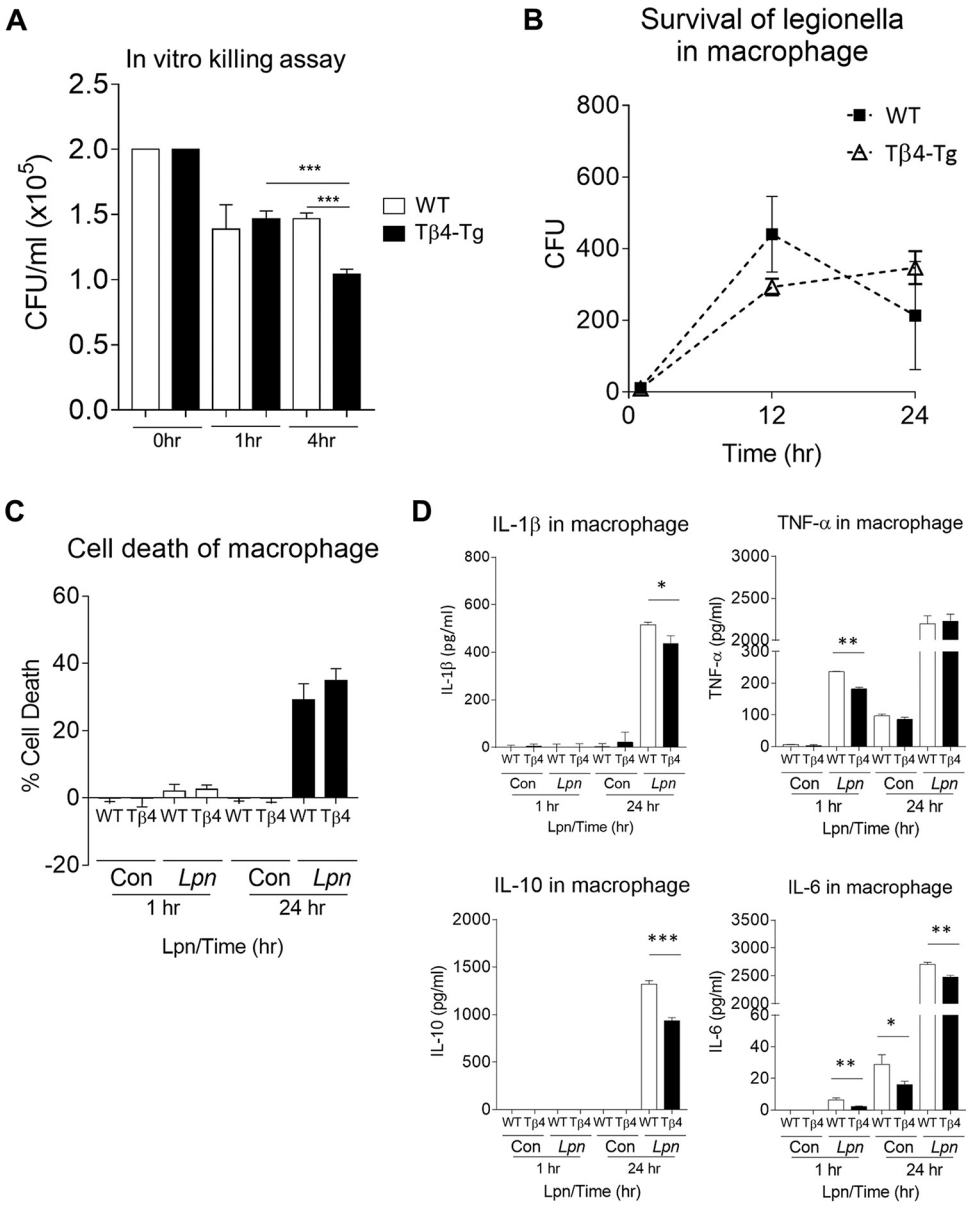

FIG 4 Tβ4-Tg macrophages produced lower levels of cytokines during *L. pneumophila* infection. (A) Tβ4-Tg BAL fluid showed less *L. pneumophila* CFU at 4 h postinfection, indicating that Tβ4 enhanced antibacterial activity. BAL fluid was collected from naive WT and Tβ4-Tg mice, mixed with *L. pneumophila*, and then *L. pneumophila* CFU were determined at 1 h and 4 h postinfection. Each group contained 5 samples ($n = 5$). The experiment was repeated three times. (B) WT and Tβ4-Tg macrophages were infected with *L. pneumophila* at 25 MOI (multiplicity of infection), which showed a more clear survival assay result, and then *L. pneumophila* CFU were counted at the indicated time points. The bacterial CFU were not significantly different at 1 h, 12 h, and 24 h postinfection. The data shown are representative of 3 independent experiments. (C) The cell death rate of infected Tβ4-Tg macrophages was quite similar to that of WT macrophages. The data shown are representative of three independent experiments. (D) Concentrations of IL-1β, IL-10, TNF-α, and IL-6 were determined in supernatants of WT and Tβ4-Tg macrophages using cytokine bead array (BD Biosciences, San Jose, CA) at 1 h and 24 h postinfection. Tβ4-Tg macrophages produced less inflammatory cytokines during *L. pneumophila* infection. The data shown are representative of 3 independent experiments. The error bars are SDs and the data are biological repeats: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ were considered significant (one-way ANOVA followed by pairwise Tukey test).

macrophages, Toll-like receptor (TLR) activation is related to Tβ4 production, suggesting that Tβ4 might play a role in macrophages against *L. pneumophila* infection (28). Based on this, we next determined the cell death rate of, and inflammatory cytokine production from, WT and Tβ4-Tg macrophages during a macrophage survival assay.

