의학박사 학위논문

# Study of type I interferon responses during *Orientia tsutsugamushi* infection

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# ABSTRACT

Type I Interferons (TI IFNs) induced by microbial infections play a pivotal role in protective immune responses. Recently, various bacterial pathogens have been shown to cause TI IFN production. However, the effect of TI IFNs induced by bacteria on immune responses is quite controversial. *Orientia tsutsugamushi*, an obligate intracellular bacterium that causes scrub typhus, can also induce TI IFNs during mammalian infection, but the effect of TI IFNs on immune responses against *O. tsutsugamushi* remains elusive. Here, I studied the molecular details of the induction of TI IFNs during *O. tsutsugamushi* infection *in vitro* and the role of TI IFNs in generating adaptive immune responses against the bacterial pathogen *in vivo*. Induction of TI IFNs during *O. tsutsugamushi* infection was consistently observed in mouse embryonic fibroblasts (MEF) and bone marrow-derived macrophages (BMDM). Using various MEF and BMDM derived from specific transgenic knockout (KO) mice, I searched for signaling molecules required for induction of TI IFNs by *O. tsutsugamushi* infection. Studies showed that IKK-α, RIG-I, cGAS, MAVS, and STING are required for the induction of TI IFNs, but signaling adaptors involved in TLR and NOD signaling are dispensable. *In vitro* replication of *O. tsutsugamushi* in MEF or BMDM deficient in a gene encoding the TI IFN receptor

(IFNAR) did not significantly change when compared to wild type cells. In addition, the survival rate of TI IFNAR KO mice lethally challenged with *O. tsutsugamushi* was similar to that of wild type mice, indicating that T1 IFN responses do not play a significant role in protective immunity during primary infection of *O. tsutsugamushi*. Nevertheless, it is interesting to note that early antibody responses against 56 kD type specific antigen (TSA56), a major outer membrane protein, were significantly delayed in IFNAR KO mice when compared to wild type. These findings correlate with a delayed alteration of follicular architecture in the spleen as well as a delay in active differentiation of follicular helper T ($T_{FH}$) cells in the spleen of IFNAR KO mice infected with *O. tsutsugamushi,* suggesting that T1 IFNs may affect the generation of specific antibody responses via promoting the differentiation of naïve $CD4^+$ T cells into $T_{FH}$ cells in secondary lymphoid organs. Also, $CD8^+$ memory T cell responses against *O. tsutsugamushi* antigen are significantly reduced in IFNAR KO mice whereas $CD4^+$ memory T cell responses remain intact. Therefore, T1 IFNs produced by *O. tsutsugamushi* infection do not significantly affect protective immunity during primary infection, but they might promote the generation of specific adaptive immune responses by supporting the differentiation of $T_{FH}$ cells as well as enhancing the generation of $CD8^+$ memory T cell responses.

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# INTRODUCTION

Interferons (IFNs), discovered by Isaacs and Lindenmann in 1957 (Isaacs and Lindenmann 1957), play a significant role in both innate and adaptive immune responses. IFNs are classified into three groups, type I, II and III, based on their receptors. Type 1 interferons (TI IFNs), which include IFN-α, -β, -ω, -κ, -τ, and – ζ, bind a heterodimeric transmembrane receptor termed the IFNα receptor (IFNAR) (Ivashkiv and Donlin 2014), and are involved in anti-viral immune responses by inhibiting viral replication in host cells (Pestka 2007). TI IFNs also regulate innate and adaptive immune responses through direct and indirect mechanisms that affect the activation, migration, differentiation, and survival of multiple subsets of immune cells (Fuertes et al. 2013). IFN-α and -β are expressed in various cell types, whereas the other TI IFNs are induced in restricted subsets of cell types and species. IFN-γ, the only TII IFN is recognized by interferon γ receptor (IFNGR), and also contributes to diverse innate and adaptive immune responses. TII IFNs are produced by specialized subsets of immune cells such as $CD4^+$ and $CD8^+$ T cells, NKT cells, and NK cells (Boehm et al. 1997). IFN-γ activates innate immune cells such as macrophages and promotes cell-mediated T cell responses (Nathan et al. 1984, Lees 2015). TIII IFNs, currently comprised of IFN-$\lambda_1$, $\lambda_2$, $\lambda_3$, and $\lambda_4$have recently been identified, and they signal through receptors containing IFNLR1 and IL-10R2 (Kotenko et al. 2003). TIII IFN receptors has a more restricted expression and the functional role of TIII IFNs are currently poorly defined (Borden et al. 2007).

Expression of TI IFNs is initiated when cells recognize diverse pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs) (Kawai and Akira 2006). PRRs include Toll-like receptors (TLRs) and various intracellular biosensors (Figure 1A) (Akira, Uematsu, and Takeuchi 2006). TLRs are mainly expressed in myeloid immune cells, such as macrophages and dendritic cells, and are associated with plasma or endosomal membranes. Among the TLRs, TLR4 is present in the plasma membrane and recognizes bacterial lipopolysaccharide (LPS) to trigger TI IFN signaling through TIR-domain-containing adapter-inducing interferon-β (TRIF) (Yamamoto et al. 2003). TLR3 also recognizes dsRNAs in endosomes and transmits signals through an interaction with TRIF to induce TI IFNs. ssRNAs and DNAs present in endosomes are sensed by TLR7 or TLR9, which signals through myeloid differentiation primary response gene 88 (MyD88) for the production of TI IFNs (Kawai and Akira 2007). These endosome-associated TLRs induce TI IFN expression in an endosomal acidification-dependent manner (Kawai and Akira 2007). Nucleotide-binding oligomerization domain-containing proteins (NODs) are another family of intracellular receptors that are involved in the recognition of peptidoglycan moieties such as D-glutamyl-meso-diaminopimelic acid (DAP) or muramyldipeptide (MDP) (Moreira and Zamboni 2012). NOD1 and NOD2 are expressed in the cytosol of many cell types and induce inflammatory cytokines including TI IFNs upon binding to PAMP ligands via the activation signaling pathways dependent on receptor-interacting serine/threonine-protein kinase 2 (RIP2) and mitochondria anti-virus signaling protein (MAVS)

(Moreira and Zamboni 2012). TI IFN responses can also be induced by RNAs primarily derived from intracellular pathogens, via recognition by several intracellular PRRs such as retinoic acid-inducible gene 1 (RIG-I) and Melanoma Differentiation-Associated protein 5 (MDA5) (Kawai and Akira 2007). RIG-I and MDA5 utilize MAVS as a signaling adaptor to initiate T1 IFN responses. In contrast, TI IFN activation in response to cytosolic DNAs is mediated by stimulator of interferon genes (STING), an endoplasmic reticulum (ER)-localized protein (Ishikawa and Barber 2008). STING dimerizes and promotes the activation of TANK-binding kinase 1 (TBK1), which phosphorylates the transcription factor IRF3 to initiate TI IFN expression. Cytosolic DNAs are recognized by various PRRs including interferon-inducible protein 16 (IFI16) in human and IFI204 in mouse (Patrick, Bell, and Watson 2016). STING is also a direct sensor of prokaryotic cyclic dinucleotides, as well as cyclic GMP–AMP (cGAMP), which is synthesized following DNA binding to the cytosolic receptor cGAMP synthase (cGAS) or secreted by bacterial pathogens (Hornung et al. 2014) .

Each PRR recognizes specific ligands derived from pathogenic microorganisms and shares signaling adaptor molecules with other PRRs (Figure 1A). The signaling cascades required for TI IFN induction generally merge at the TBK1 complex and IκB kinase (IKK) complex, which activate the transcription factors, interferon regulatory factors (IRFs) and NF-κB, respectively (Figure 1A). The NF-κB pathway is a core transcription factor involved in the production of proinflammatory cytokines such as TNF-α and IL-6.

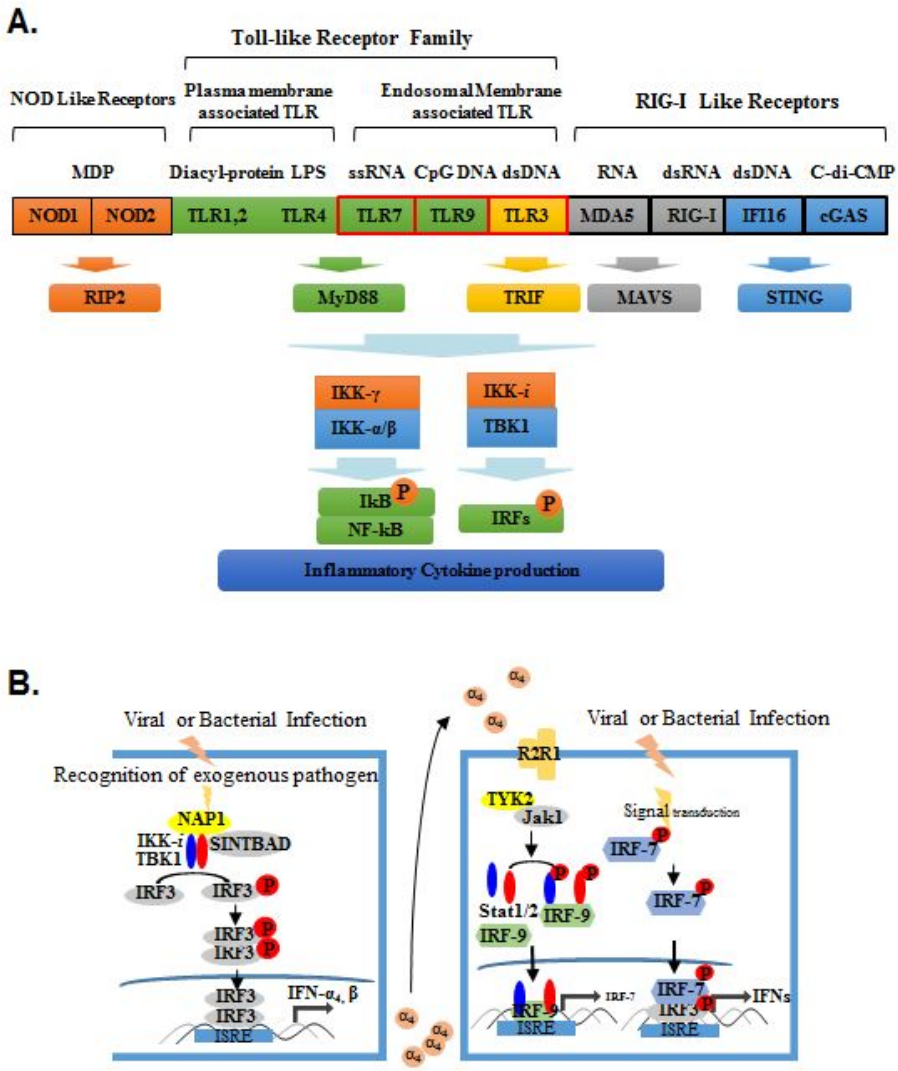


**Figure 1. Pattern Recognition Receptors (PRRs) and down-stream signaling pathways involved in the production of TI IFNs. (A) PRRs recognize specific ligands derived from microorganisms and their downstream signaling pathways induce inflammatory cytokines. (B) T1 IFNs induced by pathogen infections can amplify TI IFN responses via autocrine and paracrine manners through sequential signaling from TI IFN receptors.**

Upon PAMP recognition by PRRs, TNF receptor-associated factor 6 (TRAF6) and TAK1-mediated pathway activates the NEMO/IKK-α/β complex, which induces phosphorylation and polyubiquitination of IκB, resulting in the degradation of IκB and activation of NF-κB (Kawai and Akira 2007). For the production of TI IFNs, the TBK-1/IKKε complex specifically induces phosphorylation and nuclear translocation of IRFs, which in turn promote the transcription of TI IFNs (Kawai and Akira 2007). Upon primary stimulation, TI IFNs, especially IFN-$\alpha_4$ and β, are secreted by pathogen-infected cells and bind to IFNAR, which is composed of IFNAR1 and IFNAR2 subunits (Fensterl and Sen 2009). The association of TI IFNs with IFNAR induces hetero-dimerization of signal transducer and activator of transcription (STAT) 1 and 2 via the Jak/STAT pathway and complex formation of IRF9 and STAT1/2 heterodimer (Trinchieri 2010). This transcriptional complex moves into nucleus, binds to IFN-stimulated response element (ISRE) and further induces T1 IFN responses and IFN-inducible genes.

