A Doctorial Dissertation

Regulation of Tumor Necrosis Factor-Alpha Gene Expression and Signal Transduction during *Orientia tsutsugamushi* Infection

by

Ji-Hyun Yun

Department of Medicine

Graduate School

Cheju National University

June, 2006

Orientia tsutsugamushi 감염 시 신호전달과 Tumor Necrosis Factor-Alpha 유전자 발현의 조절

지도교수 고 영 상

윤 지 현

이 논문을 의학 박사학위 논문으로 제출함

2006년 6월

윤지현의 의학 박사학위 논문을 인준함

심사위원장 _____ 印

위 원 _____ 印

위 원 _____ 印

위 원 _____ 印

위 원 _____ 印

제주대학교 대학원

2006년 6월

Regulation of Tumor Necrosis Factor-Alpha Gene Expression and Signal Transduction during *Orientia tsutsugamushi* Infection

Ji Hyun Yun

(Supervised by Professor Young-Sang Koh)

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of
Docotor of Philosophy
in Medicine

Date Approved: _____

_____

_____

_____

_____

_____

Department of Medicine
Graduate School
Cheju National University
June, 2006

ABSTRACT

Orientia tsutsugamushi, an obligate intracellular bacterium, is the causative agent of scrub typhus. The disease is histopathologically characterized by inflammatory manifestations, indicating that orientiae induce mechanisms that amplify the inflammatory response. To understand the pathogenesis of scrub typhus, I examined the induction of tumor necrosis factor alpha (TNF-α) after infection with *O. tsutsugamushi* in mice, peritoneal macrophage, and macrophage cell line. Peak expression of TNF-α gene was observed between 4 and 8 days after infection in mice. The TNF-α mRNA were induced and showed a transitory peak for 6 to 24 h after infection in macrophage cell line. TLR4 defective mice were found to lack the ability to respond to *O. tsutsugamushi* as measured by secretion of TNF-α by macrophages. Western blot analysis of cell lysates indicates that extracellular signal-regulated kinase 1/2 (ERK1/2), Jun N-terminal kinase 1/2 (JNK1/2), and p38 mitogen activated protein kinases (MAPKs) become phosphorylated, and hence activated in *O. tsutsugamushi*-stimulated macrophages. Selective inhibitors of ERK1/2 (PD98059), JNK1/2 (SP600125), and p38 (SB203580) MAPK pathways could all completely prevent TNF-α secretion. However, these drugs did not prevent either bacterial internalization and invasion into the host cells or TNF-α processing and secretion. Host TNF-α production via p38 and JNK pathways by this bacterium was found to be regulated by post-transcriptional mechanism, mainly by translational control. In contrast, ERK pathway mainly control the transcription step of TNF-α gene expression. Orientia inactivation by heat did not abolish induction of TNF-α

production. However, inhibition of cellular invasion by treatment of host cell with cytochalasin D led to a diminished TNF-α induction, suggesting requirement of invasion by bacteria for this host cell response. In conclusion, our data indicate that MAPKs pathways are required to induce maximal TNF-α production in host cells during *Orientia tsutsugamushi* infection.

Key words: *Orientia tsutsugamushi,* TNF-α, ERK, JNK, p38, signal transduction, regulation of gene expression

CONTENTS

	Page
ABSTRACT	i
CONTENTS	iii
LIST OF TABLE	v
LIST OF FIGURES	vi
LIST OF ABBREVIATIONS	viii
INTRODUCTION	1
MATERIALS AND METHODS	8
1. Reagents	8
2. Bacteria	8
3. Mice	10
4. Cell culture and infection	11
5. Cytotoxicity test	12
6. Indirect immunofluorescent antibody (IFA) test	12
7. RNase protection assay	13
8. Semiquantitative reverse transcriptase(RT)-PCR analysis	14
9. ELISA assay	15
10. Western blot analysis	16
RESULTS	18
1. *O. tsutsugamushi* induces TNF-α gene expression *in vivo* and *in vitro*	18

2. *O. tsutsugamushi*-stimulated TNF-α production is independent of TLR4 and LPS 21
3. *O. tsutsugamushi*-stimulated TNF-α gene expression does not require eukaryotic protein synthesis 23
4. Various inhibitors block *O. tsutsugamushi*-stimulated TNF-α secretion specifically 25
5. *O. tsutsugamushi* activates MAPKs pathways during infection 31
6. *O. tsutsugamushi*-stimulated TNF-α production is regulated via ERK pathway by transcriptional mechanism but regulated via p38 or JNK pathways by post-transcriptional and translational mechanism 34
7. *O. tsutsugamushi* induces NF-κB activation, but blockage of NF-κB activation by PDTC did not reduce *O. tsutsugamushi*-stimulated TNF-α induction 37
8. Bacterial invasion but not viability was required for *O. tsutsugamushi*-stimulated TNF-α production 40

DISCUSSION 43

REFERENCES 48

ABSTRACT IN KOREAN 55

ACKNOWLEDGMENTS 56

LIST OF TABLES

Page

Table 1. Effects and working concentration of inhibitors used in this study 9

LIST OF FIGURES

Page

Figure 1. TNF-α mRNA exprssion in cells from infected mice and J774A.1 19
Figure 2. Time course of TNF-α protein production 20
Figure 3. Effect of polymyxin B or TLR4 mutation on *O. tsutsugamushi*-stimulated TNF-α production in macrophage 22
Figure 4. Effect of cycloheximide (CHX) on *O. tsutsugamushi*-stimulated TNF-α gene expression in J774A.1 24
Figure 5. Effect of various inhibitors on *O. tsutsugamushi*-stimulated TNF-α production in J774A.1 26
Figure 6. Effect of *O. tsutsugamushi* or/and each inhibitor on the viability of J774A.1 cells 27
Figure 7. Effect of cytochalasin D or TPCK on the bacterial invasion into J774A.1 28
Figure 8. Effect of PD98059 or SB203580 on the bacterial invasion into J774A.1 29
Figure 9. Effect of SP600125 on the bacterial invasion into J774A.1 30
Figure 10. Time-dependent phosphorylation of MAPKs of J774A.1 by *O. tsutsugamushi* infection 32
Figure 11. Effects of various MAPK inhibitors on *O. tsutsugamushi*-induced phosphorylation of MAPKs in J774A.1 33

Page

Figure 12. Effect of PD98059 or SB203580 on *O. tsutsugamushi*-stimulated TNF-α production in J774A.1 35

Figure 13. Effect of SP600125 on *O. tsutsugamushi*-stimulated TNF-α production 36

Figure 14. Time course of IκBα degradation during *O. tsutsugamushi* infection and effects of TPCK or PDTC on *O. tsutsugamushi*-stimulated IκBα degradation 38

Figure 15. Effect of TPCK or PDTC on *O. tsutsugamushi* stimulated TNF-α production in J774A.1 39

Figure 16. Effect of cytochalasin D (CD) or heat-inactivated *O. tsutsugamushi* (HOT) on *O. tsutusgamushi*-induced TNF-α production and IκBα degradation in J774A.1 42

Figure 17. Model of signaling and gene regulatory mechanisms of TNF-α biosynthesis during *O. tsutsugamushi* infection 46

LIST OF ABBREVIATIONS

AP-1	Activator protein 1
ATF-2	Activating transcription factor 2
BSA	Bovine serum albumin
CD	Cytochalsin D
CBP	CREB binding protein
CHX	Cycloheximide
c-FOS	v-fos oncogene cellular homolog
c-Myc	Avian myelocytomatosis virus oncogene cellular homolog
CREB	cAMP response element-binding protein
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
dNTP	deoxynucleotide triphosphate
ELISA	Enzyme linked immunosorbent assay
Elk-1	Ets-like transcription factor
ERK	Extracellular signal-regulated kinases
Eta-1	Early T-lymphocyte activation 1
Ets-1	E26-AMV virus oncogene cellular homolog, a transcription factor
HBSS	Hank's balanced salt solution
HRP	Horseradish peroxidase
HSF-1	Heat shock factor 1
HSP27	Heat shock protein 27

ICU	Infected-cell counting units
IFN-ɣ	Interferon-gamma
IκB	Inhibitor of nuclear factor kappa B
IKK	IκB kinase
IL-8	Interleukin 8
IRAK	IL-1R-associated kinase
IP-10	IFN-ɣ-inducible 10 protein
JNK	N-terminal c-Jun kinases
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
MEF2	Myocyte enhancer factor 2
MEK	MAPK/Erk kinase
MIP-1 α	Macrophage inflammatory proteins 1 α
MIP-1 β	Macrophage inflammatory proteins 1 β
MIP-2	Macrophage inflammatory proteins 2
MK2	MAPK activated protein kinase 2
MK3	MAPK activated protein kinase 3
MKP-1	MAPK phosphatase-1
MNK	MAP kinase interacting kinase
MSK	Mitogen and stress activated kinases
MTT	Thiazolyl blue tetrazolium bromide
MyD88	Myeloid differentiation primary-response protein 88
NOD	Nucleotide-binding site and leucine-rich repeat (NBS-LRR) domains

NF-AT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor-kappa B
NK cell	Natural killer cell
Op	Osteopontin
p300	A histone acetyltransferase
p53	Tumor suppressor protein that protects from DNA damage
PAMP	Pathogen-associated molecular pattern
Pax	Paxillin
PB	Polymyxin B sulfate
PBS	Phosphate-saline buffer
PD	PD98059
PDTC	Pyrrolidinedithiocarbamate
PEC	Peritoneal exudate cells
PRAK	p38 regulated activated kinase
PRR	Pattern recognition receptor
PVDF	Polyvinylidene fluoride
RANTES	Regulated upon activation, normal T-cell expressed and secreted
RIP2	Receptor interacting protein 2
RSK	Ribosomal protein S6 kinase
SB	SB203580
SP	SP600125
STAT	Signal transducer and activator of transcription
Syk	A tyrosine-protein kinase