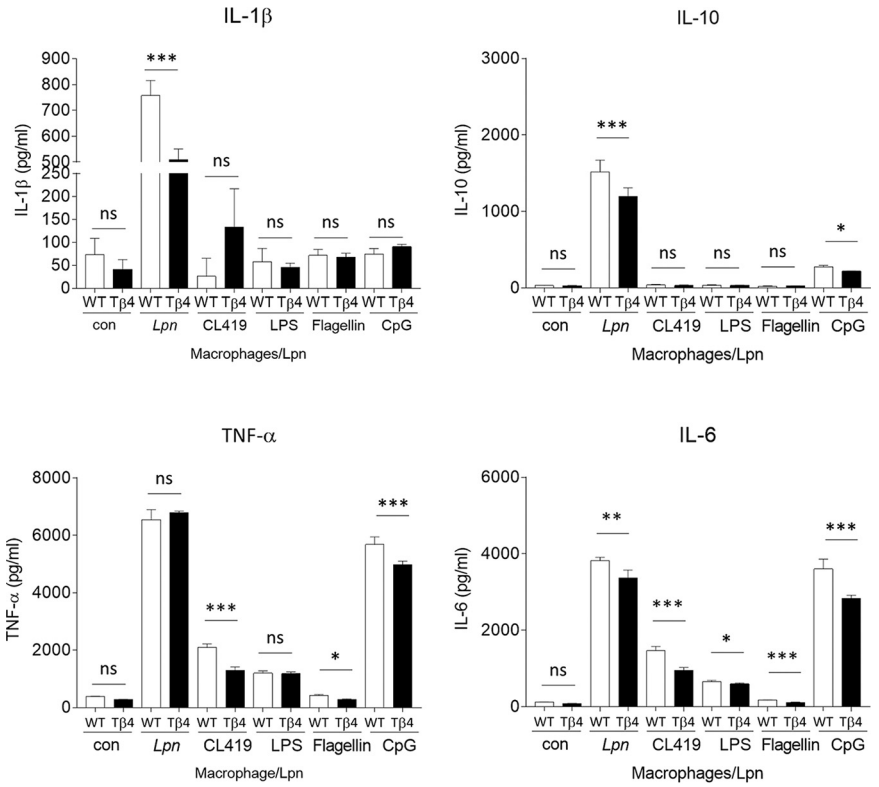

FIG 5 Wild-type and Tβ4-Tg macrophages were infected with *L. pneumophila* or stimulated with tlr2, tlr4, tlr5, and tlr9 ligands (CL419, LPS, flagellin, and CpG). Then, concentrations of IL-1β, IL-10, TNF-α, and IL-6 were determined in supernatants of WT and Tβ4-Tg macrophages using cytokine bead array (BD Biosciences, San Jose, CA) at 1 h and 24 h postinfection. Tβ4-Tg macrophages produced less inflammatory cytokines than WT macrophages during *L. pneumophila* infection, as well as after stimulation with tlr ligands. The data shown are representative of 3 independent experiments. One-way ANOVA was performed, followed by pairwise Tukey test. Significance is represented with asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

While the cell death rate of infected Tβ4-Tg macrophages was quite similar to that of WT (Fig. 4C), reduced levels of IL-1β, IL-10, TNF-α, and IL-6 were found in the supernatants from *L. pneumophila*-infected Tβ4-Tg macrophages (Fig. 4D). The lower cytokine levels in Tβ4-Tg macrophages did not affect the number of surviving bacteria (CFU), implying that *in vivo* killing by Tβ4 was the main contributor to the reduced bacterial count in Tβ4-Tg mouse lungs (Fig. 1C and 4B). In addition, nitric oxide (NO) production was significantly decreased in Tβ4-Tg bone marrow-derived macrophages in response to *L. pneumophila* infection (Fig. S2). Together, these data demonstrate that the anti-inflammatory effect of Tβ4 might have contributed to the protection against inflammatory tissue damages, rather than directly killing *L. pneumophila*.