Even though the functional roles of TI IFN responses during viral infection have been well established, the effect of TI IFNs induced by bacterial infections is still controversial. In 1963, Sueltenfuss and his colleagues showed that TI IFNs induced by duck hepatitis virus inhibits replication of *Chlamydia psittaci* in chicken embryos, suggesting that TI IFN responses might inhibit the replication of intracellular bacteria (Sueltenfuss and Pollard 1963). Since then, a number of groups have reported that TI IFN responses could be either beneficial or detrimental to *in vivo* bacterial infections (Trinchieri 2010, McNab et al. 2015). For example, IFNAR KO

mice are more susceptible to *Streptococcus* and *Staphylococcus* infection compared to wild type mice (Mancuso et al. 2007, Martin et al. 2009), whereas *Listeria monocytogenes* and *Salmonella enterica* Typhimurium have decreased survival rates in IFNAR KO mice (O'Connell et al. 2004, Robinson et al. 2012). In patients co-infected with influenza virus and *Streptococcus pneumonia*, morbidity and mortality are significantly increased when compared to patients infected with only *Streptococcus pneumonia* (Nakamura, Davis, and Weiser 2011), suggesting that TI IFN responses amplified by viral co-infection might increase susceptibility to subsequent bacterial infection. Nevertheless, the detailed mechanisms of how TI IFN responses contribute to immune responses against bacterial infections still remain elusive.

*Orientia tsutsugamushi*, the causative agent of scrub typhus, is an obligate intracellular bacterium belonging to the *Rickettsia* family (Min et al. 2008). Scrub typhus is endemic throughout the Asia-Pacific rim, extending from Russia Far East and Korea in the north, to northern Australia in the south, Afghanistan in the west, and Japan and the western Pacific islands in the east (Kelly et al. 2009). Since the report of thousands of scrub typhus cases during World War II (Alexander et al. 1972), it has been estimated that one million new cases of scrub typhus arise annually in the endemic region (Valbuena and Walker 2012). In addition, the rapid increase of scrub typhus incidence as well as sporadic outbreaks in endemic countries, make it a serious public health issue (Kelly et al. 2009, Paris et al. 2013).

Even though the clinical symptoms of scrub typhus are quite variable, fever, headache, myalgia, and rash are common in the early stage of the

disease. Early diagnosis and proper antibiotic treatment with doxycycline or chloramphenicol can resolve infection within a week. However, scrub typhus patients often develop severe complications, such as respiratory distress syndrome, acute renal failure, meningitis, gastrointestinal tract bleeding, and multiorgan failure, due to delayed diagnosis and antibiotic treatment (Paris et al. 2013, Kelly et al. 2009). Several studies also report that scrub typhus cases are poorly responsive to antibiotics (Watt et al. 1996). Despite the wide range of approaches that have been attempted in the past 70 years, all have failed to develop an effective prophylactic vaccine (Ha et al. 2015).

The primary pathogenesis of scrub typhus is mediated by broad vasculitis and perivasculiti, accompanying infiltration of mononuclear cells into multiple organs, including the skin, lung, brain, kidney, and heart (Moron et al. 2001). Histological analysis of fatal human cases reveal disseminated bacterial infection in endothelial cells and associated inflammation in the affected organs (Moron et al. 2001). The increase of $CD8^+$ T cells (Ikeda, Takahashi, and Yoshida 1994), as well as rise of granzymes, IFN-γ, and IP-10 (de Fost et al. 2005), in recovered patients suggest that cytotoxic T cell responses might play an important role in the removal of infected cells during the acute stage of scrub typhus. Recently, it was also shown that massive T cell apoptosis was detected in the acute phase of scrub typhus and a preferential increase of $CD8^+$ T cells with activated phenotypes was observed in both acute and convalescent phases (Cho et al. 2012). According to a recent immunohistochemical analysis of eschar lesions in patients, antigen-presenting phagocytes might initiate inflammatory responses during the acute

phase of *O. tsutsugamushi* infection (Paris et al. 2012). Interestingly, *O. tsutsugamushi* can target dendritic cells to exploit the sentinel cells as replication reservoirs and delay or impair the functional maturation of DCs during bacterial infection (Choi et al. 2013). In addition, it has been reported that the levels of proinflammatory cytokines, such as TNF-α, IFN-γ and IL-1β, were elevated in patients' sera in a manner that correlated with disease severity during the acute stage (Iwasaki et al. 2010, Kramme et al. 2009, Iwasaki et al. 1997). Consistently, higher levels of chemokines (MIP-1α, MIP-2, and MCP-1) and cytokines (IFN-γ, IL-12, IL-10, and TNF-α) have been observed in more susceptible mice (C3H/HeN) when compared to resistant mice (BALB/c) during bacterial infection (Ge and Rikihisa 2011, Valbuena and Walker 2012). In addition to the pro-inflammatory responses, induction of TI IFNs and IFN-stimulated genes has been reported in dendritic cells and monocytes infected with *O. tsutsugamushi* (Gorvel et al. 2014, Tantibhedhyangkul et al. 2013, Tantibhedhyangkul et al. 2011). Nevertheless, the molecular details of how TI IFNs are induced by *O. tsutsugamushi* infection have never been studied. In addition, the specific role of TI IFNs during bacterial infection remains poorly defined (Hanson 1991). Therefore, I studied the molecular mechanisms and signaling pathways involved in the production of TI IFNs and the specific role of TI IFN responses, particularly focusing on the regulation of specific adaptive immune responses, during *O. tsutsugamushi* infection. Studying the role of TI IFN responses during *O. tsutsugamushi* infection may not only expand our understanding of the immunopathogenesis of scrub typhus but may also help us to develop better

therapy and preventive vaccine.

# MATERIALS AND METHODS

## 1. Ethics statement

Animal experiments were approved by the Seoul National University Institutional Animal Care and Use Committee (SNU IACUC, Permit No. SNU-100414-1) and performed in strict accordance with the recommendations in the National Guideline for the care and use of laboratory animals.

## 2. Mice

IFN alpha Ro/o 129/Sv/Ev mice (B&K universal Ltd, Hull, UK) were back-crossed with C57BL/6J more than 7 generations. C57BL/6 IFNAKR KO and C57BL/6 mice (Orient Bio, Seongnam, Korea) were housed and maintained in the specific pathogen-free facility at Seoul National University College of Medicine. C57BL/6 MyD88, TRIF, or RIP2 KO mice were graciously provided by Dr. Jong-hwan Park in Kun-yang University. C57BL/6 MAVS KO mice were provided by Dr. Shizuo Akira in Osaka University.

## 3. Cell culture

L929 cells (mouse fibroblast, CCL-1) were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco Invitrogen, Grand Island, NY, USA)

containing 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) (Gibco Invitrogen, Grand Island, NY, USA), 100 μg of streptomycin per ml, 100U of penicillin per ml in humidified 5% CO2 atmosphere at 37 ˚C.

## 4. Preparation of mouse embryonic fibroblasts

Mouse embryonic fibroblasts (MEF) were isolated from C57BL/6J, IFNAR KO, and MAVS KO mice. Embryos were isolated at E13.5 and were chopped and treated with 0.5% Trypsine-EDTA for 5min at 37˚C. Then, trypsinized cells were washed three times with DMEM containing 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) (Gibco Invitrogen, Grand Island, NY, USA), 100 μg of streptomycin per ml, 100U of penicillin per ml. Washed cells were resuspended with DMEM containing 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) (Gibco Invitrogen, Grand Island, NY, USA), 100 μg of streptomycin per ml, 100U of penicillin per ml and incubated in a humidified 5% $CO_2$ atmosphere at 37°C. Isolated MEFs were subcultured every other day.

## 5. Preparation of bone marrow-derived macrophages

Bone marrow-derived macrophages (BMDM) were generated from the bone marrow of 6- to 12-week-old C57BL/6J Wild type, IFNAR, MyD88, TRIF, or RIP2-deficient mice. The bone marrow cells were flushed out of the femurs and tibias with serum-free DMEM. The single cell suspension was then filtered through a nylon cell strainer (70-μm Nylon mesh; BD Pharmingen, Franklin Lakes, NJ, USA), washed twice with serum-free DMEM. Bone

marrow cells from the femur and tibia were cultured for 4 days in 10% L929 culture media (as a source of M-CSF)-containing DMEM containing 4 mM glutamine and 10% FBS. In every infection study, we confirmed that more than 90% of the cells were infected with *O. tsutsugamushi* after 2 h of incubation.

## 6. Preparation of *O. tsutsugamushi*

The Boryong strain of *O. tsutsugamushi* was purified using a modified Percoll gradient purification method (Brass et al. 2009). *O. tsutsugamushi* was propagated in L929 cells. At 3 to 4 days postinfection, infectivity was determined using an indirect immunofluorescence assay. When more than 90% of cells were infected, cells were harvested by centrifugation at 500× *g* for 4 min. The cell pellet was resuspended with 6.5 ml of Tris-sucrose (TS) buffer (33 mM Tris-Cl [pH 7.4], 0.25 M sucrose) and the cells were homogenized using 100 strokes of a Polytron homogenizer (Wheaton Inc., Millville, NJ, USA), followed by centrifugation at 200× *g* for 5 min. The supernatant was stored in liquid nitrogen until use. To determine the infectivity titer of the inoculum, L-929 cell layers in 24-well tissue culture plates were inoculated with fivefold serially diluted rickettsia samples. After 3 days of incubation, the cells were collected, fixed, and stained as described below. The ratio of infected cells to counted number of cells was determined microscopically, and Infected-cell-counting units (ICU) were calculated as follows: [(total number of cells used for infection) × (percentage of infected cells) × (dilution of the *O. tsutsugamushi* suspension)]/100. For infection assays, $1.0 \times 10^7$ ICU

of *O. tsutsugamushi* were used to infect cells cultured in 6-well plates containing $1.0 \times 10^6$ host cells.

## 7. Immunofluorescence assay

*O. tsutsugamushi* was stained with monoclonal antibody (KI-37) against p56 major outer membrane. Immunofluorescence assays were performed as described previously (Brass et al. 2009). In brief, the infected monolayer of cells was washed three times with PBS, fixed in PBS containing 4% paraformaldehyde for 10 min at room temperature, and permeabilized in a 0.2% Triton X-100 solution. Subsequently, cells were incubated for 30 min at 37°C with primary antibodies. After incubation, cells were washed three times and incubated at 37°C with anti-mouse IgG conjugated with Alex488 (Invitrogen, Grand Island, NY, USA) diluted in PBS. After incubation, cells were washed three times and covered with immersion oil (Vector Lab, CA, USA). Cells were examined under an Olympus FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan). Images of cell sections were analyzed and processed using the Olympus Fluoview software (Olympus, Tokyo, Japan).

## 8. siRNA knockdown

MEFs were transfected using 20nM of nonsilencing control siRNA or siRNA against cGAS (Sense: CUG AAC ACU GGC AGC UAC U and Antisense A GUA GCU GCC AGU GUU CAG) or IF16 (Sense: UGG GCA AAC UGA UUG CGU U (dTdT) and Antisense: A ACG CAA UCA GUU UGC CCA

(dTdT)) and Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA). 18 hours after transfection, the media was replaced with complete DMEM and transfected cells were maintained for 2 days. After incubation, cells were infected with *O. tsutsugamushi* at 1450 x g for 5 min and harvested for RNA extraction after infection.