TBST	Tris buffered saline containing 0.1% Tween-20
TEPM	Thioglycollate exudate peritoneal macrophages
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
TNFR	TNF receptor
TRAF	Tumor necrosis factor receptor-associated factors
TPCK	*N*-tosyl-L-phenylalanine chloromethyl ketone

INTRODUCTION

Scrub typhus (tsutsugamushi disease), caused by an intracellular bacterium *Orientia tsutsugamushi*, is one of the most prevalent febrile illnesses in South Korea (Chang *et al.*, 1990). Tsutsugamushi disease is characterized by fever, rash, eschar, pneumonitis, meningitis, and disseminated intravascular coagulation, which leads to severe multiple organ failure in untreated cases (Allen *et al.*, 1945; Chi *et al.*, 1997). This disease extends from northern Japan and far eastern Rusia in the North, to northern Australia in the south, and to Pakistan and Afganistan in the west (Oaks *et al.*, 1983). In addition to the three prototype strains, Gilliam, Karp and Kato, more than 30 antigenically distinct serotypes are present in the definite geographic region (Enatsu *et al.*, 1999). The bacteria is approximately 0.5 ㎛ wide and 1.2-3.0 ㎛ long (Tamura *et al.*, 1991). Phylogenetic analysis of 16S rRNA sequence homology proved that *O. tsutsugamushi* locates in the Proteobacteria α-subdivision and this suggests that *O. tsutsugamushi* is a member of Gram-negative bacteria (Ohashi *et al.*, 1995). But this bacteria has little or no peptidoglycan or lipopolysaccharide components (Amano *et al.*, 1987). Bacterial doubling time is about 8-9 h (Kawamura *et al.*, 1995). It propagates in host cytoplasm by binary fission (Tamura *et al.*, 1988). This bacterium infects a variety of host cells *in vitro* and *in vivo*, including macrophages, polymorphonuclear leukocytes (PMN), lymphocytes, and endothelial cells, where it replicates in the cytoplasm without being surrounded by a phagolysosomal membrane (Ng *et al.*, 1985; Rikihisa *et al.*, 1979; Cho *et al.*, 2000). *O. tsutsugamushi* causes local inflammations accompanying eschars at the site of infection, which then spread systemically (Burnett

et al., 1980). Inflammation is initiated by *O. tsutsugamushi*-infected macrophages and endothelial cells in the dermis.

Analysis of early immunologic responses to *O. tsutsugamushi* infection in mice showed that macrophage-mediated cellular immunity is essential for resolution of this infection (Nacy *et al.*, 1981), since macrophages are one of the potent effector cells that are able to remove bacterial pathogens. Resistance to the lethal effects of acute rickettsial infection is under unigenic dominant control by the *Ric* locus (Groves *et al.*, 1980). Macrophages infiltrate both susceptible (Ric^s) and resistant (Ric^r) mouse strains in response to *O. tsutsugamushi* Gilliam infection (Nacy *et al.*, 1981; Jerrells *et al.*, 1981). Susceptible mice died within 2 weeks of infection, whereas resistant strains showed that inflammatory response was significantly reduced over 2 weeks and survived the infection (Nacy *et al.*, 1981; Jerrells *et al.*, 1981). Early host inflammatory responses seem to play a key role in determining the fate of the host infected with *O. tsutsugamushi* (Nacy *et al.*, 1981; Jerrells *et al.*, 1981). The explanation for a susceptible/resistant mouse phenotype to *O. tsutsugamushi* infection was provided by the analysis of the early T-lymphocyte activation 1 (*Eta*-1)/osteopontin (*Op*) gene, which maps to the *Ric* locus (Groves *et al.*, 1980; Patarca *et al.*, 1989). The expression of Eta-1 represents an essential early step in the pathway that leads to Th1 immunity (Ashkar *et al.*, 2000). Eta-1/Op has been thought to enchance resistance to rickettsial infection by affecting the ability of macrophages to migrate to sites of infection and/or to express bacterial activity (Patarca *et al.*, 1989).

In general, inflammatory cytokines have been known to determine the fate of T-cell development (Hedrick *et al.*, 1999). Proinflammatory cytokines and chemokines are the main factors responsible for the

recruitment of distinct leukocytes into inflamed tissues during inflammatory disease (Baggiolini *et al.*, 1994). Members of the CC chemokine subfamily such as regulated upon activation, normal T-cell expressed and secreted (RANTES), macrophage inflammatory proteins 1 α /β (MIP-1 α/β), monocyte chemoattractant protein 1 (MCP-1) preferentially attract monocytes and lymphocytes, while those of the CXC chemokine subfamily such as interleukin 8 (IL-8) and MIP-2 are potent neutrophil attractants (Baggiolini *et al.*, 1994). The chemokines RANTES, MIP-1α, MIP-1β were found to efficient chemoattractants for Th1 cells whereas MCP-1 provokes Th2 cell development (Siveke *et al.*, 1998). Thus, chemokine profile induces the kinetics of infiltration of different kinds of inflammatory cells. It may be important that regulation of cytokine gene expression and the resulting macrophage activation can contributes to host immune response during *O. tsutsugamushi* infection. Recently, the chemokine and cytokine family produced during infection by *O. tsutsugamushi* has been reported. The mRNAs levels that were upregulated included RANTES, MIP-1 α/β, MCP-1, MIP-2, interferon-gamma-inducible 10 protein (IP-10), TNF-α after *O. tsutsugamushi* infection in murine macrophages (Cho *et al.*, 2000; Koh, 2001). Gene induction in mice was followed by the secretion of various chemokines and cytokine proteins including TNF-α and interferon-gamma (IFN-ɣ) (Koh *et al.*, 2004; Yun *et al.*, 2005). *O. tsutsugamushi* stimulates TNF-α production in a macrophages cell line (Choi *et al.*, 1998) as well as in mice. It was found that the spleen cells from mice immunized with *O. tsutsugamushi* produced TNF-α in response to *O. tsutsugamushi* antigens, and TNF-α activity was found in the sera of immune mice after injection with the rickettsial antigen (Jerrells *et al.*, 1994). Significant increases in TNF-α levels in serum were observed during the

convalescent phase in patients (Iwasaki *et al.*, 1997). Cytokine hyper-production is associated with host susceptibilty by *O. tsutsugamushi* infection (Yun *et al.*, 2005). It was known that TNF-α plays a critical role in host defense, immune regulation and inflammatory responses. Whereas TNF-α has numerous beneficial roles in immune regulation, TNF-α has been implicated in the pathogenesis of both acute and chronic inflammatory disease (Beutler *et al.*, 1986). However, mechanisms which are involved in *O. tsutsugamushi*-induced TNF-α induction are little investigated.

Innate immunity is triggered when pathogen-associated molecular patterns (PAMPs) that are shared by group of microbial pathogens are recognized by pattern recognition receptors (PRRs) in host (Medzhitov *et al.*, 1997). Microbial products such as lipopolysaccharide (LPS) induce production of cytokines and other proteins of the inflammatory response. TNF-α is one of the most potent cytokines and plays a central role in various immune and inflammatory phenomena (Vassalli, 1992; Pasparakis *et al.*, 1996). TNF-α is produced by various cell types including macrophages, lymphocytes and natural killer (NK) cells. Mitogens such as LPS or PMA/ionomy stimulate TNF-α induction in various cell types. The most powerful inducer of TNF-α production by macrophages is the bacterial LPS (sanghera *et al.*, 1996; Medvedev *et al.*, 1998). Microbial products as ligands bind to receptors, which initiate the signal transduction cascade leading to TNF-α production.

Toll-like receptors (TLRs) are one of the PRRs and the signaling receptors and play a central role in early host defense against microbial pathogens. Different TLRs can recognize PAMPs. For example, TLR4 is involved in recognition of LPS components of Gram-negative bacteria, TLR2 detects Gram-positive cell wall components, bacterial

lipoproteins/lipopeptides, mycobacterial components and zymosan of fungi. TLR1, TLR2 and TLR4 are located on the cell surface and are recruited to phagosomes after activation. In contrast, TLR3, TLR7 and TLR9 are not expressed on the cell surface and are involved in the recognition of nucleic acid-like structures (Matsumoto *et al.*, 2003; Ahmad-Nejad *et al.*, 2002; Heil *et al.*, 2003). These signaling pathways control the expression of genes of the inflammatory response at both transcriptional and post-transcriptional levels.

Signal transduction relies on mitogen-activated protein kinase (MAPK) and nuclear factor-kappa B (NF-κB) pathways. Once MAPK were activated, they phosphorylate and regulate a variety of transcription factors. MAPKs include extracellular signal-regulated kinases (ERK1/ERK2), p38 MAPK, N-terminal c-Jun kinases (JNKs). ERK is activated by a variety of receptors involved in growth and differentiation. Activated ERK translocate to the nucleus where it phosphorylate a variety of transcription factors regulating gene expression. Activated ERK1 and ERK2 phosphorylate numerous substrates in all cellular compartments including various membrane proteins such as CD120a, a tyrosine-protein kinase (Syk), and calnexin, and nuclear substrates such as paxillin (Pax6), nuclear factor of activated T-cells (NF-AT), ets-like transcription factor (Elk-1), myocyte enhancer factor 2 (MEF2), v-fos oncogen cellular homolog (c-Fos), avian myelocytomatosis virus oncogene cellular homolog (c-Myc), and signal transducer and activator of transcription 3 (STAT3), and cytoskeletal proteins such as neurofilaments and paxillin, and several MAPKAPKs (MKs) (Chen *et al.*, 2001). Ribosomal protein S6 kinases (RSKs), mitogen and stress activated kinases (MSKs), and MAP kinase interacting kinases (MNKs) represent three kinase subfamilies of ERK1/2 substrates. While MSKs and MNKs have been shown to be activated by

both the ERK1/2 and p38 pathways, RSK family members are exclusively activated by the ERKs (Frodin *et al.*, 2000).