Given that macrophages provide an intracellular habitat for *L. pneumophila*, we investigated which TLRs are stimulated during *L. pneumophila* infection. To this end, we measured the production of inflammatory cytokines in WT and Tβ4 macrophages after treatment with various TLR ligands *in vitro*. While CpG (tlr9) produced less IL-10, TNF-α, and IL-6 in Tβ4-Tg macrophages, TNF-α and IL-6 were reduced when Tβ4-Tg macrophages were treated with CL419 (tlr2) and flagellin (tlr5) (Fig. 5). LPS (tlr4) decreased only IL-6 in Tβ4-Tg macrophages. Thus, anti-inflammatory effects of Tβ4 are likely mediated by the reduced activation of multiple TLR receptors in Tβ4 transgenic mice during *L. pneumophila* infection. In contrast, the rate of cell death was not significantly different in either WT or Tβ4-Tg macrophages (Fig. S3).

Tβ4-Tg mice were protected from lethal challenge by *L. pneumophila* in the sepsis model. In order to test the function of Tβ4 as a systemic anti-inflammatory agent, we investigated whether Tβ4-Tg mice could be protected from *L. pneumophila*-

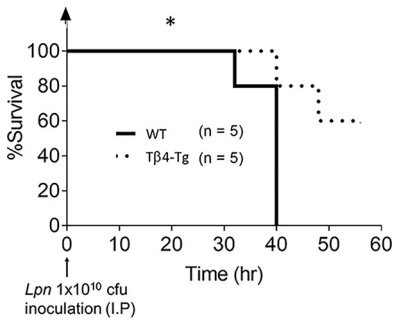

FIG 6 Tβ4-Tg mice showed resistance in the mouse model of *L. pneumophila*-injected sepsis. WT and Tβ4-Tg mice were inoculated intraperitoneally with *L. pneumophila* (1 to 2×10^{10} CFU) to examine the effect of thymosin β4 in *L. pneumophila*-induced septic shock ($n = 5$ mice/group). The experiment was repeated three times. *, $P < 0.05$ was considered significant (log-rank test in GraphPad Prism 5).

induced sepsis. After intraperitoneal injection of *L. pneumophila*, all WT mice died within 40 h of infection. However, 80% of Tβ4-Tg mice survived until 40 h, and 60% were alive until 60 h postinfection. These data, indicating increased resistance to *L. pneumophila* infection in Tβ4-Tg mice, validate the systemic anti-inflammatory effect of Tβ4 (Fig. 6).