## 9. Immunoblot analysis

Cells were lysed with lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), and 2 mM EDTA). Cellular proteins separated by SDS–polyacrylamide gel electrophoresis were electrotransferred to PVDF membranes and subjected to immunoblot analysis using the indicated antibodies. Anti-IκB (L35A5, Cell signaling Technology, Danvers, MA, USA), anti-actin (C4, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), Horseradish peroxidase–conjugated anti-mouse or rabbit antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), were used for immunoblot analysis. The immune-reactive bands were detected using enhanced chemiluminescence reagents (Ab frontier, Seoul, South Korea). Signal intensities were analyzed by Quantity One software (Bio-Rad, Hercules, CA, USA).

## 10. RNA purification and RT-qPCR

Total RNA was extracted from cells using Trizol reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. ~1 μg of total

RNA was reverse transcribed by Eco-dry cDNA Synthesis kit containing poly-dT primer (Clonetech, Mountain View, CA, USA). The quantification of cDNA was performed with gene specific primers (Table 1) using Power SYBR green PCR Master Mix (Applied Biosystems, Grand Island, NY, USA) and processed using ABI 7500 (Applied Biosystems, Grand Island, NY, USA). The primer sequences are listed in Table 1. The relative level of gene expression was calculated by the $2^{-dCt}$ or the ddCt method, where actin was used for normalization. The RT-qPCR graphs represent the average of at least two independent experiments.

| | | |
|---|---|---|
| β-Actin | Forward | 5'-GTGACGTTGACATCCGTAAAGA-3' |
| | Reverse | 5'-GCCGGACTCATCGTACTCC-3' |
| IFN-β | Forward | 5'-ATGGTGGTCCGAGCAGAGAT-3' |
| | Reverse | 5'-CCACCACTCATTCTGAGGCA-3' |
| TNF-α | Forward | 5'-TCCCCAAAGGGATGAGAAGTT-3' |
| | Reverse | 5'-GTTTGCTACGACGTGGGCTAC-3' |
| IFI204 | Forward | 5'-GAGCAAGGCGGCTAAGGAA-3' |
| | Reverse | 5'-GCTGTGGAGTATTGGTGACTG-3' |

| | | |
|---|---|---|
| cGAS | Forward | 5’-GAGGCGCGGAAAGTCGTAA-3’ |
| | Reverse | 5’-TTGTCCGGTTCCTTCCTGGA-3’ |

**Table 1. Primers for RT-qPCR**

## 11. Purification of recombinant antigen

Recombinant TSA56 proteins were purified from *E.coli* BL21 (DE3) harboring a recombinant plasmid encoding each bacterial protein. Following induction with isopropyl β-D-thiogalactoside (IPTG) (0.1 mM, Duchefa, Zwijndrecht, Netherlands) at 16°C for 16 h, the proteins were purified using Ni-nitrilotriacetic acid His-resin (Qiagen, Calrsbad, CA, USA), according to manufacturer’s instructions. The purified proteins were dialyzed against phosphate-buffered saline (PBS) in an Aside-A-Lyzer Dialysis Cassette (Therrmo scientific, Rockford, IL, USA) overnight at 4°C.

## 12. Enzyme-linked immunosorbent assay

To determine the titer of antibodies specific to TSA56 in the sera of infected mice, immunoassay plates (96-well plates; Nunc, Rochester, NY, USA) were coated with 100 μl of purified antigen at a concentration of 5 μg/ml at 4°C overnight. The plates were then blocked for 2 h at room temperature with PBS containing 1% BSA. 100 μl of serum samples serially diluted by 2-fold were incubated for 2 h at room temperature. After washing with PBS containing 0.05% Tween20 (PBST), horseradish peroxidase (HRP)-conjugated goat anti-

mouse IgM, $IgG_1$, or $IgG_{2c}$ (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added and plates were incubated for 2h at room temperature. Wells were washed with PBST and incubated with 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase substrate solution (KPL, Gaithersburg, MD, USA) for 10 min. The reactions were stopped by addition of 1M phosphoric acid solution. Absorbances were measured at 450 nm using a microplate reader (Beckman Coulter Inc., Fullerton, CA, USA).

## 13. Type I IFN bioassay and luciferase reporter assay

Cell-culture supernatants from stimulated cells or serum from infected mice were overlayed on top of L929 IFN reporter cells containing the ISRE-luciferase construct (Jiang et al., 2005) and incubated for 4 h (48- or 96- well plate). The reporter cells were lysed in Passive Lysis Buffer (Promega, Madison, WI, USA) for 30 min at room temperature, mixed with firefly luciferin substrate (Promega, Madison, WI, USA ), and florescence was measured on an illuminometer (Beckman Coulter Inc., Fullerton, CA, USA ).

## 14. Flow cytometry

Splenocytes were stained with antibodies against the indicated surface molecules after blocking Fc receptors with anti-CD16/32 (2.4G2; BD Pharmingen, Franklin Lakes, NJ, USA). CD44 (IM7), CD279 (PD-1, RMP1-30), CD19 (6D5; Biolegend, San Diego, CA, USA), CD3 (145-2CD11), CD4 (RM4-5), CD69 (H1.2F3), CD8 (53-6.7; esBioscience, San Diego, CA, USA), CXCR5 (2G8), CD62L (MEL-1; BD Pharmingen, Franklin Lakes, NJ, USA),

were used for flow cytometric analysis. For intracellular detection of IFN-γ, $1x10^6$ splenocytes were stimulated with 10 μg rTSA56 and 1 μg Golgiplug (BD Pharmingen, Franklin Lakes, NJ, USA) for 16 hours in humidified $CO_2$ atmosphere at 37°C. Stimulated cells were stained with surface markers. Surface-stained cells were fixed and permeabilized with Fixation and Permeabilization Solution (BD Bioscience, Franklin Lakes, NJ, USA), followed by incubation with IFN-γ (XMG1.2; BD Pharmingen, Franklin Lakes, NJ, USA). Fluorescence intensities of the stained molecules were examined on a FACS Fortessa II flow cytometer (BD Pharmingen, Franklin Lakes, NJ, USA). Data were analyzed using Flowjo software (Tree Star, Ashland, OR, USA).

## 15. Fluorescent immunohistochemisty

Isolated tissues were fixed for 2–4 h in 4% paraformaldhehyde (Sigma-Aldrich, St. Louis, MO, USA) and placed in 30% sucrose. Tissues were embedded in optimal cutting temperature medium, then frozen and cut into sections 10–20 μm in thickness. The samples were fixed in chilled Acetone and dehydrated in PBS. Fixed samples were stained with anti-CD3 (Abcam, Cambridge, UK), anti-CD4 (GK1.5, BioLegend, San Diego, CA, USA), anti-B220 (RA3-6B2, BD Pharmingen, Franklin Lakes, NJ, USA), PNA (Sigma-Aldrich, St. Louis, MO, USA), followed blocking with Ultravision protein block (Thermo Fisher Scientific, Waltham, MA, USA). Cells were examined under an Olympus FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan). Images of cell sections were analyzed and

processed using the Olympus Fluoview software (Olympus, Tokyo, Japan).

## 16. Statistical analysis

The data was analyzed using the Graph Pad Prism 5.01 software. Statistical analysis of all the experimental data except survival rate was performed using the two-tailed Student's t-test with 95% confidence interval. Data are expressed as the mean ± standard deviation. Statistical analysis of survival rates were performed using the Mantel-Cox Log Rank test. A $p$-value of $< 0.05$ was considered statistically significant.

# RESULT

**Induction of T1 IFNs by *O. tsutsugamushi* infection**

To test whether TI IFNs are induced by *O. tsutsugamushi*, BMDM and MEF were infected with *O. tsutsugamushi* at a multiplicity of infection (MOI) of 4 and harvested at different time points. Induction of *ifnb* and *tnfα* was analyzed by quantitative RT-PCR. TI IFNs secreted by the infected cells were also measured by incubating infected cells with L929 cells containing a luciferase construct under the control of interferon stimulated response element promoter (ISRE) in heat-inactivated culture medium. *ifnb* mRNA expression in *O. tsutsugamushi*-infected BMDM peaked at 4 h after infection and rapidly declined thereafter (Figure 2A). TI IFNs secreted into infection media also increased up to 4 h after infection and then gradually decreased. In MEF, *O. tsutsugamushi* also induced *infb* and *tnfa* mRNA expression at 4 hours after infection (Figure 2B). ISRE-promotor activity was increased by addition of infection media derived from MEF culture and activity was observed up to 24 hours after infection. Consistently, IFN-β was also detected

in the infection media of BMDM as measured by ELISA (Figure 2C) as well as in the sera of infected mice during *in vivo* infection of *O. tsutsugamushi* as measured by TI IFN bioassay (Figure 2D). The level of TI IFN peaked at 12 days after infection in mice challenged with 5 x $LD_{50}$.

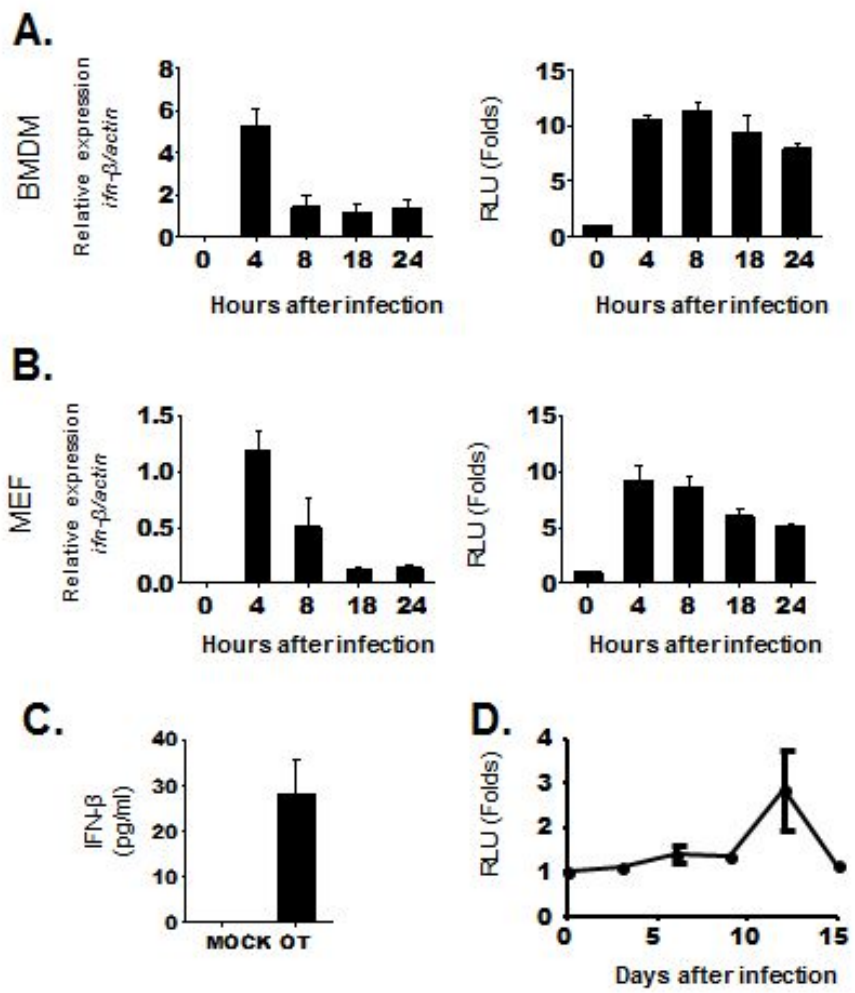


**Figure 2 Induction of T1 IFNs by *O. tsutsugamushi*.**

BMDM (A) and MEF (B) were infected with *O. tsutsugamushi* and the expression of IFN-β mRNA was measured by RT-PCR analysis. Secreted TI IFNs were also assessed by ISRE-responsive luciferase bioassays using the infection media. (C) The level of IFN-β was detected by ELISA assay at 12 hours after *O. tsutsugamushi* infection in BMDM. (D) T1 IFN in serum collected from *O. tsutsugamushi* infected mice was detected by T1 IFN bioassay (D). The data is representative of 2-3 independent experiments.