JNK and p38 MAPK are activated by a variety of stimuli including environmental stresses and inflammatory cytokines. p38 MAPK is involved in regulation of MK2, MK3, and several transcription factors including activating transcription factor 2 (ATF-2), Stat1, Max/Myc complex, MEF-2, NF-κB, E26-AMV virus oncogene cellular homolog, a transcriptiion factor (Ets-1), tumor suppressor protein that protects from DNA damage (p53), Elk-1, and cAMP response element-binding protein (CREB) via MSK1 (Kyriakis *et al.*, 2001;Roux *et al.*, 2004). p38 also activates MSK1/2, MNK1, p38 regulated activated kinase (PRAK), heat shock protein 27 (HSP27) (Roux *et al.*, 2004). JNK translocate to the nucleus where it can regulate the activity of multiple transcription factors. A well-known substrate for JNKs is the transcription factor c-Jun. Several other transcription factors have been shown to be phosphorylated by the JNKs, such as ATF-2, NF-ATc1, heat-shock facotr 1 (HSF-1), and STAT3 (Chen *et al.*, 2001; Kyriakis *et al.*, 2001).

NF-κB function as transcription factors that control genes regulating biological processes including innate and adaptive immunity, inflammtion, stress responses and B cell development. After activation by upstream signals, inhibitor of nuclear factor kappa B [IκB] kinase complex (IKK complex) phosphorylates the IκB leading to a polyubiquitination and preteasome-mediated degradation. And then NF-κB is activated and translocates to nucleus where, either alone or in combination with other transcription factor families including activator protein 1 (AP-1: c-JUN and c-FOS), Ets and STAT, they induce target gene expression.

However pattern recognition receptors and signal transduction mechanisms involved in *O. tsutsugamushi*-induced TNF-α production have

not yet been investigated. To address this issue, signal transduction and regulation of TNF-α gene expression during *O. tsutsugamushi* infection was investigated in this study.

MATERIALS AND METHODS

1. Reagents

Lipopolysaccharide (LPS, at a final concentration of 1 ㎍/ml) derived from *Escherichia coli* (Sigma, MO, USA) was used. In the inhibition assays, J774A.1 cells were preincubated with *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK, Sigma), pyrrolidinedithiocarbamate (PDTC, Sigma), PD98059 (PD, Calbiochem, CA, USA), SB203580 (SB, Calbiochem) and SP600125 (SP, Sigma) for 1 h before *O. tsutsugamushi* was inoculated. J774A.1 cells were also pretreated for 1 h with cycloheximide (CHX, Sigma) or cytochalsin D (CD, Sigma) before *O. tsutsugamushi* was inoculated. Monensin (Becton Dickinson Biosciences, CA, USA) was added to J774A.1 cells for 1 h before infection of O. *tsutsugamushi*. Also inhibitors were maintained during the course of inhibition assays. To exclude the possible LPS contamination in the medium or in the inoculum, polymyxin B sulfate (PB, Sigma) was added to the cell culture to neutralize LPS. Heat-inactivated bacterial inoculum was obtained by heating *O. tsutsugamushi* at 56℃ for 30 min. The effects and working concentration of inhibitors used in this study are shown in Table 1.

2. Bacteria

O. tsutsugamushi Boryong and Gilliam (Seoul National University

Table 1. Effects and working concentration of inhibitors used in this study.

Inhibitor	Effects	Working conc.
Polymyxin B	Binds to the lipid A portion of bacterial LPS and change the membrane permeability	30 ㎍/㎖
Cytochalasin D	Inhibitor of actin polymerization	1 ㎍/㎖
Cycloheximide	Inhibits eukaryotic protein synthesis	50 ㎍/㎖
TPCK	Blocks the LPS-/cytokine-induced activation of NF-κB (proteasome inhibitor)	50 ㎛M
PDTC	Prevents induction NO synthetase (anti-oxidants) Inhibits NF-κB activation	25 ㎛M
PD98059	Inhibitor of MEK (MEK1/2)	50 ㎛M
SB203580	Inhibitor of p38	20 ㎛M
SP600125	Inhibitor of JNK (JNK1/2)	20 ㎛M
Monensin	Blocks the intracellular protein transport processes	2 ㎛M

Colleage of Medicine, Korea) was propagated in monolayers of L-929 cells as described previously (Kim *et al.*, 1993). When more than 90% of the cells were infected, as determined by an indirect immunofluorescent-antibody technique (Chang *et al.*, 1990), the cells were collected, homogenized with a glass Dounce homogenizer (Wheaton Inc., NJ, USA), and centrifuged at 500 × *g* for 5 min at 4℃. The supernatant was recovered and the stored in liquid nitrogen until use. The infectivity titer of the inoculum was determined (Tamura *et al.*. 1981). Five-fold serially diluted oriential samples were inoculated onto L-929 cell layers on 24-well tissue culture plates. After 3 days of incubation, the cells were collected, fixed, and stained (Chang *et al.*, 1990). The ratio of infected cells to the counted number of cells was determined microscopically, and infected-cell counting units (ICU) of the oriential sample were calculated as followed (Tamura *et al.* 1981): ICU = (total number of cells used in infection) × (percentage of infected cells) × (dilution rate of the orientiae suspension)/100. A total of 1×10^6 to 1×10^8 ICU of *O. tsutsugamushi* was used to infect cultured cells.

3. Mice

Specific pathogen free, female C3H/HeN and C3H/HeJ mice purchased from SLC Inc. (Japan) were kept in the animal facility located in the Cheju National University College of Medicine. Utmost precautions were taken so the mice remained free from infection environmental pathogens. All mice used for peritoneal macrophages were 6 to 8 weeks old. Animal procedures were performed according to the guidelines of the Laboratory Animal Care Committee of Cheju National University College of Medicine.

4. Cell culture and infection

J774A.1 cells and L-929 cells were obtained from the American Type Culture Collection and cultured in Dulbecco's modified eagle medium (DMEM, GIBCO, NY, USA) containing 10% heat-inactivated fetal bovine serum (FBS, GIBCO), 100 U of penicillin G per ml, 100 ㎍ of streptomycin per ml (DMEM-10) in a humidified 5% CO_2 atmosphere at 37℃. J774A.1 cells cultured in six-well plates for the preparation of total RNA, in 24-well plates for the measurement of TNF-α production, and in 40-mm dishes for the examination of MAP kinase activity.

Thioglycollate exudate peritoneal macrophages (TEPM) were obtained from mice that had been injected intraperitoneally 3 days previously with 1 ml of 4% Brewer's thioglycollate broth (Difco Laboratories, MI, USA) by peritoneal lavage with 10 ml of phosphate-saline buffer (PBS). The cells were pelleted, washed with cold PBS and resuspended in DMEM-10. The cells were counted on a hemacytometer and then plated in 24-well plates (Corning Inc., NY, USA). Nonadherent cells were removed after 2 h of incubation in a humidified 5% CO_2 atmosphere at 37℃ by washing the cells three times with cold PBS. Adherent macrophages were then cultured in a humidified 5% CO_2 atmosphere at 37℃ for 18 h before initiation of each experiment.

For RNase protection assay, infected mice were sacrificed by cervical dislocation, and peritoneal exudate cells (PEC) were harvested by washing the peritoneal cavity with 5 ml of Hanks' balanced salt solution (HBSS, GIBCO) containing 10 U/ml heparin. Peritoneal lavage fluid obtained from the two washings were pooled, spun in a centrifuge at 520 × *g* for 5 min at 4℃, and supernatants were removed and stored at -70℃ for cytokine analysis. Cell pellets were washed once with cold HBSS,

and resuspended in PBS. PEC suspension was snap-frozen in liquid nitrogen, and stored at -70℃ for RNA analysis.

5. Cytotoxicity test

The effect of the inhibitors on the J774A.1 cell growth was determined by measuring the metabolic activity using a Thiazolyl blue tetrazolium bromide (MTT) assay (Carmichael *et al.*, 1987). J774A.1 cells were dispensed to 48-well culture plates at a concentration of 1×10^5 cells/ml, and incubated for 24 h at 37℃ in 5% CO_2. After the cells were treated for 24 h or 6 h with inhibitors, 200 ㎕ of MTT (Sigma) was added to each well and cells continuously were incubated at 37℃ for 4 h. During this incubation period, viable cells convert MTT to a water-insoluble formazan dye. The plates were centrifuged at 500 × *g* for 20 min at room temperature and supernatants were removed carefully. 250 ㎕ of dimethyl sulfoxide (DMSO, Sigma) was added to each well and the plates were gently rocked to resolve the formazan dye. Absorbance was measured at wavelength of 540 nm. The results were calculated the percentage of inhibition or viability.

6. Indirect immunofluorescent antibody (IFA) test

O. tsutsugamushi Boryong-infected cells were collected for IFA staining (Chang *et al.*, 1990). The infected cells were centrifuged at 500 × *g* for 5 min and supernatants were removed. And then the cells were resuspended with PBS. Repeat the process two times for a total of three

washes. The cells were resuspended with PBS and were mixed well by vortexing. The cells were smeared onto spot slides and fixed with acetone. Human sera reacting with *O. tsutsugamushi* were kindly provided by Dr. Ik-Sang Kim, Seoul National University College of Medicine. The cells on slides were reacted with this sera at 37℃ for 30 min. After three washes with PBS, goat anti-human IgG-FITC conjugate (Caltag, Burlingame, CA) was added, and incubated at 37℃ for 30 min. After three washes, counter-staining was performed with 0.003% evans blue (Sigma) solution in PBS. After wash with distilled water, the stained cells were mounted with glycerol and examined with a fluorescene microscope.