DISCUSSION

In this study, we investigated the role of Tβ4 in host defense against *L. pneumophila* in pulmonary infection, as well as in a sepsis model, using Tβ4-Tg mice. Tβ4-Tg mice were found to be more resistant to *L. pneumophila*-induced pulmonary infection and the overexpressed peptide protected the host from *L. pneumophila*-induced sepsis. Tβ4-Tg mice showed approximately 100-fold lower bacterial count (CFU) in the lungs than corresponding WT controls on day 3 after intranasal *L. pneumophila* infection, and their BAL fluid killed more *L. pneumophila in vitro* than that of WT mice. However, this *L. pneumophila* infection model of Tβ4-Tg mice has some limitations in that the model does not provide other scopes, such as loss-of-function, that would be shown in a Tβ4 knockout (KO) mouse model. It is important to study the loss-of-function state for Tβ4 to gain insight into the physiological role of the molecule. We would like to generate Tβ4 KO mice and study the outcome of *L. pneumophila* infection, but that it is beyond the scope of this study. As our Tg studies provide gain-of-function by ectopically introducing human Tβ4 in mice, we can project the therapeutic benefit of Tβ4 in the *L. pneumophila* disease in human. Our data demonstrate that overexpression of human Tβ4 in mice induced antimicrobial and anti-inflammatory functions against *L. pneumophila* infection in the lung, hence providing a scientific rationale for the use of Tβ4 as a therapeutic modality in the clinic. Tβ4-Tg mice ubiquitously expressing constant levels of Tβ4 may have limitations for projecting what would happen in humans in the course of Legionnaires' disease. Although clinical trials using Tβ4 peptides are under way in the treatment of eye injuries, dermal wounds, repair of the heart following myocardial infarction, and healing of the brain following stroke, trauma, or neurological diseases (29), their precise role *in vivo* against *L. pneumophila* infection would be best characterized by generating a loss-of-function mutant or a Tβ4 gene-deficient mice.

Furthermore, fewer immune cells infiltrated into BAL fluids and lungs compared to WT counterparts. *In vitro*, Tβ4-Tg macrophages demonstrated reduced cytokine production against *L. pneumophila* and TLR ligands, although they did not provide any survival advantage to *L. pneumophila*. Together, these data suggest that soluble Tβ4 possesses strong antimicrobial and anti-inflammatory properties that can be harnessed to treat acute pneumonia and sepsis caused by *L. pneumophila* infection in humans. Our data are in line with recent studies that reported that Tβ4 could reduce inflammatory cytokine induction and enhance survival in the mouse model of sepsis (30). Tβ4

supports actin polymerization by sequestering actin monomer and transferring it to profilin. *L. pneumophila* interferes with eukaryotic cells by disturbing actin cytoskeleton dynamics. VipA, an *L. pneumophila* virulence factor, is associated with actin filaments and interferes with organelle trafficking pathways by associating with early endosomes through its N-terminal region, and with actin polymerization via its C-terminal region (31–33). Therefore, it might be assumed that Tβ4 could prevent the function of VipA of *L. pneumophila* at the membrane-proximal level by supporting the actin cytoskeleton.

In addition, Tβ4 suppressed TLR proinflammatory pathways (34) and inhibited neutrophil chemotaxis *in vitro* (35), contributing to the anti-inflammatory effect in various clinically relevant disease models. Thus, the lower bacterial count (CFU) in Tβ4-Tg lungs, together with the anti-inflammatory activity of Tβ4, minimized pulmonary inflammation while simultaneously killing *L. pneumophila* in the infected lungs. In histological studies, hyaline membranes were less obvious in Tβ4-Tg lungs compared to WT lungs. Interstitial infiltration of inflammatory cells was reduced, along with lower levels of necrotic abscess, hence suggesting the protective role of Tβ4 against substantial destruction of pulmonary infrastructure during intranasal *L. pneumophila* infection. Therefore, the lower pulmonary bacterial count (CFU) due to bactericidal activity, as well as the anti-inflammatory effect of Tβ4, leads to less production of proinflammatory cytokines. TNF-α, a potent mediator of inflammation and antimicrobial immunity, is produced mainly by activated macrophages but also by many other cell types, such as $CD4^+$ lymphocytes, NK cells, neutrophils, mast cells, eosinophils, and neurons (36). As shown in real-time PCR analysis and a macrophage survival assay, TNF-α expression was suppressed at the early time points of *L. pneumophila* infection, implying that Tβ4 inhibits TNF-α induction and its downstream NF-κB activation in pulmonary immune cells during *L. pneumophila* infection (36, 37).