**Induction of T1 IFN by *O. tsutsugamushi* is mediated by an IKKα-dependent pathway**

In order to assess the role of the NF-κB pathway in TI IFN production by *O. tsutsugamushi* infection, I first examined the activation of NF-κB by measuring the expression TNF-α mRNA, as this is primarily regulated by the transcription factor, and the degradation of IκB in *O. tsutsugamushi*-infected MEF or BMDM. Cells were harvested at the indicated times after infection and the level of TNF-α transcripts and IκB protein were measured by quantitative RT-PCR and immunoblotting, respectively (Figure 3A and 3B). The level of TNF-α mRNA peaked at 4 hour after infection in both MEF and BMDM. IκB also rapidly degraded within 30 min after infection and gradually recovered thereafter. IκB in BMDM was completely degraded at 10 min after infection and recovered at 60 min (Figure 3A), whereas it degraded more slowly (maximum degradation at 30 min after infection) and fully recovered at 90 min after infection in MEF (Figure 3B). Next, the role of the NF-κB pathway in T1 IFN induction was assessed by

using IKK-α KO MEF. TI IFN expression in infected IKK-α WT and KO MEF was determined by RT-PCR and ISRE promoter bioassays (Figure 3C). The expression of IFN-β and TNF-α mRNAs were barely detected in IKK-α KO MEF after infection in contrast to wild type MEF which showed rapid induction of both transcripts. In addition, luciferase activity driven by TI IFNs was dramatically impaired in IKK-α KO MEF. These results confirmed that TI IFNs responses induced by *O. tsutsugamushi* infection require IKK-α which activates NF-κB transcription factor.

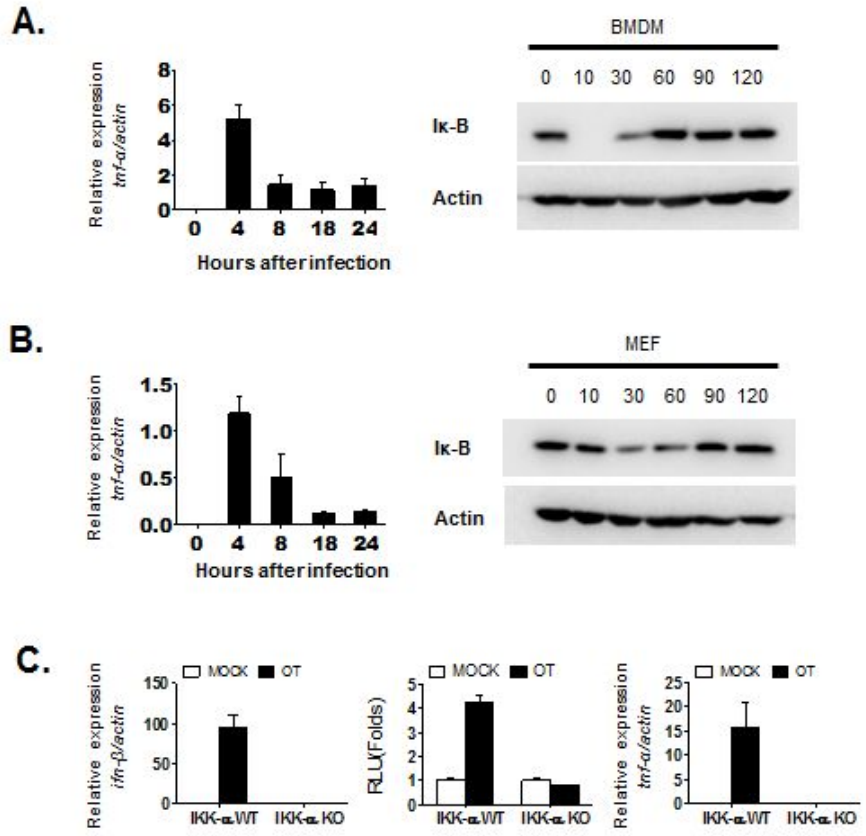


**Figure 3. Induction of T1 IFN by *O. tsutsugamushi* is dependent on an IKKα-dependent pathway**

The degradation of IκB was assessed in *O. tsutsugamushi*-infected BMDM (A) and MEF (B) by western blotting. The expression of TNF-α mRNA, induced via the NF-κB pathway, was also determined by RT-PCR analysis using total RNAs isolated from BMDM and MEF. (C) The level of T1 IFNs triggered by *O. tsutsugamushi* was measured in IKK-α WT and KO MEF via RT-PCR analysis and TI IFN bioassay. Induction of TNF-α mRNA was also measured by RT-PCR analysis as a control. The data is representative of 2-3 independent experiments.

**MyD88, TRIF, and RIP2 are dispensable for T1 IFN induction by *O. tsutsugamushi* in BMDM**

Since *O. tsutsugamushi* induces the production of T1 IFNs in BMDM, we investigated which signaling adaptor molecules are involved in the production T1 IFNs by *O. tsutsugamushi*. MyD88, TRIF, and RIP2 KO BMDM was infected with *O. tsutsugamushi* at 4 moi and T1 IFNs production was determined by RT-PCR and TI IFN bioassay at 4 hours after infection. Compared to WT BMDM, there were no significant changes in the level of IFN-β mRNAs in MyD88, TRIF, or RIP2 KO BMDM (Figure 4). Consistently, ISRE promoter activity in the presence of infection media showed similar level of luciferase activity in the KO BMDM and WT BMDM. These results indicate that these three signaling adaptors, which mediate the TLRs and NOD pathways, are dispensable for the induction of TI IFNs responses during *O. tsutsugamushi* infection. However, I could observe that induction of TNF-α mRNA by *O. tsutsugamushi* is severely impaired only in

MyD88 KO BMDM (Figure 4A), suggesting that the TLR-MyD88 pathway might be play a significant role in NF-κB-mediated induction of TNF-α expression, whereas TRIF and RIP2 adaptors play a minor role.

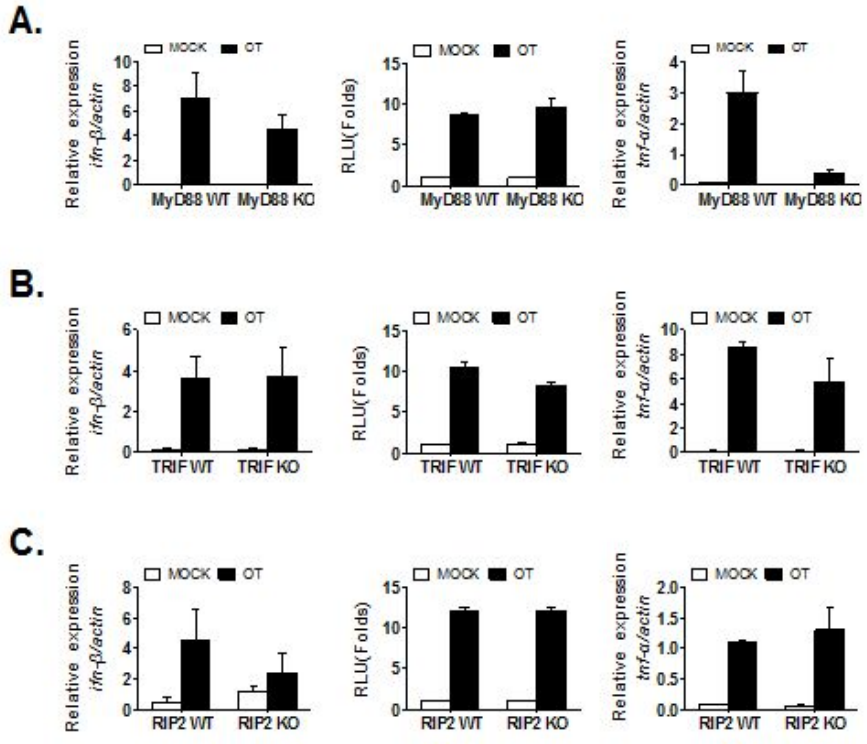


**Figure 4. MyD88, TRIF, and RIP2 are dispensable for T1 IFN induction by *O. tsutsugamushi* in BMDM.**

BMDM isolated from the indicated KO mice were infected with *O.*

*tsutsugamushi* at 4 moi and harvested at 4 hours after infection. Total RNA was used for RT-PCR analysis to determine IFN-β and TNF-α mRNA levels. L929 cells containing luciferase construct regulated by ISRE promoter were incubated with infection media. (A) MyD88 WT and KO BMDM, (B) TRIF WT and KO BMDM, (C) RIP2 WT and KO BMDM. The data is representative of 2-3 independent experiments.

**Induction of T1 IFN by *O. tsutsugamushi* is mediated by MAVS and STING-mediated pathways.**

Since the primary signaling adaptors that normally play significant roles in inflammatory responses in innate phagocytes,, MyD88, TRIF, and RIP2, are not significantly involved in the induction of TI IFN, I investigated potential role of well-known intracellular PRRs, MAVS and STING, in *O. tsutsugamushi*-mediated TI IFN responses. As shown in Figure 5, induction of T1 IFN was drastically impaired in both MAVS and STING-deficient MEF when infected with *O. tsutsugamushi*, whereas it was significantly upregulated in WT MEFs. In addition, *O. tsutsugamushi* infection failed to induce TNF-α mRNA in KO MEFs, indicating that MAVS and STING-mediated signaling are required for the NF-κB-mediated induction of inflammatory cytokines in non-phagocytic host cells.

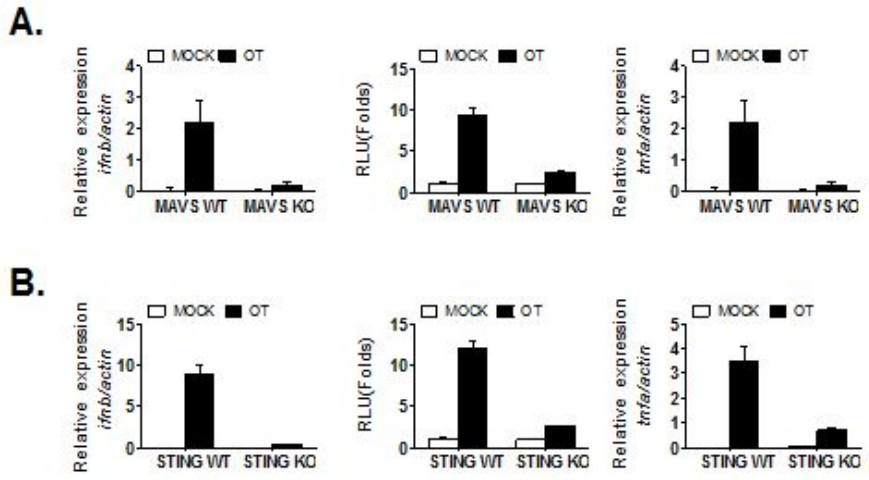


**Figure 5. Role of MAVS and STING-mediated pathways in TI IFN responses induced by *O. tsutsugamushi*.**

The expressions of IFN-β and TNF-α mRNA were measured by RT-PCR analysis using total RNA isolated from *O. tsutsugamushi*-infected MEFs. L929 cells containing the construct of luciferase under the control of ISRE promoter were incubated in cultured media for 4 hours for TI IFN bioassay. MAVS WT and KO MEF (A) and STING WT and KO MEF (B) were infected at 4 moi of *O. tsutsugamushi* and used for the analysis. The data is

representative of 2-3 independent experiments.