7. RNase protection assay

Total RNA was extracted from spleen tissue or PEC from uninfected and infected C3H/HeN mice using Trizol reagent (GIBCO) and was quantitated spectrophotometrically. Detection and quantitation of murine TNF-α mRNA was performed with the RiboQuant multiprobe RNase protection assay kit from PharMingen (CA, USA). In brief, a mixture of [^{32}P]UTP-labeled antisense riboprobes was generated from a panel of different cytokine or chemokine template DNAs. These panels also included templates for the murine housekeeping gene encoding L32 to ensure equal loading of total RNA onto the gels. Total RNAs from each sample (30 ㎍ each) were hybridized overnight at 56℃ with 3×10^6 cpm of the ^{32}P-labeled antisense riboprobe mixture. After hybridization, the samples were digested with a mixture of RNases A and T1. Nuclease-protected RNA fragments were precipitated with ethanol. The samples were resolved on a 5% polyacrylamide sequencing gel. The

bands were observed after autoradiography. The specific bands were identified on the basis of their individual mobilities compared with the undigested probes. The band intensities shown in autoradiography were digitized by scanning the images and analyzed using a Gel Doc 2000 Gel Documentation System and Quantity One software (Bio-Rad, CA, USA). The densitometric intensity was normalized with respect to the intensities of the band for the housekeeping genes, L32.

8. Semiquantitative reverse transcriptase (RT)-PCR analysis

Total RNA was prepared with SV total RNA isolation system (Promega, WI, USA) according to the manufacturer's instructions. Amount of RNA was determined spectrophotometrically. After about 1 ㎍ total RNA extracted from each sample was treated with DNase (Promega), it was subjected to first-strand cDNA synthesis at 42℃ for 30 min in a 20 ㎕ reaction mixture containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 5 mM $MgCl_2$, 1 mM deoxynucleotide triphosphate mixture (dNTP mixture), 1 u/㎕ RNasin, 0.5 ㎍ Oligo$(dT)_{15}$ primer, and 15 u/㎍ AMV Reverse Transcriptase (Promega). The cDNA was heated at 95℃ for 5 min and diluted with nuclease-free water. The diluted cDNA were subjected to PCR amplification in a 25 ㎕ reaction mixture containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM dNTP mixture, 0.1 ㎛M each primer, and 1.25 U of *Taq* DNA polymerase (Promega) in a GeneAmp PCR system 9600 (Perkin-Elmer, CT, USA). Primer sequences of TNF-α were as follows: 5'-GCG ACG TGG AAC TGG CAG AAG-3' (forward primer) and 5'-TCC ATG CCG TTG GCC AGG AGG-3' (reverse primer), giving a 340 bp

product. Primer sequences of β-actin were as follows: 5'-TGG AAT CCT GTG GGA TCC ATG AAA C-3' (forward primer) and 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3' (reverse primer), giving a 349 bp product. The reaction mixture was prepared as a master mixture to minimize reaction variation. One PCR cycle consisted of denaturation at 95℃ for 30 s, annealing at 55℃ for 30 s, and extension at 72℃ for 1min. During the first cycle, the 95℃ step extended to 2 min, and on the final cycle the 72℃ step extended to 5 min. 10 ㎕ of the PCR products were electrophoresed in a 2% agarose gel containing 0.5 ㎍ of ethidium bromide per ml. 100bp DNA ladder (Promega) was used 1 ㎍/lane as a molecular size marker. The amplified DNA fragment in the gels were identified and the densities of the bands were analyzed using a Gel Doc 2000 Gel Documentation System and Quantity One software (Bio-Rad). The densitometric intensity was normalized by comparing the ratio of TNF-α bands with that of β-actin.

9. Enzyme linked immunosorbent assay (ELISA).

J774A.1 cells and peritoneal macrophage were dispensed to plates at a concentration of 2×10^5 cells/ml and cultured for 24 h. After 6 h infection of *O. tsutsugamushi* with and without inhibitors, supernatants were collected from culture dishes and were centrifuged at 500 × *g* for 15 min at 4℃. In some cases, J774A.1 cells were infected for 2 h with each inhibitor in the absence or presence of monensin. And then bacterial inocula were removed from the cells and the cells were incubated for 4 h with each new medium and inhibitor in the absence or presence of monensin. For measurement of TNF-α levels inside cultured cell, the

cells were lyzed in lysis buffer (the same buffer for western blot analysis). The cell-free supernatants and cell lysate were analyzed for TNF-α using murine TNF-α ELISA kits (R & D systems, MN, USA) according to the manufacturer's instructions. The lower limits of detection of these assays were less than 5.1 pg/ml.

10. Western blot analysis

J774A.1 cells were dispensed to 40 mm culture dishes (Nunc, Roskilde, Denmark) at a concentration of 1×10^6 cells/ml and cultured for 24 h. After infection with *O. tsutsugamushi* with or without inhibitors or LPS or plain medium as a control, J774A.1 cells were collected at indicated time point and washed twice with ice-cold PBS. And then the cells were lyzed in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, IGEPAL CA-630, 2 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10 mM NaF, 1 mM DTT, 1 mM PMSF, 1 mM aprotinin, 1 mM leupeptin, 1 mM pepstatin A) by incubation on ice for 30 min. Lysates were then centrifuged at 13,000 × *g* for 15 min at 4℃. Protein concentration in each sample was determined using a Bicinchoninic acid protein assay kit (Sigma). A protein sample (30 ㎍) was electrophoresed in 10% SDS-polyacrylamide gels, transfered to a polyvinylidene fluoride (PVDF) membrane (Millipore, MA, USA), and the membrane was blocked in tris buffered saline containing 0.1% Tween-20 (TBST) supplemented with 5% milk for 1 h at room temperature. The membrane was incubated overnight with 1/1000-diluted primary polyclonal antibodies in 5% w/v bovine serum albumin (BSA), 1X TBS, 0.1% Tween 20 at 4℃ with gentle shaking. Antibodies were as follows: phospho-p44/42 MAP Kinase (ERK1/2), p44/42 MAP Kinase, phospho-p38

MAP Kinase, p38 MAP Kinase, phospho-SAPK/JNK, SAPK/JNK and IκBα (Cell Signaling Technology, Inc., MA, USA). After washing, the membrane was incubated with a horseradish peroxidase (HRP)-linked anti-rabbit IgG (Cell Signaling Technology, Inc.) as secondary antibody at a 1/2000 dilution for 1 h at room temperature. Immunoactive bands were detected using the WEST-ZOL plus Western blot detection system (iNtRON Biotechnology, Korea) according to the manufacturer's instructions.

RESULTS

1. *O. tsutsugamushi* induces TNF-α gene expression *in vivo* and *in vitro*.

To determine whether *O. tsutsugamushi* stimulates TNF-α gene expression *in vivo* and *in vitro*, mRNA and protein levels of TNF-α during infection were analyzed in mice and murine J774A.1 cell (Figures 1 and 2). Before and after infection of mice with *O. tsutsugamushi*, the level of TNF-α mRNA in PEC and spleen was assayed at each time point by a RNase protection assay (Figures 1A and B). TNF-α transcript was constitutively expressed at low level in PEC from uninfected mice (Figure 1A). TNF-α mRNA induced as early as 2 day after infection, increased by 8 day, and peaked at 8 day after infection (Figure 1A). TNF-α mRNA increased as early as 2 day after infection, peaked at 4 day, and began to decrease or approach uninfected baseline levels by 8 day (Figure 1B). The induction fold of TNF-α gene expression in splenic tissue was significantly lower than that in PEC. This suggests that TNF-α induction during *O. tsutsugamushi* infection may be a localized event.

J774A.1 cells, murine macrophage cell lines, were infected with *O. tsutsugamushi* and the mRNA level of TNF-α was analyzed by semiquantitative RT-PCR (Figure 1C and D). The mRNA for TNF-α increased as early as 1 h after infection, persisted by 24 h then began to decrease by 48 h (Figure 1C and D). The induction of TNF-α gene expression increased significantly at the initial stage of infection. This indicates that upregulation of TNF-α gene expression during *O. tsutsugamushi* infection occured rapidly.

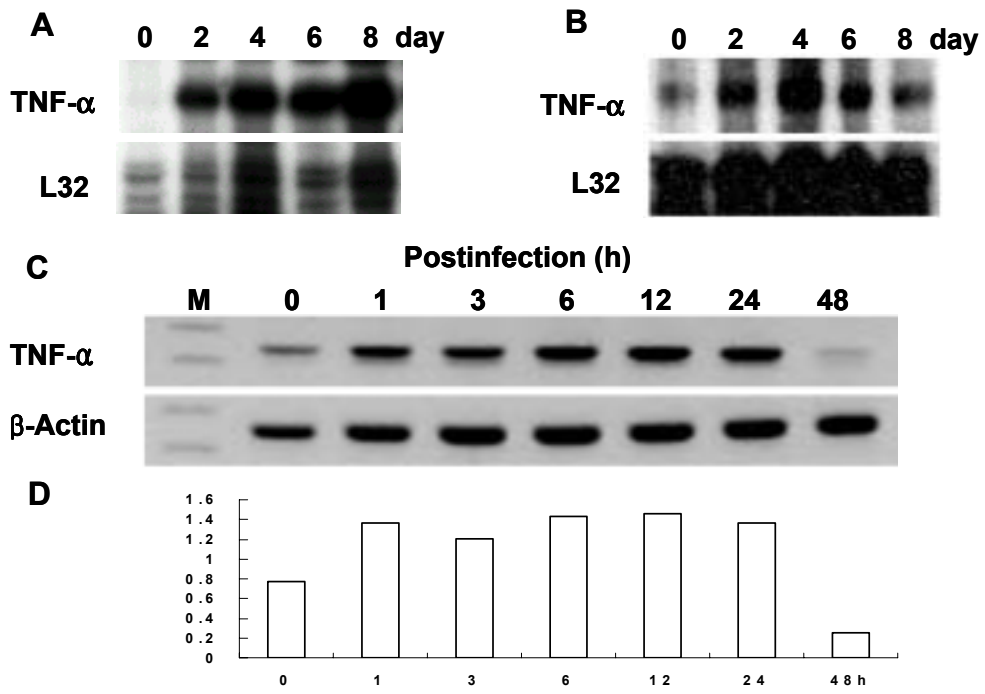

Figure 1. TNF-α mRNA expression in cells from infected mice and J774A.1. TNF-α mRNA expression of peritoneal exudate cells (PEC) (A) and splenic tissue (B) removed from C3H/HeN mice after infection *O. tsutsugamush*. At various times after infection of mice, total RNA was extracted from PEC and spleen, and mRNA expression was determined by RNase protection assay. L32, a murine ribosomal protein. (C) Determination of TNF-α mRNA induction in J774A.1 treated with *O. tsutsugamush*. Kinetics of *O. tsutsugamush*-stimulated TNF-α mRNA level induced by the infection of *O. tsutsugamushi* was analyzed by RT-PCR at each time point. M, 100-bp DNA ladder. (D) The band intensities were determined and normalized with mRNA level of β-actin.