In mouse macrophages, *L. pneumophila* is normally sensed by its inflammasome Nlrc4 (Ipaf) and by Naip5, which recognize the 35-amino acid carboxy terminus of flagellin, resulting in caspase-1 activation and *L. pneumophila* clearance in phagolysosomes, whose formation is otherwise avoided by *L. pneumophila* in human monocytes and macrophages, as well as in permissive A/J mice (38–41). Since NO production in macrophages, BALs, and lungs of Tβ4 mice was lower than that in WT (Fig. S2), despite their lower bacterial counts (CFU), the intracellular messenger does not seem to control *L. pneumophila* bacterial loads either in Tβ4-Tg macrophages *in vitro* or in Tβ4-Tg mouse BAL fluids and lungs *in vivo* (42). The lower number of pulmonary *L. pneumophila* counts in Tβ4-Tg lungs could be mainly due to *in vivo* killing of *L. pneumophila* by Tβ4, as already shown in *in vitro* studies of *E. coli* and *S. aureus* (18). However, it is possible that the effect of Tβ4 could be more indirect, by inducing other antibacterial mediators present at larger quantities in BAL fluid from Tg mice. Finally, Tβ4-Tg mice demonstrated resistance to sepsis caused by intraperitoneal *L. pneumophila* injection, thereby proving that Tβ4 protect the mice from systemic shock-induced death. TLR2, TLR4, TLR5, and TLR9 are usually activated by various components of *L. pneumophila* and inhibited by Tβ4 for the production of IL-1β, IL-10, TNF-α, and IL-6. These data suggest that anti-inflammatory effects of Tβ4 are likely mediated via inhibition of TLR receptor activation during *L. pneumophila* infection, primarily by inhibiting overexpression of proinflammatory IL-1β and TNF-α, essential for the induction of sepsis (43, 44). Therefore, Tβ4 cannot only protect mice from *L. pneumophila*-induced acute lung infection, but also functions as an antisepsis agent.

MATERIALS AND METHODS

Mice. Male C57BL/6 mice were purchased from Orient Biotech (Seoul, South Korea). Tβ4-Tg mice were generated as described previously (21). Briefly, a human Tβ4 fragment was subcloned into an EcoRI/EcoRI site of the ATB vector in pCAGGS. Injectable DNA containing mouse *albumin* enhancer/promoter, hTβ4 cDNA, and *SV40* poly A sequences was obtained by the removal of phagemid sequences from pCAGGS. DNA was microinjected into fertilized mouse eggs of C57BL/6. Injected eggs were transferred into pseudopregnant recipients. Potential Tβ4-Tg founders were analyzed by PCR from mouse tail DNA using oligonucleotide primers forward 5′-accatgttcatgccttcttctt-3′ and reverse 5′-gttcaatcgtttctttggaagg-3′. Briefly, genomic DNA was obtained from tail biopsy specimens of each mouse and digested with proteinase K

(20 mg/ml) for 16 h at 55°C. The reaction volume was 25 μl, containing 200 μM each of the regular deoxynucleoside triphosphates (dNTPs), 2.5 units *Taq* DNA polymerase, and 10 pmol of each primer. Thirty-one thermal cycles were performed (denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension for 30 s at 72°C). Eight- to twelve-week-old male mice were used in all experiments under specific pathogen-free conditions according to guidelines from the Institutional Animal Care and Use Committees of Sejong University and Korea University, South Korea (approval number SJ20181103).