**RIG-I and cGAS are the potential sensors for the induction of T1 IFNs in host cells infected with *O. tsutsugamushi*.**

MAVS and STING are the key signaling adaptors for the induction of T1 IFN after recognition of intracellular nucleic acids by intracellular PRRs. Therefore, I investigated whether well-known intracellular PRRs, such as RIG-I, cGAS, and IFI, are involved in TI IFN responses by *O. tsutsugamushi* infection. As shown in Figure 6A, induction of TI IFN responses was severely impaired in RIG-I KO MEF in contrast to WT MEF. I also further examined the potential role of other intracellular sensors, cGAS and IFI204, after knockdown with specific siRNA in MEF (Figure 6B and 6C). Endogenous cGAS and IFI204 expression was efficiently suppressed by specific siRNA when compared to those of control siRNA (~80 and ~60 % respectively) as measured by RT-PCR analysis. The induction of IFN-β and TNF-α mRNA by *O. tsutsugamushi* infection was reduced up to 60% in cGAS knockdown MEF when compared to control MEF (Figure 6B). In contrast, the levels of IFN-β and TNF-α transcripts were elevated in IFI204 knockdown MEF upon *O. tsutsugamushi* infection as efficiently as in control MEF (Figure 6C). These

results suggest that the RIG-I/MAVS pathway and cGAS/STING pathway might play a significant role in TI IFN responses during *O. tsutsugamushi* infection in non-phagocytic cells. In addition, both pathways might contribute to inflammatory cytokine responses, as seen in TNF-α responses, mediated by NF-κB activation during bacterial infection.

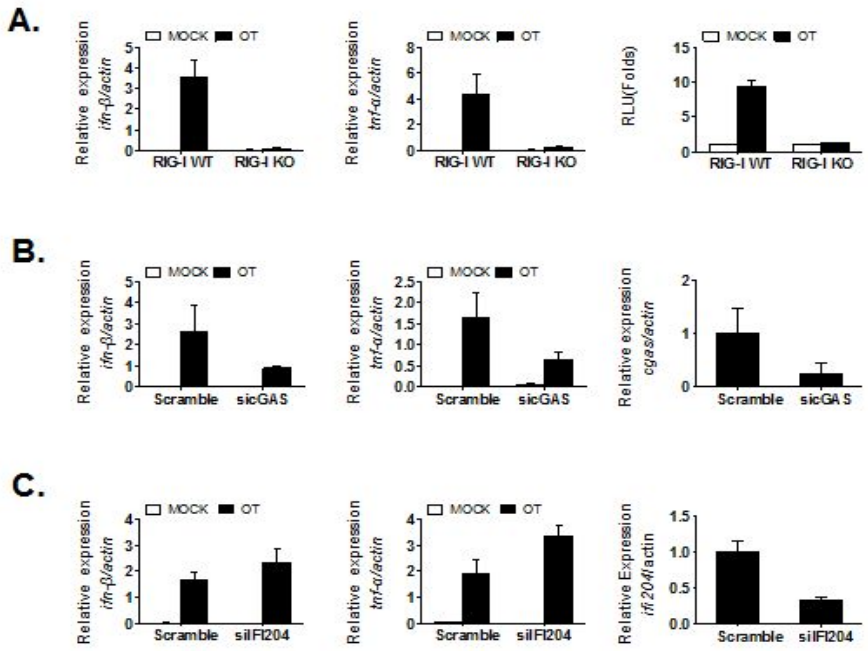


**Figure 6. RIG-I and cGAS are required for the induction of T1 IFNs by *O. tsutsugamushi*.**

(A) RIG-I WT and KO MEF were infected with *O. tsutsugamushi* at 4 moi and harvested at 4 hours after infection. Total RNA was used for RT-PCR analysis to determine IFN-β and TNF-α mRNA. L929 cells containing the construct of luciferase under the control of ISRE promoter were incubated with culture media for 4 hours for TI IFN bioassay. MEF was also transfected

with 100 μM of siRNA against cGAS (B), IFI204 (C), or scrambled control siRNA and then incubated for 24 hours. Knockdown efficiency was confirmed by RT-PCR analysis using total RNA extracted from the cells (third panels of 6B and 6C). The level of IFN-β and TNF-α transcripts were assessed as in Figure 6A. The data is representative of 2-3 independent experiments.

**Replication of *O. tsutsugamushi* is not significantly affected in IFNAR KO cells.**

To investigate the potential role of T1 IFNs on the replication of *O. tsutsugamushi in vitro*, MEF and BMDM derived from IFNAR WT and KO mice were infected with the bacteria. Bacterial growth within the host cells was assessed by measuring 56kDa type-specific antigen (TSA56), a major membrane protein of *O. tsutsugamushi.* As shown in Figure 7, the level of TSA56 gradually increased up to 4 days after infection in both cell types, indicating efficient growth of *O. tsutsugamushi* in MEF and BMDM. The level of TSA56 increased in IFNAR KO MEF and BMDM as efficiently as in WT cells. These results indicate that replication of *O. tsutsugamushi* within host cells is not significantly affected by TI IFN responses generated during bacterial infection. These findings were consistently observed in both phagocytic cells, BMDM, as well as in non-phagocytic cells, MEF (Figure 7).

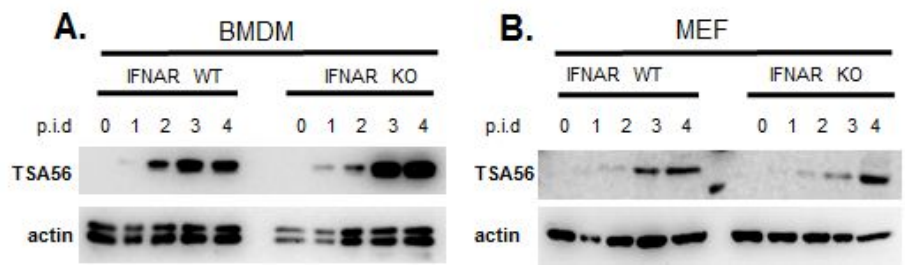


**Figure 7. Replication of *O. tsutsugamushi* is not significantly affected in IFNAR KO cells.**

IFNAR WT and KO BMDM (A) or MEF (B) were infected with *O. tsutsugamushi* at 4 moi and harvested at the indicated times after infection for the analysis of intracellular bacterial growth. The level of TSA56, a major outer membrane protein of *O. tsutsugamushi*, was measured as the indicator of bacterial growth by immunoblot. β-actin was used as a protein loading control.

**The mortality and morbidity of mice infected with *O. tsutsugamushi* are not significantly changed in the absence of IFNAR.**

The effect T1 IFN responses induced by *O. tsutsugamushi* infection *in vivo* was assessed using WT and IFNAR KO mice. Mice were infected with 1 x 50% lethal dose ($LD_{50}$) or 5 x $LD_{50}$ of *O. tsutsugamushi* and disease severity was monitored by measuring body weight and mortality. With both infection doses, no significant differences between WT and IFNAR KO mice were observed (Figure 8). These results show that TI IFN responses induced by *O. tsutsugamushi* do not significantly affect disease morbidity and mortality during *in vivo* infection.

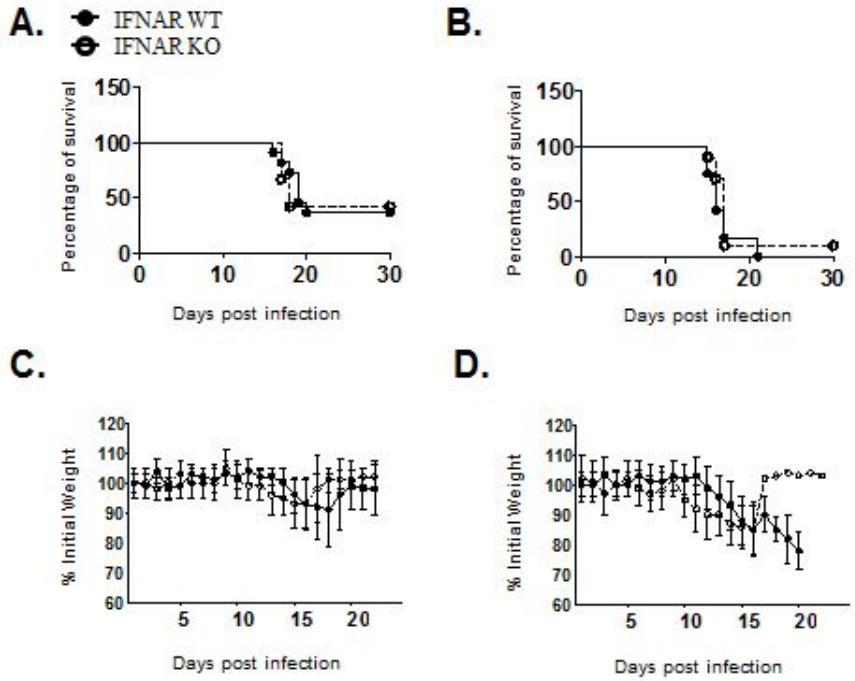


**Figure 8. The mortality and morbidity of mice infected with *O. tsutsugamushi* are not significantly changed in the absence of IFNAR.**

IFNAR WT or KO mice (n=5) were infected with 1 x (A, C) or 5 x (B, D) LD50 and monitored for the rate of survival (A, B) and the change of bodyweight (C, D) every day.

**Lymphocyte responses in IFNAR KO mice infected with *O. tsutsugamushi*.**

Even though the morbidity and mortality were not significantly different between WT and IFNAR KO mice upon primary infection with *O. tsutsugamushi*, I sought to confirm the potential role of TI IFN responses in adaptive immune responses using IFNAR KO mice. First, spleens were collected at different days after infection and splenomegaly, which could be observed during systemic infection of *O. tsutsugamushi,* was inspected. A gradual increase in the volume of spleens was generally observed in all the infected mice and the degree of splenomegaly was similar in both WT and IFNAR mice (Figure 9A). When the number of total mononuclear cells in spleens of infected mice was quantified, I observed a decline at day 3 after infection and increase at day 6 and 9 after infection (Figure 9B). There was no significant difference in the change of total splenocytes between WT and IFNAR mice. In the case of $CD19^+$ B cells, the total counts in infected spleens remained constant up to day 7 and increased at day 10 (Figure 9C). However, the total counts of $CD4^+$ T cells (Figure 9D) and $CD8^+$ T cells (Figure 9E) were elevated at day 7 and then declined at day 10 after infection.

Nevertheless, overall kinetic changes of $CD19^+$ B cells, $CD4^+$ T cells, as well as $CD8^+$ T cells were not significantly different in the spleens of IFNAR WT and KO mice upon *O. tsutsugamushi* infection.

Changes in the follicular structures of spleens during *O. tsutsugamushi* infection were also examined after H&E staining. Massive alteration and disintegration of lymphocyte follicles were observed at day 6 after infection in the spleens of WT mice, whereas these phenomena were observed at day 9 after infection in IFNAR KO mice (Figure 10A). Disorganization of the microstructures of lymphoid follicles in spleens were further examined by using immunofluorescent staining of B cell and T cell zones (Figure 10B). Disorganization of typical follicular structure, T cell zone surrounded by B cell zone, in spleens was often observed at day 6 after infection in WT mice, whereas structural changes in spleens were rather delayed in IFNAR mice and generally observed at day 9 after infection. These results suggest that TI IFN responses may affect the structural changes in secondary lymphoid organs, and thereby affect the quality of adaptive immune responses, despite not significantly influencing the total counts of lymphocytes during *O. tsutsugamushi* infection.