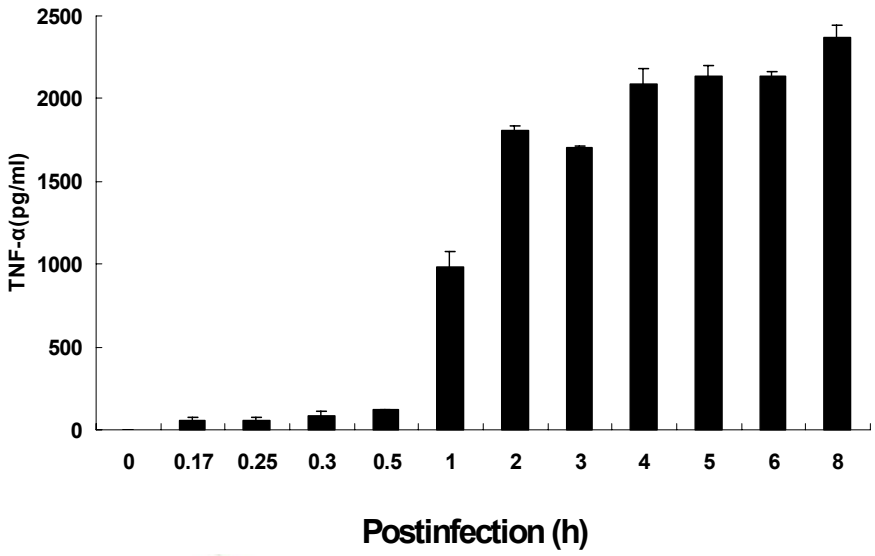

Figure 2. Time course of TNF-α protein production. Secreted TNF-α protein in macrophage J774A.1 cells infected with *O. tsutsugamushi* was measured. Supernatants were colleted at indicated time after the infection of *O. tsutsugamushi* and were tested for the presence of TNF-α protein using a ELISA kit. Mean ± SD from duplicate experiments.

The secretion of TNF-α protein was determined in *O. tsutsugamushi*-infected J774A.1 cells by ELISA assay (Figure 2). TNF-α protein from uninfected cells was undetectable (Figure 2). TNF-α secretion increased from 1 h after infection and persisted by 8 h (Figure 2). These results show that the production pattern of TNF-α protein correlats with mRNA induction pattern of TNF-α gene expression (Figure 1C and D).

2. *O. tsutsugamushi*-stimulated TNF-α production is independent of TLR4 and LPS.

To determine whether *O. tsutsugamushi*-stimulated TNF-α production is due to exogenous LPS contaimination or endogenous LPS components of the bacteria, TNF-α level was determined from cells treated with LPS or *O. tsutsugamushi* with or without polymyxin B (PB). PB is an inhibitor of LPS signaling. Therefore, LPS-stimulated TNF-α production was dramatically reduced in the presence of PB (Figure 3A). However, level of TNF-α production in the *O. tsutsugamushi*-infected cells with PB was not reduced, compared with that in *O. tsutsugamushi*-infected cells without PB (Figure 3B). These results reveal that possible LPS contamination in *O. tsutsugamushi* inoculum was not cause for stimulated TNF-α production and demonstrate that *O. tsutsugamushi* has no LPS.

Although *O. tsutsugamushi* activates adaptive immunity, the major PAMPs which play in the activation is not known. Since there is no LPS and very little (or no) peptidoglycan, it is likely that these bacteria do not activate TLR4. To ascertain whether *O. tsutsugamushi* activates TLR4 or not, peritoneal macrophage derived from both C3H/HeN ($Tlr4^{wild-type}$)

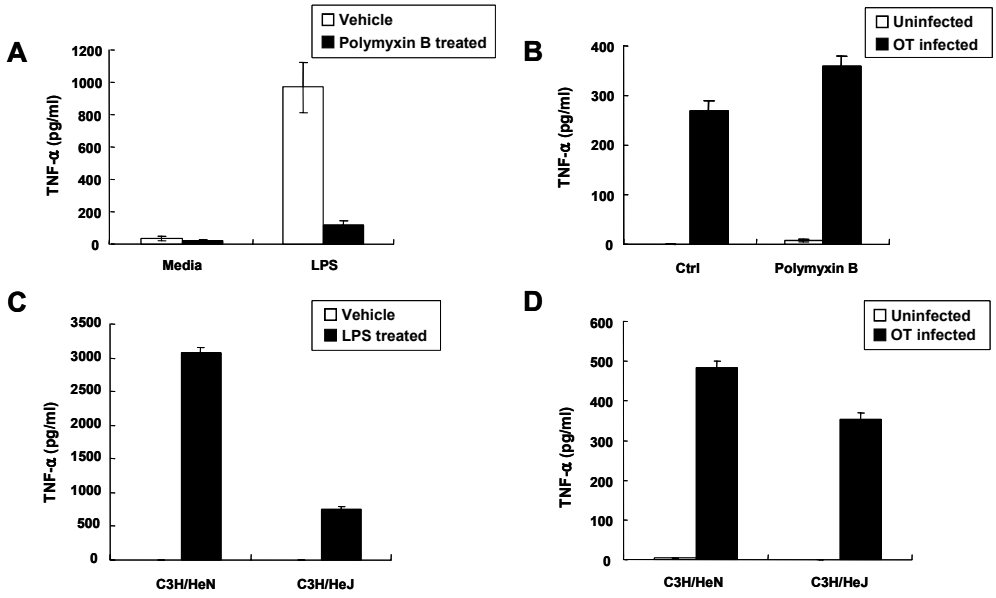

Figure 3. Effect of polymyxin B or TLR4 mutation on *O. tsutsugamushi*-stimulated TNF-α production in macrophage. (A and B) Determination of TNF-α production in J774A.1 cells treated for 6 h with LPS (1 ㎍/ml) or *O. tsutsugamushi* (OT) in the absence or presence of polymyxin B (30 ㎍/ml). (C and D) TNF-α production in infected C3H/HeN (*Tlr4*$^{wild-type}$) and C3H/HeJ (*Tlr4*$^{mutant-type}$) macrophages. Macrophages were stimulated with LPS or *O. tsutsugamushi*. Supernatants were collected and tested using a ELISA kit. Mean ± SD from duplicate experiments.

and C3H/HeJ (*Tlr4*$^{mutant-type}$) mice were stimulated with LPS or *O. tsutsugamushi* (Figure 3C and D). C3H/HeJ mice have mutation in TLR4 and are hyporesponder to LPS stimulation. So, LPS-stimulated TNF-α production in C3H/HeJ macrophages was greatly reduced compared with C3H/HeN macrophages (Figure 3C). However, *O. tsutsugamushi*-stimulated TNF-α production in C3H/HeJ macrophages did not decrease greatly compared with C3H/HeN macrophages (Figure 3D). These results suggest that TLR4 is probably not involved in *O. tsutsugamushi*-stimulated TNF-α production.

3. *O. tsutsugamushi*-stimulated TNF-α gene expression does not require eukaryotic protein synthesis.

TNF-α has its own receptor and can bind to the receptor and then can amplify the signal. Finally a variety of chemokines and cytokines are produced. To examine whether TNF-α induction was a direct consequence of *O. tsutsugamushi* infection, the cells were treated with cycloheximide (CHX), an inhibitor of eukaryotic protein synthesis. In the CHX-treated *O. tsutsugamushi*-infected cells, TNF-α production decreased, whereas the mRNA level of TNF-α did not decrease compared with the level of *O. tsutsugamushi*-infected cells without CHX (Figure 4). These results suggest that TNF-α induction was originated from *O. tsutsugamushi* infection and did not require newly synthesized host proteins.

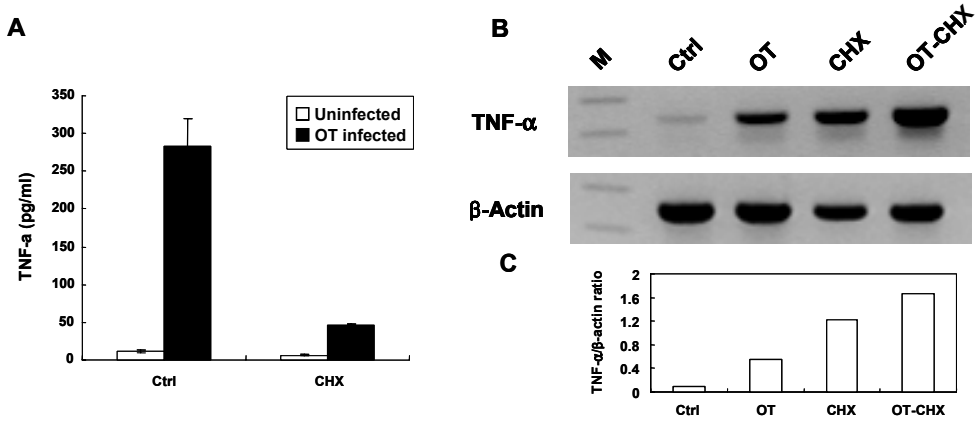

Figure 4. Effect of cycloheximide (CHX) on *O. tsutsugamushi*-stimulated TNF-α gene expression in J774A.1. (A) Determination of TNF-α secretion in J774A.1 cells infected *O. tsutsugamushi* with or without CHX. Cell-free supernatants were collected and tested using ELISA kit. Mean ± SD from three duplicate experiments. (B) Determination of TNF-α mRNA induction by RT-PCR analysis. (C) The band intensities were determined and normalized with mRNA level of β-actin. Ctrl, medium alone. OT, *O. tsutsugamushi*-infected cells. OT-CHX, *O. tsutsugamushi*-infected cells in the presence of CHX. M, size marker.