Bacteria and infection. *L. pneumophila* serogroup 1 (ATCC 33152, Philadelphia-1) (45) was grown on buffered charcoal yeast extract (BCYE) agar plates at 37°C for 2 to 3 days. The bacteria were washed with sterile phosphate-buffered saline (PBS), diluted to the appropriate bacterial CFU in suspension, and enumerated by spreading the inoculum on BCYE plates. In pulmonary infection, mice were anesthetized and intranasally inoculated with *L. pneumophila* (1.0×10^9) in 20 μl PBS (46–48). In a survival test, after anesthetization mice were intraperitoneally inoculated with *L. pneumophila* (0.5 to 1.0×10^{10}) in 300 μl PBS, and their death and survival were determined over time (46).

Bronchoalveolar lavage, lung homogenates, and bacterial CFU. Five hundred microliters of ice-cold PBS were injected into lungs and collected into 10-ml conical tubes. Pelleted bronchoalveolar lavage (BAL) fluid cells from all four washes were combined to analyze the cells (49, 50). Lavage fluid was incubated for 5 min in ammonium-chloride-potassium (ACK) buffer to lyse contaminating red blood cells and washed with warm Roswell Park Memorial Institute (RPMI) medium. Total BAL fluid cell counts were determined with a hemocytometer. Lungs were removed from mice, halves of them were minced and processed using glass slides to obtain cell suspensions. Suspensions were passed through nylon membrane to remove large pieces of tissues, incubated in ACK buffer, washed in RPMI, and centrifuged at $700 \times g$ for 10 min to collect lung cells at room temperature (51). BAL fluid and lung cells were stained and analyzed by flow cytometry. The bacteria in lung halves were enumerated on plates after serial dilutions, which were both intracellular and extracellular. In an *in vitro* assay using WT and Tβ4-Tg BAL fluids, fluids were collected from both naive WT and Tβ4-Tg mice, then *L. pneumophila* (2×10^5/ml) was added to both WT and Tβ4-Tg (500 μl) and incubated *in vitro* at 37°C with shaking. After 1 h and 4 h incubations, 25 μl of the mixture of *L. pneumophila* and BAL fluid was removed, diluted in PBS, and plated on BYCE plates to enumerate surviving intracellular and extracellular *L. pneumophila* CFU.

Flow cytometry. The immune cells were stained with phycoerythrin (PE) rat anti-mouse Ly-6G (Gr-1) antibody (clone RB6-8C5, eBioscience), Alexa fluor 700 rat anti-mouse CD11b antibody (clone M1/70, eBioscience), APC mouse anti-mouse NK1.1 antibody (clone PK136, BD Biosciences), PE-Cy7 hamster anti-mouse TCRβ chain antibody (clone H57-597, BD Biosciences) and APC-Cy7 rat anti-mouse CD4 antibody (BD Biosciences) for 15 min in fluorescence-activated cell sorting (FACS) buffer (PBS, 1% bovine serum albumin [BSA], and 0.01% NaN_3). FcγR-blocking antibody α-CD16/32 (2.4G2) (Bio X Cell, West Lebanon, NH) was added to prevent nonspecific binding. Stained cells were analyzed with FACS Canto (BD Biosciences, Franklin Lakes, NJ) and data were processed with FlowJo (Tree Star) software. Cytokine concentrations in supernatants from infected WT and Tβ4-Tg macrophages were determined using BD Cytometric Bead Array kit (BD Biosciences, Franklin Lakes, NJ). PMNs, macrophages, and monocytes in lung homogenates were stained using the above fluorochrome-conjugated monoclonal antibodies. Each of the above stained immune cells was further stained with 5 μl of 7-amino-actinomycin D (7-AAD) in 100 μl PBS (BD Biosciences). After a 5-min incubation at room temperature, the stained cells were added with 300 μl PBS and analyzed with FACS Canto (BD Biosciences, Franklin Lakes, NJ) and the data were processed with FlowJo (Tree Star) software.