Since delayed structural changes were observed in the microstructures of lymphoid follicles in the spleens of IFNAR KO mice infected with *O. tsutsugamushi*, differentiation of follicular helper T cells ($T_{FH}$) was examined. The kinetic changes in the fraction of $CXCR5^+PD1^+$ $T_{FH}$ cells among activated $CD4^+CD44^+$ T cells were assessed by flowcytometry (Figure 11). In IFNAR WT mice, the fraction of $T_{FH}$ among activated $CD4^+$ T cells in

spleen gradually increased upon *O. tsutsugamushi* infection, whereas it was static until day 7 after infection in IFNAR KO mice and then elevated at day 10 after infection (Figure 11). The differentiation of $T_{FH}$ cells from $CD4^+$ T cells during the first week following bacterial infection was significantly different between IFNAR WT and KO mice and the delay in $T_{FH}$ cells in the spleen might correlate with the delayed structural changes in lymphoid follicles of IFNAR KO mice (Figure 10).

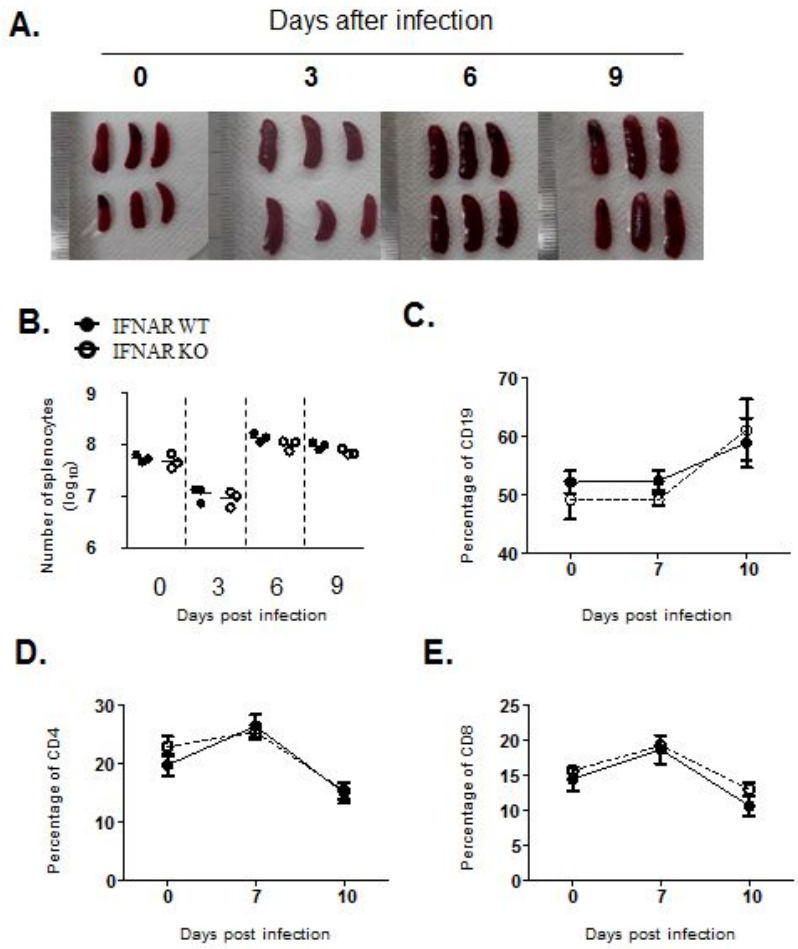


**Figure 9. Lymphocyte responses in IFNAR KO mice infected with *O. tsutsugamushi*.**

(A) Spleens were collected from infected mice at the indicated times after *O. tsutsugamushi* infection and their volumes were measure and compared. (B) Total splenocytes were harvested at the indicated times after *O. tsutsugamushi* infection and counted after tryphan-blue staining. (C ~ D) The kinetic changes in total $CD19^+$ B cells, $CD4^+$ T cells, as well as $CD8^+$ T cells were measured by flow cytometry.

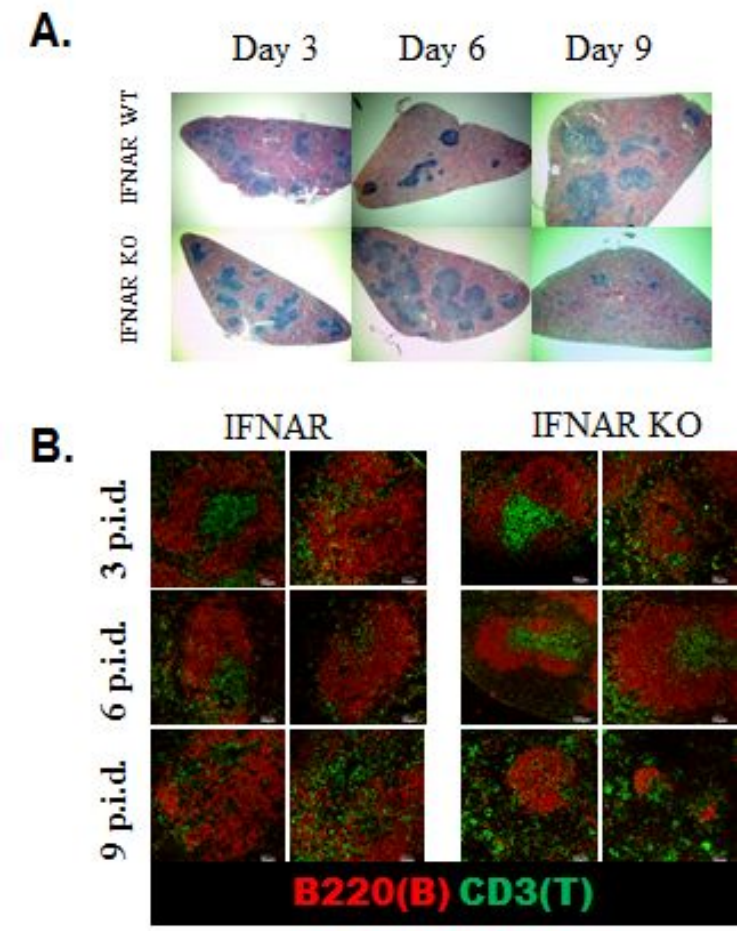


**Figure 10. The alteration of spleen architecture by *O tsutsugamushi* infection.**

(A) Spleens were collected from IFNAR WT and KO mice at the indicated times after infection and changes in follicular structures were examined after H&E staining. (B) Structures of lymphoid follicles comprised of B cells (red) and T cells (green) were examined in spleens derived from IFNAR WT and KO mice at the indicated times after infection. Immunofluorescent microscopy was performed after staining of fixed spleen sections with the indicated antibodies.

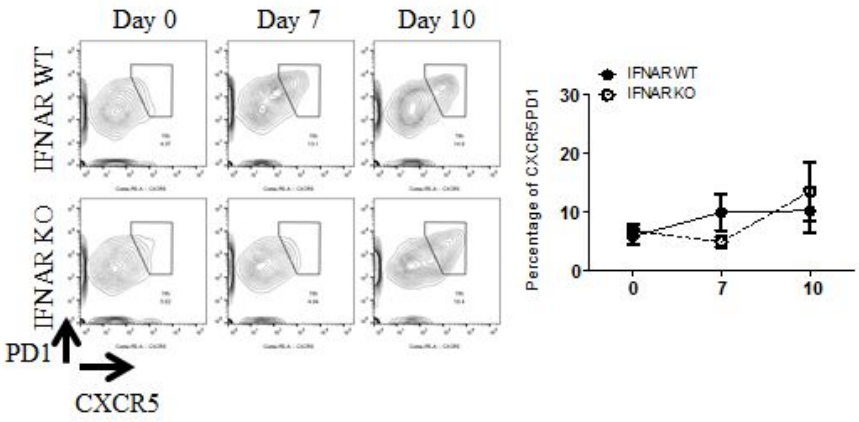


**Figure 11. Differentiation of follicular helper T cells in mice infected with *O. tsutsugamushi*.**

The differentiation of $T_{FH}$ cells in spleens of infected mice was examined by flowcytometry after staining the splenocytes with the indicated antibodies. The fraction of $CXCR5^{+}PD1^{+}$ $T_{FH}$ cells was gated on $CD4^{+}CD44^{+}$ T cells in spleens of IFNAR WT and KO mice at the indicated times after infection. FACS analysis of $CXCR5^{+}PD1^{+}$ $T_{FH}$ cells is presented (left panels) and the kinetic changes of $T_{FH}$ cells from triplicate experiments are summarized in right graph.

**Antibody responses in IFNAR KO mice infected with *O. tsutsugamushi.***

$T_{FH}$ cells play a major role in the germinal center reaction of B cells in secondary lymphoid organs and contribute to the development of specific antibody responses. Since I could observe delayed differentiation of $T_{FH}$ cells in IFNAR KO mice when compared to WT mice during *O. tsutsugamushi* infection, development of specific antibody responses against bacterial antigen were examined. Specific antibodies against a major outer membrane proteinof *O. tsutsugamushi*, TSA56, were titrated using the sera of infected mice at different time points (Figure 12). I also titrated the specific antibody responses of different isotypes, including IgM, $IgG_1$, and $IgG_{2c}$. At day 6 and 7 after infection, all immunoglobulin isotypes against TSA56 in the sera of IFNAR WT mice were relatively higher than those of IFNAR KO mice. However, the levels of immunoglobulins became similar at days 10 and 12 after infection in both IFNAR WT and KO mice. These results indicate that T1 IFN responses might affect the development of specific antibody responses during early course of infection, potentially via contributing to the

differentiation of antigen-specific $T_{FH}$ cells in secondary lymphoid organs. In order to measure the longevity of the specific antibody responses, mice challenged with 5 x $LD_{50}$ were treated with antibiotics for a week from day 5 after infection and cured of infection. At days 21 and 28 after the initial challenge, antigen specific antibody responses were further assessed by ELISA. The levels of specific antibody responses at days 21 and 25 were generally increased in all the isotypes tested (Figure 12B). However, I could not observe any significant difference in antibody responses between IFNAR WT and KO mice.

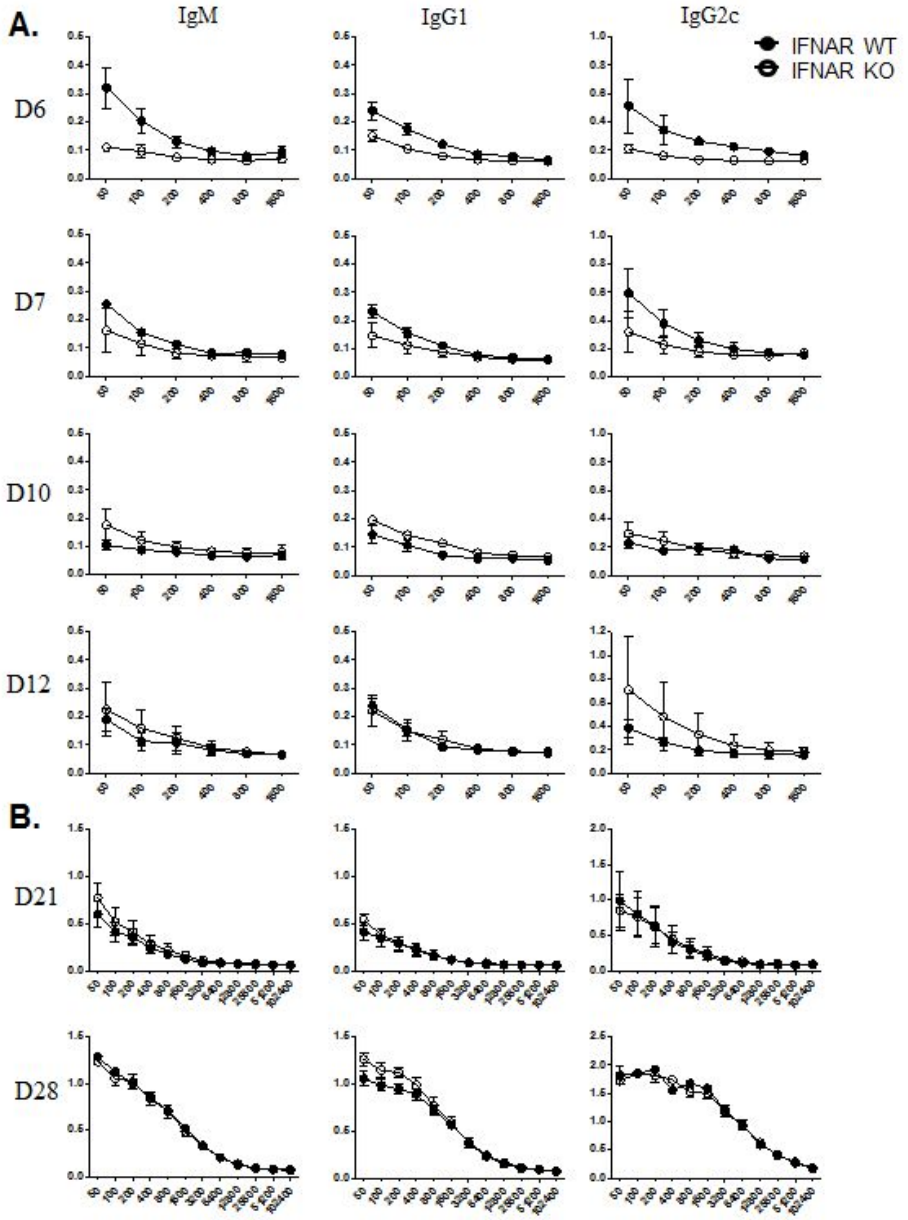


**Figure 12. Antibody responses in IFNAR KO mice infected with *O. tsutsugamushi*.**

(A) The level of specific antibody responses against TSA56 in sera of IFNAR WT and KO mice was titrated by ELISA. Sera from the infected mice were collected at the indicated times and serially diluted for the titration of the