4. Various inhibitors block *O. tsutsugamushi*-stimulated TNF-α secretion specifically.

The effect of various inhibitors on TNF-α secretion in J774A.1 cells was assessed by ELISA assay (Figure 5). In the cells treated with cytochalasin D, the level of TNF-α secretion was dramatically reduced (Figure 5A). When TPCK was treated with the concentration of 50 μM, TNF-α production decreased greatly (Figure 5B). Treatment of MAPK inhibitors, PD98059, SB203580 and SP600125, also inhibited *O. tsutsugamushi*-stimulated TNF-α production (Figure 5C, D, and E). These results suggest that reduction in *O. tsutsugamushi*-stimulated TNF-α secretion was probably due to the blockage of signal pathways by each inhibitor.

To confirm whether the inhibitors used in this study have no inhibitory effect on the cell growth and hence the reduced *O. tsutsugamushi*-stimulated TNF-α secretion is not due to inhibitory effect of the drugs on cell viability, cytotoxicity tests were conducted (Figure 6). The indicated concentration of each inhibitor does not lead to significant cell death. The cell viability of inhibitor-treated cells approached the level of control group, except PDTC-treated cells (data not shown). PDTC has a cytotoxicity effect to some extent on the cells when treated with *O. tsutsugamushi* together. These results demonstrate that inhibitory effect on the cell growth by each inhibitor was not responsible for reduction in *O. tsutsugamushi*-stimulated TNF-α production.

To investigate inhibitors used in this study have inhibitory effect on bacterial invasion into host cells and hence the reduced *O. tsutsugamushi*-stimulated TNF-α production is due to reduction in bacterial invasion or not, IFA test was performed (Figures 7 to 9).

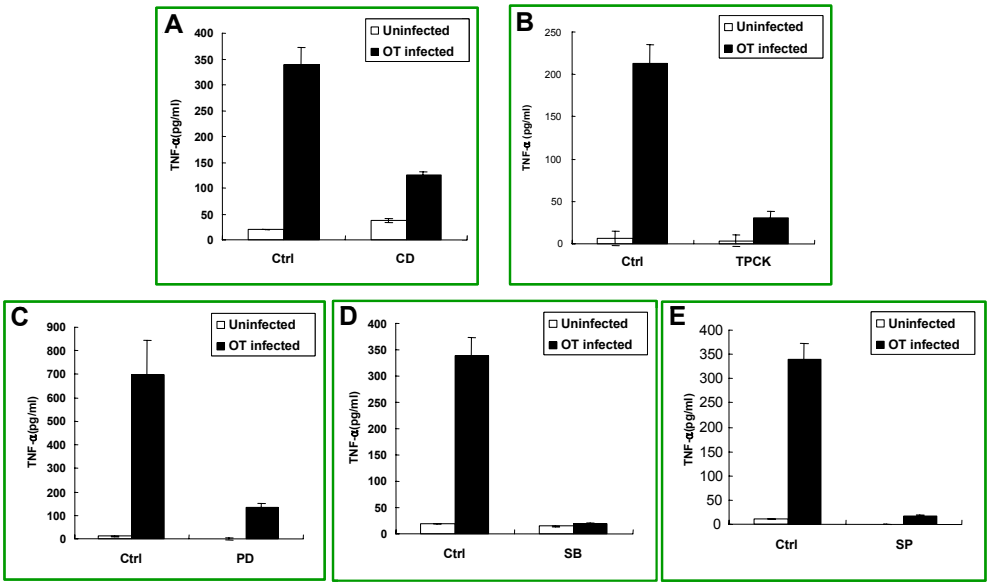

Figure 5. Effect of various inhibitors on *O. tsutsugamushi*-stimulated TNF-α production in J774A.1. J774A.1 cells were pretreated for 1 h with each inhibitor and were infected with *O. tsutsugamushi* in the presence of each inhibitor. Cell-free supernatants were collected and tested using ELISA kit. Mean ± SD from duplicate experiments. Ctrl, medium alone. CD, *O. tsutsugamushi*-infected cells in the presence of CD. TPCK, *O. tsutsugamushi*-infected cells in the presence of TPCK. PD, *O. tsutsugamushi*-infected cells in the presence of PD. SB, *O. tsutsugamushi*-infected cells in the presence of SB.

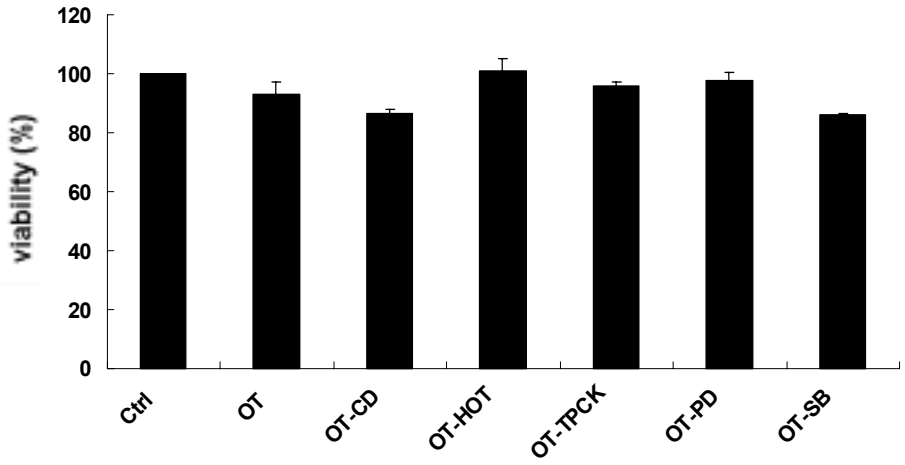

Figure 6. Effect of *O. tsutsugamushi* or/and each inhibitor on the viability of J774A.1 cells. J774A.1 cells were pretreated for 1 h with each inhibitor and then were infected with *O. tsutsugamushi* in the presence of each inhibitor. After infection, J774A.1 cells were incubated 24 h with new medium in the presence of each inhibitor. Cell viability was measured by MTT assay. Medium alone (Ctrl), *O. tsutsugamushi* (OT) or *O. tsutsugamushi*-infected cells in the presence of cytochalasin D (OT-CD, 1 ㎍/ml), heat-inactivated (OT-HOT), TPCK (OT-TPCK, 50 ㎛M), PD (OT-PD, 50 ㎛M) and SB (OT-SB, 20 ㎛M).

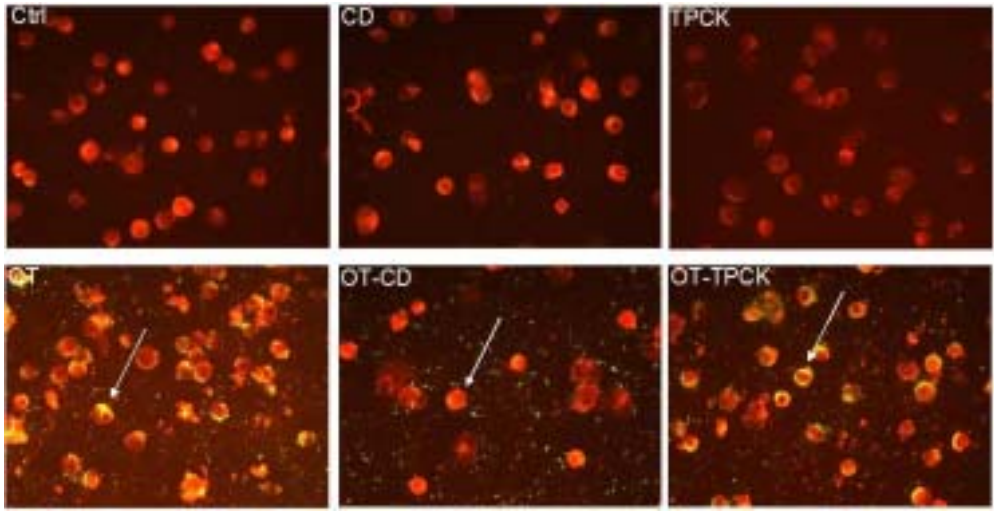

Figure 7. Effect of cytochalasin D or TPCK on the bacterial invasion into J774A.1. J774A.1 cells were treated with medium (Ctrl) or infected with *O. tsutsugamushi* (OT) with or without CD or TPCK (CD, TPCK, OT-CD, OT-TPCK). IFA test were performed. Stained cells were examined with a fluorescene microscope. J774A.1 cells are in red color and *O. tsutsugamushi* is in green color.

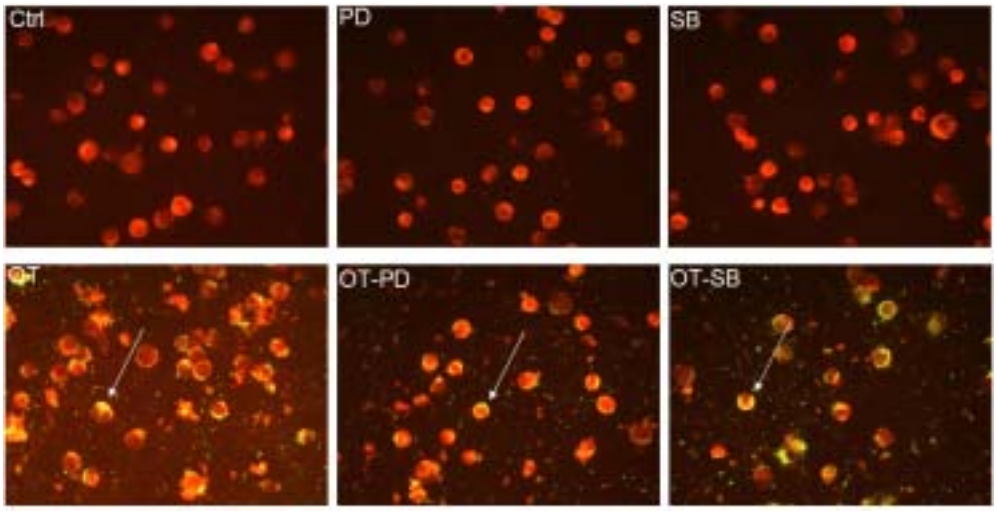

Figure 8. Effect of PD98059 or SB203580 on the bacterial invasion into J774A.1. J774A.1 cells were treated with medium (Ctrl) or infected with *O. tsutsugamushi* (OT) with or without PD98059 or SB203580 (PD, SB, OT-PD, OT-SB). IFA test were performed. Stained cells were examined with a fluorescene microscope. J774A.1 cells are in red color and *O. tsutsugamushi* is in green color.