Real-time PCR. Real-time PCR was conducted as previously described (52). Total RNA of WT and Tβ4-Tg lungs were extracted with TRIzol Reagent (Invitrogen, CA, USA). cDNA was synthesized using a TOPscript cDNA synthesis kit (Enzynomics, Daejeon, South Korea). Real-time PCR was performed with SYBR Green (Bio-Rad, CA, USA) on a StepOnePlus (Applied Biosystems, CA, USA). Gene expression was normalized to the levels of GAPDH mRNA, and relative expression levels were calculated according to the threshold cycle $\Delta\Delta C_T$ method.

Histology. WT and Tβ4-Tg mice were sacrificed on days 3 and 7 after pulmonary infection and their lungs were formalin fixed, embedded in paraffin, and stained with hematoxylin and eosin (H&E). H&E-stained lungs were examined under an Olympus CKX41 inverted microscope (Olympus, Tokyo, Japan); six fields were photographed for each lung out of 4 mice in each of 4 groups (WT_3D, WT_7D, Tβ4-Tg_3D, and Tβ4-Tg_7D) and scored for the severity of inflammation. We used a scoring system based on the methods published in a recent report by Straughen et al., with slight modification (53). Three categories (necrotic abscess, hyaline membrane disease, and interstitial inflammation) were examined to measure lung histopathological scores. Scores range from 1 (the least severe inflammation) to 6 (the most severe inflammation). Scores from 3 categories (necrotic abscess, hyaline membrane disease, and interstitial inflammation) were summed so that the maximum score (the most severe inflammation) for each field would be 18. The total severity scores of 3 categories ranging between 3 and 18 were plotted.

Lung slices were stained with rabbit polyclonal anti-thymosin β4 antibody (Abcam, Cambridge, MA, USA) or rabbit polyclonal anti-*L. pneumophila* antibody (Invitrogen, Carlsbad, CA, USA) and then thymosin β4 and *L. pneumophila* were visualized using diaminobenzidine (DAB) (Thermo Scientific, Rockford, IL, USA) and counterstained with hematoxylin. At days 3 and 7, the histology of DAB-stained lungs was analyzed under 100× and 400× magnification and 4 or 2 fields were photographed for each lung and scored for levels of Tβ4 or *L. pneumophila* (scores from 1 [lowest] to 5 [highest]), after which the populations were plotted.

Macrophage survival assay and *in vitro* survival assay. Bone marrow-derived macrophages (BMDMs) were prepared by culturing bone marrow from tibia and femur of mice in RPMI with macrophage colony-stimulating factor (M-CSF) for 5 to 6 days (54). BMDMs were plated at 1×10^5 per well in

96-well plates overnight, infected with *L. pneumophila* in various multiplicity of infections (MOIs) for 1, 12, and 24 h, and lysed with Tween 20 in H_2O for enumeration of surviving intracellular and extracellular bacteria at the indicated time points. We show data using one of the various MOIs, 25 MOI, which showed more clear survival assay results. Supernatants of infected macrophages were frozen to determine cytokine levels using flow cytometry and cell death using a lactate dehydrogenase (LDH) assay kit (TaKaRa-Clontech, Shiga, Japan).

Measurement of cytokine levels using cytometric bead arrays. BMDMs were plated at 1×10^5 per well in 96-well plates overnight, then infected with 25 MOI *L. pneumophila* prior to treatment with various TLR ligands, CL419 (tlr2) (1 μg/ml), LPS (tlr4) (100 ng/ml), flagellin (tlr5) (100 ng/ml), or CpG (tlr9) (500 nM). Cytokine concentrations in the supernatants of the control-infected or TLR ligand-treated infected WT and Tβ4-Tg macrophages were determined at 24 h using BD Cytometric Bead Array kits (BD Biosciences, Franklin Lakes, NJ).

Statistical analysis. One-way analysis of variance (ANOVA) followed by pairwise Tukey test was used to determine the statistical significance of the results. Data points represent average $\pm$ standard deviation (S.E.M.); *, $P \leq 0.05$; **, $P \leq 0.01$; and ***, $P \leq 0.001$ were considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.3 MB.

SUPPLEMENTAL FILE 3, PDF file, 0.3 MB.

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We report no conflicts of interest.

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