indicated isotype antibodies specific to TSA56. (B) IFNAR WT and KO mice infected with 5 x $LD_{50}$ of *O. tsutsugamushi* were treated with doxycycline at 5 days after infection and cured of infection. Sera from the recovered mice were collected at the indicated times and the specific antibodies against TSA56 were titrated by ELISA.

**Memory T cell responses in IFNAR KO mice infected with *O. tsutsugamushi.***

Finally, to investigate the potential role of TI IFN responses in the development of memory T cells specific to *O. tsutsugamushi* antigens, IFNAR WT and KO mice were challenged with 5 x $LD_{50}$ of *O. tsutsugamushi* and treated with tetracycline for a week from day 5 after infection. Splenocytes were collected from recovered mice at 6 weeks after initial infection and used for the assessment of memory T cells specific to TSA56 antigen. The fraction of activation of T cells specific to the bacterial antigen was assessed by surface expression of CD69 as well as intracellular IFN-γ staining. The levels of IFN-γ positive $CD4^+$ T cells were similar in spleens of IFNAR WT and KO mice (Figure 13A). However, the level of $CD8^+$ T cells specifically responsive to TSA56 antigen was significantly higher in WT mice when compared to those of IFNAR KO mice (Figure 13B). IFN-γ positive $CD8^+$ T cells in IFNAR KO mice were reduced by ~ 35% when compared to those of WT mice. These results clearly suggest that TI IFN responses play a specific role in the development of $CD8^+$ memory T cells, but not for $CD4^+$ T cells, during *O. tsutsugamushi* infection.

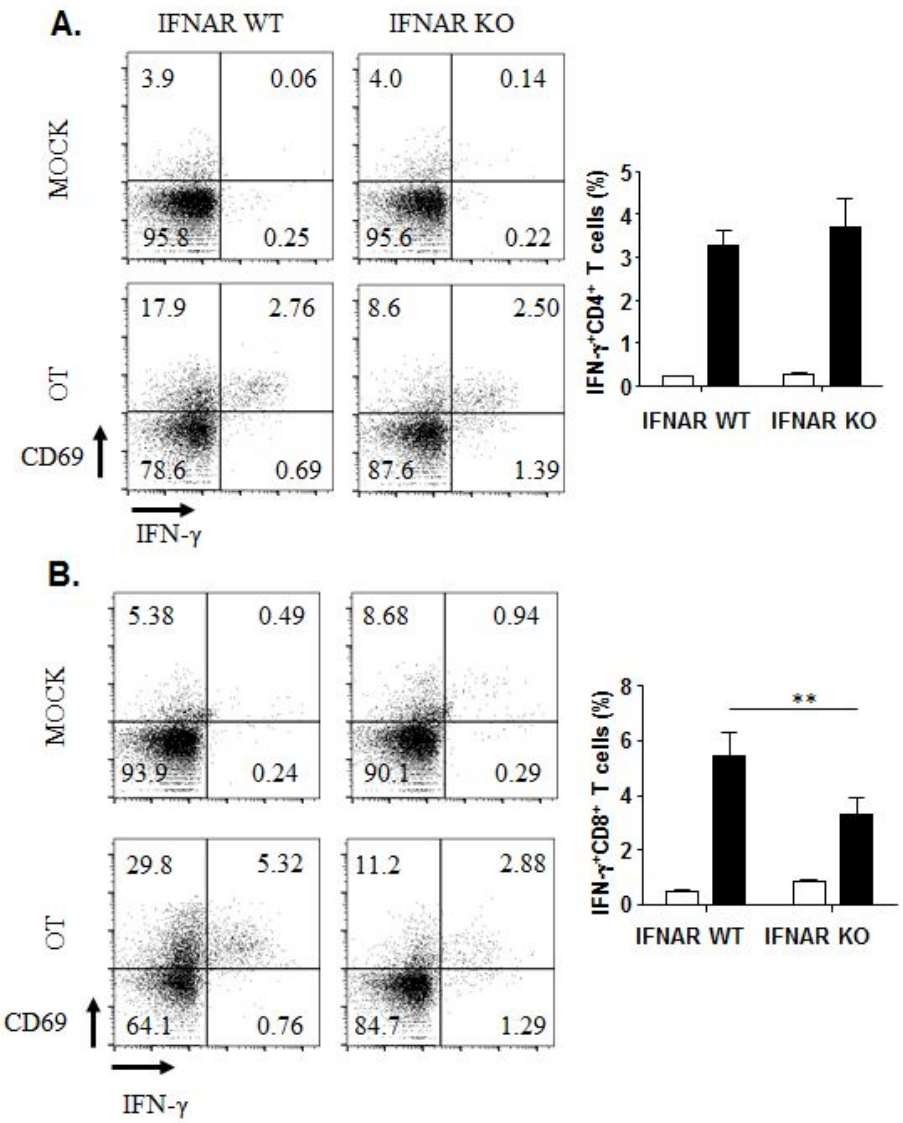


**Figure 13. Memory T cell responses in IFNAR KO mice infected with *O. tsutsugamushi*.**

IFNAR WT and KO mice infected with 5 x $LD_{50}$ of *O. tsutsugamushi* were treated with doxycycline at 5 days after infection and the splenocytes were collected from the recovered mice at 6 weeks after initial infection to assess memory T cell responses ($n$ = 3). The percentile of positive cells in each

quadrant was assessed by FACS analysis after staining with CD69 and intracellular IFN-γ in $CD4^+$ (A) and $CD8^+$ (B) T cells.

# DISCUSSION

Immune responses to infectious agents are initiated by the recognition of PAMPs by host PRRs and subsequent activation of cellular signaling pathways that induce transcriptional activation of diverse inflammatory genes. Previous studies have shown that *O. tsutsugamushi* induces strong inflammatory cytokines upon infection in diverse phagocytic cells and non-phagocytic cells and induces Th1-biased immune responses (Soong et al. 2014) (Cho et al. 2000). In addition, several recent studies reported that the intracellular pathogen significantly upregulates T1 IFNs and TI IFN-stimulated genes (ISGs) in PBMCs of scrub typhus patients (Tantibhedhyangkul et al. 2011). *Rickettsia Conorii*, belonging to Rickettsiales, also induces T1 IFNs and ISGs in human endothelial cells (Colonne, Eremeeva, and Sahni 2011, Colonne, Sahni, and Sahni 2011). These results indicate that members of Rickettsiales, including *Orientia* and *Rickettsia*, might commonly activate signaling pathways during intracellular invasion and replication within the host cytosol. Here, I confirmed that *O. tsutsugamushi* induces T1 IFN responses in phagocytic macrophages as well as in non-phagocytic fibroblasts (Figure 2). Active induction of TI IFNs during the early phase (within a few hours) of infection also supports the idea that *O. tsutsugamushi* possesses specific PAMP(s), which can be recognized by non-phagocytic cells as well as phagocytic immune cells.

Both TBK and NF-κB-related pathways need to be activated for the induction of TI IFN responses (Fensterl and Sen 2009). *O. tsutsugamushi* can

induce rapid degradation of IκB, a prerequisite for NF-κB activation, in BMDM and MEF. Degradation of IκB in BMDM upon infected with *O. tsutsugamushi* was more rapid and robust than in MEF, suggesting that phagocytic immune cells might be a major source of proinflammatory cytokines including T1 IFNs (Swiecki and Colonna 2015). Involvement of the NF-κB pathway for the induction of TI IFNs by *O. tsutsugamushi* was confirmed by using IKK-α KO MEF. The IKK complex, comprised of NEMO, IKK-α and –β, is a key component for the degradation of IκB and it was reported that IKK-α is essential for the induction of T1 IFNs by TLR-mediated signaling (Hoshino et al. 2006). As expected, the production of T1 IFNs by *O. tsutsugamushi* is completely abolished in MEF lacking IKK-α (Figure 3C). Therefore, induction of the NF-κB pathway by *O. tsutsugamushi* is critical for the production of T1 IFNs. In addition, MyD88 is required for the expression of TNF-α, but not for TI IFNs in BMDM (Figure 4A). Considering that MyD88 is involved in signaling pathways mediated by multiple TLRs, *O. tsutsugamushi* might stimulate TLR-mediated signaling for the production of inflammatory cytokines in a NF-κB dependent manner. However, TRIF and RIP2 signaling adaptors are dispensable for the induction of TNF-α in BMDM, suggesting that TRIF-mediated TLR signaling and RIP2-mediated NOD1/2 signaling may not be critical for inflammatory responses during *O. tsutsugamushi* infection although I could not exclude the possibility of redundant stimulation of signaling pathways. Nevertheless, which bacterial PAMPs stimulate TLR-MyD88 pathways still remain elusive and need to be characterized in future studies.