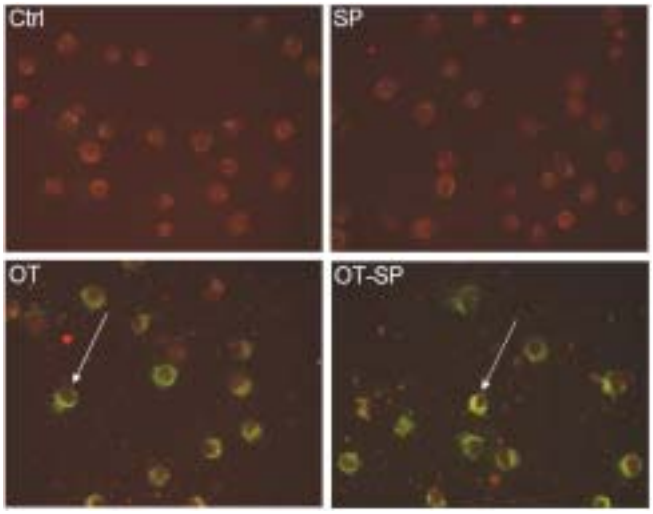

Figure 9. Effect of SP600125 on the bacterial invasion into J774A.1. J774A.1 cells were treated with medium (Ctrl) or infected with *O. tsutsugamushi* (OT) with or without SP600125 (SP, OT-SP). IFA test were performed. Stained cells were examined with a fluorescene microscope. J774A.1 cells are in red color and *O. tsutsugamushi* is in green color.

Treatment of cytochalasin D (CD) inhibited invasion of *O. tsutsugamushi* into the host cells (Figure 7). CD treatment just inhibited internalization of bacteria and did not inhibit attachment of bacteria (Figure 7). All other inhibitors did not interfere with invasion of *O. tsutsugamushi* into the cells (Figures 7 to 9). These results confirm that reduction of TNF-α secretion by drug treatment was not caused by interference of bacterial invasion.

5. *O. tsutsugamushi* activates MAPK pathways during infection.

All of the MAPK inhibitors inhibited TNF-α production. So, it is possible *O. tsutsugamushi* induces host MAPK activation during infection. To adress this issue, western blot analysis was performed. Western blot analysis of cell lysates revealed that ERKs, JNKs, and p38 MAPKs become phosphorylated, and hence activated in *O. tsutsugamushi*-stimulated macrophages (Figure 10). Activation of ERK and JNK was detected at 15 min and 30 min and that of p38 was detected only at 15 min (Figure 10).

To investigate whether MAPK phosphorylation was blocked by each inhibitor, selective inhibitors were treated to the cells infected with *O. tsutsugamushi* at indicated time and western blot analysis was performed (Figure 11). PD98059, MEK inhibitor, and SP600125, JNK inhibitor, selectively inhibited *O. tsutsugamushi*-stimulated ERK and JNK phosphorylation, respectively. However SB203580, p38 MAPK inhibitor did not block p38 MAPK phosphorylation itself. SB203580 treatment did not affect ERKs and JNKs phosphorylation.

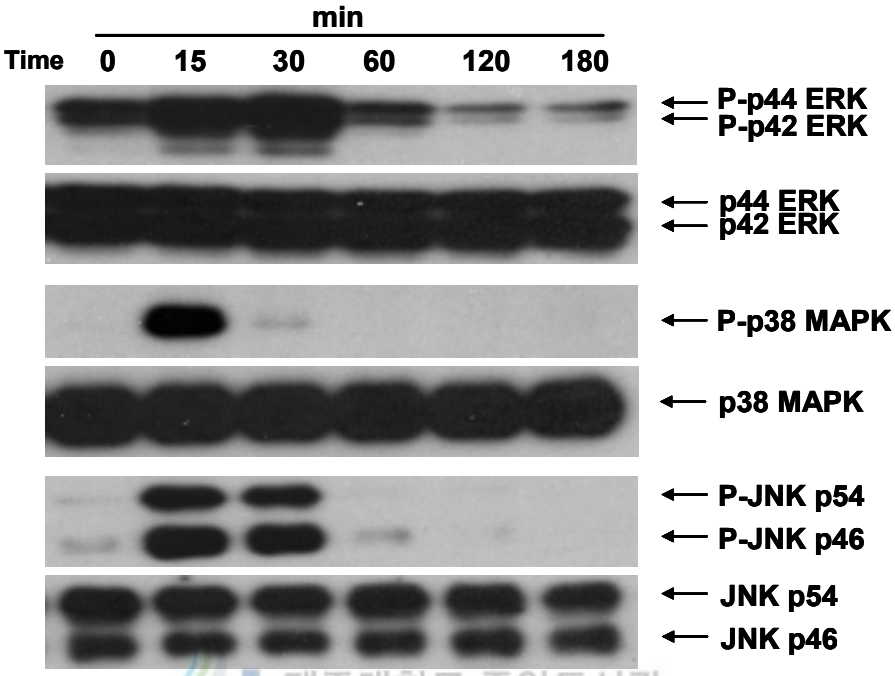

Figure 10. Time-dependent phosphorylation of MAPKs of J774A.1 by *O. tsutsugamushi* infection. J774A.1 cells were infected with *O. tsutsugamushi* at indicated time. Total cell lysates were loaded on gels (25 ㎍ of protein per lane) and subjected to SDS-PAGE and immunoblotting using rabbit polyclonal antibodies specific for the phosphorylated and control MAPKs (p44/p42 ERK, p38 MAPK, JNK p54/p46).

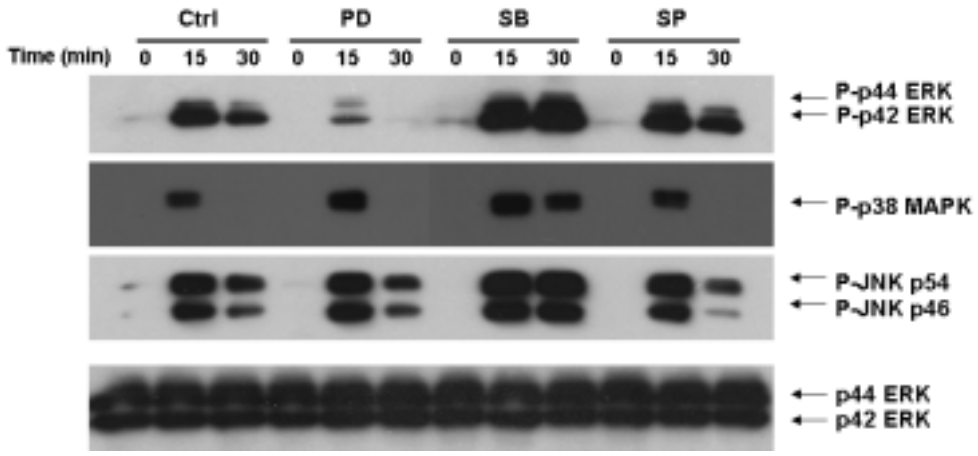

Figure 11. Effects of various MAPK inhibitors on *O. tsutsugamushi*-induced phosphorylation of MAPKs in J774A.1. J774A.1 cells were pretreated for 1 h with each inhibitor before *O. tsutsugamushi* infection. The ERK inhibitor PD98059 (PD, 50 ㎛M), the p38 inhibitor SB203580 (SB, 20 ㎛M) or the JNK inhibitor SP600125 (SP, 20 ㎛M) were used in these experiments. After infection with *O. tsutsugamushi* with or without inhibitor, total cell lysates were loaded on gels (25 ㎍ of protein per lane) and subjected to SDS-PAGE and immunoblotting using rabbit polyclonal antibodies specific for the phosphorylated and control MAPKs (p44/p42 ERK).

6. *O. tsutsugamushi*-stimulated TNF-α production is regulated via ERK pathway by transcriptional mechanism but regulated via p38 or JNK pathways by post-transcriptional and translational mechanism.

To investigate whether MAPK inhibitors have effect on TNF-α induction. MAPK inhibitors, PD98059, SB203580 and SP600125 were used. When the cells were infected with *O. tsutsugamushi* in the presence of PD98059, SB203580 and SP600125 respectively, TNF-α production was diminished significantly (Figures 12A and 13A). To ascertain whether these results were attributed to alteration in intracellular TNF-α protein synthesis or TNF-α processing and secretion, the cells also were treated with monensin which blocked the intracellular protein transport processes. In both intracellular and extracellular parts, the level of TNF-α protein decreased in the cells treated with all MAPK inhibitors. In the cells treated with each MAPK inhibitor and monensin together, TNF-α protein decreased. These results show that TNF-α secretion was not affected by MAPK inhibitors. Namely there was no alteration in TNF-α secretion (Figures 12B and 13B).