An interesting finding in this study is the involvement of intracellular nucleic acid sensors, RIG-I and cGAS, for the induction TI IFNs by *O. tsutsugamushi*. The critical role of these intracellular sensors in TI IFN responses is further supported by impaired expression of TI IFNs in MEFs lacking MAVS and STING, since these signaling adaptors are the essential mediators for RIG-I and cGAS-induced TI IFN responses, respectively. Then, what are the bacterial ligands stimulating these pathways and how are these intracellular sensors that recognize nucleic acids activated upon *O. tsutsugamushi* infection? RIG-I recognizes 5'-triphosphate blunted double stranded RNAs (Hornung et al. 2006, Pichlmair et al. 2006) as well as exogenous DNAs when transcribed by host polymerase III complex (Chiu, Macmillan, and Chen 2009). Hornung and collegues used bacterial tRNA as negative control since 5' end of bacterial tRNA was modified with pseudo-uracylation (Hornung et al. 2006). According to several reports, however, bacterial RNAs, including *Streptococcus pyogenes*, *Listeria monocytogenes*, and *Legionella pneumonia* are recognized by RIG-I (Gratz et al. 2011, Hagmann et al. 2013, Opitz et al. 2006). It was also shown that RIG-I and related genes, including MAVS, are significantly increased in the transcriptome of *O. tsutsugamushi* infected PBMC (Gorvel et al. 2014. cGAS-STING signaling pathway is participated in the T1 IFN responses via sensing of exogenous DNAs. cGAS recognizes exogenous or endogenous dsDNAs and produces cyclic-di-GAMP which in turn activates STING and induces TI IFN responses {Gao, 2013 #3194). STING also directly recognizes cyclic-di-GMP secreted by *Listeria monocytogenes* (McWhirter et al. 2009, Shu et al. 2012). Also, T1 IFNs

induced by STING signaling were measured in cells infected with *Streptococcus pneumonia* (Koppe et al. 2012), *Mycobacterium tuberculosis* (Wassermann et al. 2015), and *Listeria monocytogenes* (Archer, Durack, and Portnoy 2014). Based on these studies, T1 IFN responses induced by *O. tsutsugamushi* infection might be initiated by the recognition of bacterial nucleic acids exposed during the intracellular invasion process. It is also possible that *O. tsutsugamushi* may synthesize and secrete cyclic-di-GMP during the invasion process. *O. tsutsugamushi* encodes a *pleD*-like gene, which is involved in synthesis of the secondary messenger, cyclic-di-GMP. Even though it has not been examined whether the *pleD*-like gene of *O. tsutsugamushi* is functional, this cyclic nucleotide could be the potential ligand for stimulating STING-mediated pathways as found in the case of *Listeria monocytogenes* (McWhirter et al. 2009, Shu et al. 2012). In this study, MAVS or STING KO almost completely abolishes induction of TI IFNs by *O. tsutsugamushi* infection as reported in other studies (Hansen et al. 2014, Lippmann et al. 2011, Monroe, McWhirter, and Vance 2009, Plumlee et al. 2009). STING participates in MAVS-mediated signaling to recruit STAT6 in response to RNA viral infection (Chen et al. 2011), suggesting a crosstalk between the signaling adaptors. Nevertheless, which *O. tsutsugamushi* ligands are recognized by the intracellular nucleic sensors and how they are exposed during bacterial invasion need to be characterized in future studies.

A previous study reported that relatively low concentrations of IFN-β inhibited the growth of *O. tsutsugamushi*, whereas high concentrations of IFN-β enhanced the intracellular growth of the rickettsia in L929 cells

(Tasneen et al. 1997). In this study, the replication rate of *O. tsutsugamushi* was not significantly affected in IFNAR KO BMDM and MEF (Figure 7). In addition, *O. tsutsugamushi* infection in IFNAR KO mice resulted in similar degrees of the morbidity and mortality as that observed in WT mice (Figure 8). The *in vivo* effect of TI IFN responses on morbidity and mortality is quite diverse when using IFNAR KO mice; T1 IFNs causes protective in some bacterial infection model but, detrimental in other bacterial infection model mice (O'Connell et al. 2004, Robinson et al. 2012, Martin et al. 2009, Mancuso et al. 2007). In this experiment, it seems clear that TI IFN responses do not significantly affect the survival rate of mice infected with *O. tsutsugamushi,* at least during primary infection from reproducible assessments of disease severity upon challenges with broad ranges of infectious doses (1 ~ 1000 x $LD_{50}$). Therefore, T1 IFNs induced immune responses is unrelated with the immunopathogenesis and severity of scrub typhus, at least in the primary infection, suggesting that T1 IFNs induced *by O. tsutsugamushi* might affect adaptive immune and/or memory responses.

In order to initiate antigen-specific adaptive immune responses, antigen-specific naïve T cells need to be primed by three different types of stimulations: antigen-MHC complex (signal 1), co-stimulation (signal 2), and inflammatory cytokines (signal 3) (Crouse, Kalinke, and Oxenius 2015). Signal 3 cytokines, such as IL-12 (Makar et al. 2014) and T1 IFNs (Curtsinger et al. 2005), play a pivotal role in differentiation of naïve T cells into specific subtypes as well as clonal expansion. In humans, IFN-α enhances the frequency of IFN-γ producing $CD4^+$ T cells (Brinkmann et al. 1993).

IFNAR-dependent STAT5 activation can rescue differentiation of $T_{FH}$ cells in STAT3-deficient environment (Ray et al. 2014) and sustained T1 IFN responses suppress $T_{FH}$ development (Osokine et al. 2014). In this study, I found that differentiation of $T_{FH}$ cells is delayed in IFNAR KO mice (Figure 11) and TSA56-specific antibody production in early stage (~ 7 days) of infection is slightly reduced in IFNAR KO mice (Figure 12). However, differentiation of $T_{FH}$ cells as well as antibody production is recovered in the later stage of infection. In addition, there is no significant difference in the level of antibody responses in mice that recovered from scrub typhus, regardless of IFNAR expression (Figure 12B). Therefore, TI IFNs induced by *O. tsutsugamushi* infection do not significantly affect antibody responses against bacterial antigens.

Formation of germinal centers in lymphoid follicles is an essential process for the generation of high affinity antibodies (De Silva and Klein 2015). During various microbial infections, splenic microarchitecture transiently changes and reorganizes (Scandella et al. 2008, Racine et al. 2010, Glatman Zaretsky et al. 2012). The alteration of splenic architecture, especially in lymphoid follicles, causes a transient reduction in antibody responses. Alteration of follicular structures in spleens of *O. tsutsugamushi*-infected mice was also examined (Figure 10A). Disorganization of follicular structures during the early stage of infection was rather delayed in IFNAR KO mice and this was correlated with a reduced production of specific antibodies. In a previous study using a LCMV infection model, disruption of the T cell zone in spleens of LCMV infected mice might be mediated by cytotoxic $CD8^+$

T cells which kill LCMV-infected fibroreticular cells (Scandella et al. 2008). It is also interesting to note that the disorganization of splenic follicular structures could be observed in mice immunized with complete Freund's adjuvant, but not in mice immunized with Alum (Katakai et al. 2004), suggesting that the structural changes might be associated with the strength of innate immune responses including TI IFNs. Even though there is no direct evidence demonstrating that delayed disruption of follicular structure in spleens of IFNAR KO mice is associated with the activity of cytotoxic T cells, it is possible that the generation of cytotoxic $CD8^+$ T cells in IFNAR mice might be slower or weaker than in WT mice, thereby delaying structural changes. The specific role of $CD8^+$ T cells as well as potential infection of fiboreticular cells by *O. tsutsugamushi* in spleen are intriguing topics for further studies.

The potential role of TI IFNs responses in CD8+ T cell responses is further correlated with the reduction of memory $CD8^+$ T cell responses in IFNAR KO mice (Figure 13B). Previously, IL-12 and T1 IFNs were shown to be involved in the development of antigen-specific memory $CD8^+$ T cells (Xiao et al. 2009). Consistent with this, IFN-γ producing $CD8^+$ T cells in response to TSA56 antigen was significantly reduced in IFNAR KO mice at 6 weeks after initial infection, whereas $CD4^+$ memory T cell responses were similar in IFNAR WT and KO mice. These results suggest that TI IFNs responses induced by *O. tsutsugamushi* infection may specifically affect the generation of $CD8^+$ memory T cell responses.

In summary, I found that *O. tsutsugamushi* induces TI IFN responses

via IKK-α/NF-κB, RIG-I/MAVS, and cGAS/STING-dependent pathways, whereas signaling adaptors involved in TLR or NOD signaling, such as TRIF and RIP2, are dispensable for the induction of TI IFNs by *O. tsutsugamushi*. In addition, T1 IFNs produced by *O. tsutsugamushi* infection do not significantly affect protective immunity during primary infection, but they might promote the generation of specific adaptive immune responses via supporting the differentiation of $T_{FH}$ cells as well as enhancing the generation of $CD8^+$ memory T cell responses.

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# 국문 초록

다양한 병원체 감염에 의해 유도되는 1형 인터페론 (Type I interferons, T1 IFNs)은 바이러스 감염을 억제하는 역할을 하는 것으로 잘 알려져 있다. 최근 연구결과들에 따르면, 세균 감염에 의해서도 T1 IFN이 유도된다고 보고되고 있으나, 생산된 T1 IFN이 세균에 대한 면역반응을 어떻게 조절하고, 보호면역에 관련되어 있는 지에 대해서는 상이한 결과들이 보고되고 있어서 논란의 여지가 있다. 쯔쯔가무시병의 원인균인 *Orientia tsutsugamushi*는 세포 내 절대기생 세균으로, 최근 이 세균 감염에 의해서도 T1 IFN이 유도된다는 보고들이 있으나, 이 싸이토카인의 발현이 유도되는 기전이나 면역병리와의 연관성 여부는 알려진 바가 없다. 따라서, 본 연구에서는 *O. tsutsugamushi*에 의한 T1 IFN 생성기전과 감염 동물모델에서 이 싸이토카인이 면역반응에 어떠한 영향을 미치는지 조사하였다. 숙주세포에서 *O. tsutsugamushi* 감염에 의한 T1 IFN 생성 기전을 알아보기 위하여 다양한 유전자 결손 세포들을 사용하여, 연관 신호경로 분자들

을 동정하였으며, T1 IFN 수용체 (T1 IFNAR)가 결손 된 생쥐 감염모델을 이용하여 T1 IFN이 숙주의 면역반응과 보호면역 유도에 어떠한 영향을 미치는 지 연구하였다. 생쥐태아의 섬유세포 (mouse embryonic fibroblast; MEF)와 골수 유래 큰 포식세포 (bone-marrow derived macrophage; BMDM)에서 *O. tsutsugamushi* 감염에 의해 IFN-β의 발현이 유도되는 것을 확인하였으며, 다양한 유전자 결손세포들을 이용한 실험을 통하여 IKK-α, RIG-I, cGAS, MAVS, 그리고 STING 신호단백이 T1 IFN 유도에 필요하다는 것을 확인하였다. 하지만, T1 IFN이 결손 된 세포들에서 *O. tsutsugamushi*의 증식을 살펴본 결과, 생체 외 세포에서의 증식에는 야생형 세포와 차이가 나지 않았으며, T1 IFNR이 결손 된 생쥐도 야생형 생쥐와 거의 동일한 세균 감수성(치사율)을 보였다. 따라서 *O. tsutsugamushi*의 일차 감염 시, 유도되는 T1 IFN은 세균의 증식과 보호면역 생성에는 크게 영향을 미치지 않는 것으로 보인다. 흥미롭게도, *O. tsutsugamushi*에 감염된 T1 IFNR 결손생쥐에서는 이 세균에 대한 초기 항체반응이 야생형에 비하여 지연되는 것이 관찰되었으며, 비장에서 $T_{FH}$ 세포의 비율 증

가가 더디게 나타나는 것을 확인하였다. 또한 감염된 T1 IFNAR 결손생쥐의 이차 림프조직에서는 림프소절 (follicle) 구조의 초기변화가 지연되어 발생하는 것으로 보아, 유도된 T1 IFN이 감염초기의 $T_{FH}$ 세포 분화와 림프소절 (follicle) 구조의 변화에 영향을 미침으로서 항원특이적 항체반응을 유도하는 것에 관여하는 것으로 판단된다. 또한 IFNAR이 결핍된 생쥐에서는 $CD8^+$ 기억 T 세포 반응이 야생형 생쥐에서 보다 약하게 생성되는 것이 확인 되었는데, $CD4^+$ 기억 T 세포 반응은 유의하게 차이가 없는 것으로 보아, TI IFN 반응은 1차 감염 시 생성되는 $CD8^+$ 기억 T 세포 반응을 특이적으로 증가시키는 것으로 판단된다. 결론적으로, *O. tsutsugamushi* 감염에 의해 유도되는 T1 IFN 반응은 이 세균의 증식이나 숙주의 감수성에 크게 영향을 미치지는 않지만, 감염초기에 $T_{FH}$ 세포의 분화를 촉진하여 항원특이적 항체반응을 빠르게 유도하는데 관여하며 1차 감염 후 생성되는 $CD8^+$ 기억 T 세포 반응을 특이적으로 증가시키는 데 기여함으로서 쯔쯔가무시병에 대한 적응면역반응의 생성에 기여하고 있음을 알 수 있다.