To investigate whether MAPK inhibitors have effect on TNF-α gene induction, mRNA level was examined. PD98059, an inhibitor of MEK1/2, could reduce the level of TNF-α mRNA whereas SB203580, an inhibitor of p38 MAPK, could rather increase the level of it (Figure 12C). However, SP600125, an inhibitor of JNK, could not reduce TNF-α induction (Figure 13C). These results show that MAPK inhibitors such as SB and SP could not affect TNF-α gene expression but could affect TNF-α production. In conclusion, TNF-α production via ERK pathway could be regulated mainly by transcriptional mechanism and partly by post-transcriptional mechanism. However, TNF-α production via p38 or

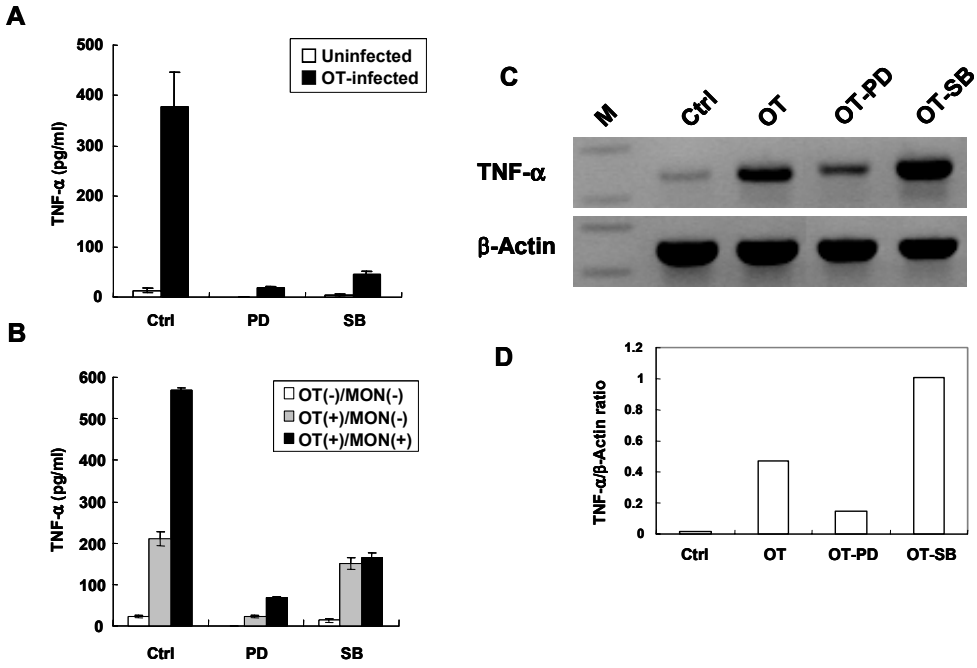

Figure 12. Effect of PD98059 or SB203580 on *O. tsutsugamushi*-stimulated TNF-α production in J774A.1. (A) Determination of TNF-α protein secretion in J774A.1 cells infected with *O. tsutsugamushi* with or without PD or SB. Cell-free supernatants were collected and tested using ELISA kit. Mean ± SD from three duplicate experiments. (B) Determination of intracellular TNF-α protein level by ELISA kit. Cells were infected with *O. tsutsugamushi* with or without PD or SB in the absence or presence of monensin. (C) Determination of TNF-α mRNA induction by RT-PCR analysis. Medium alone (Ctrl), *O. tsutsugamushi*-infected cells (OT), infected cells in the presence of PD (OT-PD) or SB (OT-SB), M, size marker. (D) The band intensities were determined and normalized with mRNA level of β-actin.

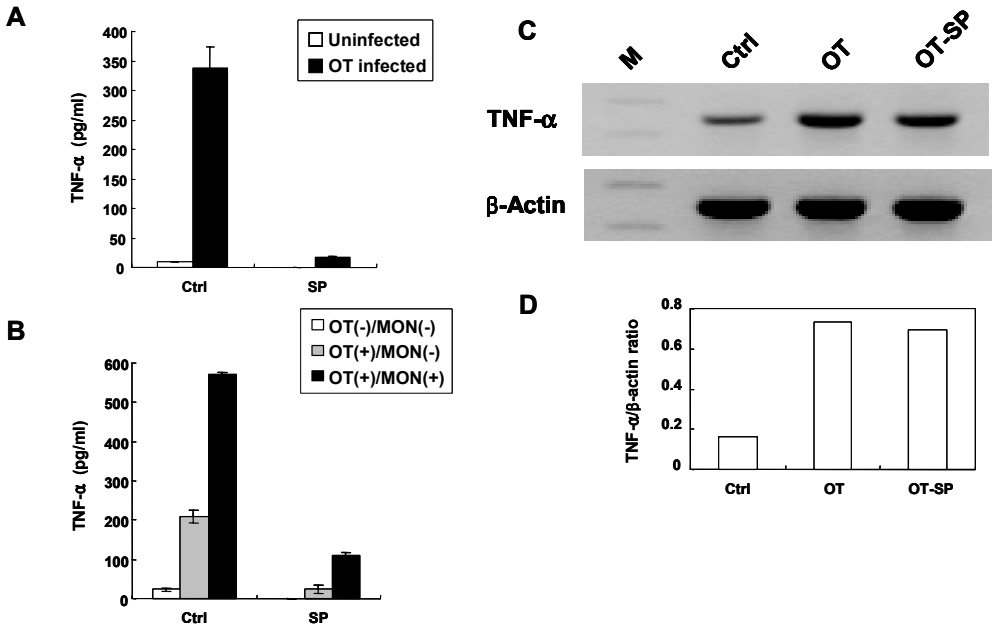

Figure 13. Effect of SP600125 on *O. tsutsugamushi*-stimulated TNF-α production. (A) Determination of TNF-α protein secretion in J774A.1 cells infected with *O. tsutsugamushi* with or without SP. Cell-free supernatants were collected and tested using ELISA kit. Mean ± SD from three duplicate experiments. (B) Determination of intracellular TNF-α protein level by ELISA kit. Cells were infected with *O. tsutsugamushi* with or without SP in the absence or presence of monensin. (C) Determination of TNF-α mRNA induction by RT-PCR analysis. Medium alone (Ctrl), *O. tsutsugamushi*-infected cells (OT), infected cells in the presence of SP (OT-SP), M, size marker. (D) The band intensities were determined and normalized with mRNA level of β-actin.

JNK pathways could be regulated mainly by post-transcriptional and translational mechanism.

7. *O. tsutsugamushi* induces NF-κB activation, but blockage of NF-κB activation by PDTC did not reduce *O. tsutsugamushi*-stimulated TNF-α induction.

The cells were infected with *O. tsutsugamushi* and NF-κB activation was examined (Figure 14A). Since degradation of IκBα represents NF-κB activation, kinetics of IκBα degradation was investigated by western blot analysis (Figure 14A). Degradation of IκBα was detected at 10 min and peaked at 15 min and 20 min, and recovered basal level at 1 h. This result means that NF-κB activation occurs rapidly during infection. To investigate whether NF-κB activation occurs in the experimental condition, selective inhibitors were treated to the cells infected with *O. tsutsugamushi* at indicated time and IκBα degradation was examined. PDTC treatment inhibited *O. tsutsugamushi*-stimulated NF-κB activation whereas TPCK treatment did not inhibit NF-κB completely in this experimental condition (Figure 14B).

To investigate whether blockage of NF-κB activation have effect on *O. tsutsugamushi*-stimulated TNF-α induction, NF-κB inhibitors, TPCK and PDTC were used. When the cells were infected with *O. tsutsugamushi* in the presence of TPCK, TNF-α production was diminished significantly (Figure 15A). However, TNF-α production was not reduced but rather increased greatly when the cells were infected with *O. tsutsugamushi* in the presence of PDTC (Figure 15A). To ascertain whether these results were attributed to alteration in TNF-α processing and secretion or

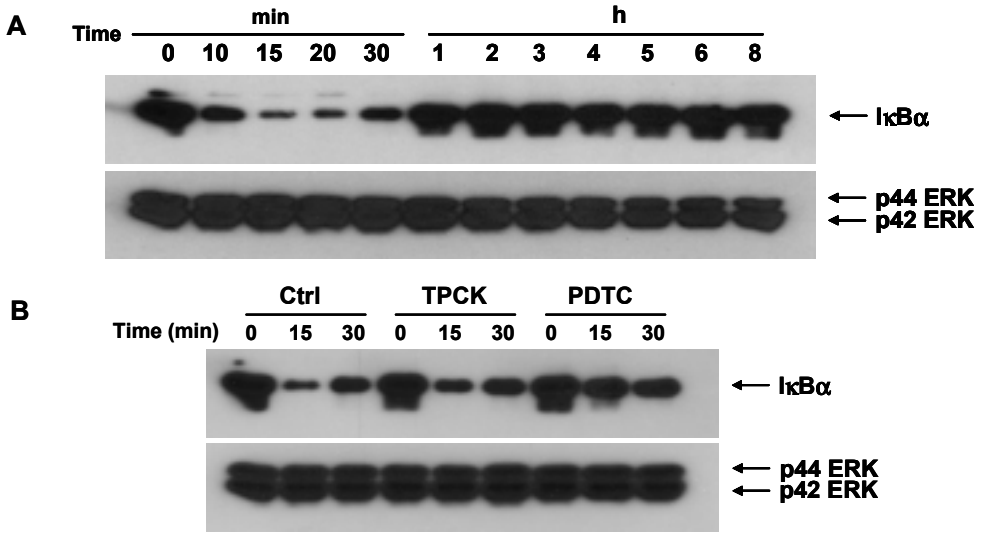

Figure 14. (A) **Time course of IκBα degradation during *O. tsutsugamushi* infection.** J774A.1 cells were infected with *O. tsutsugamushi* at indicated time, and total cell lysates were subjected to immunoblotting using rabbit polyclonal antibodies specific for IκBα and p44/p42 ERK. (B) **Effects of TPCK or PDTC on *O. tsutsugamushi*-stimulated IκBα degradation.** J774A.1 cell were pretreated for 1 h with each inhibitor before *O. tsutsugamushi* infection. Total cell lysates were loaded on gels (25 ㎍ of protein per lane) and subjected to immunoblotting using rabbit polyclonal antibodies specific for IκBα and p44/p42 ERK.

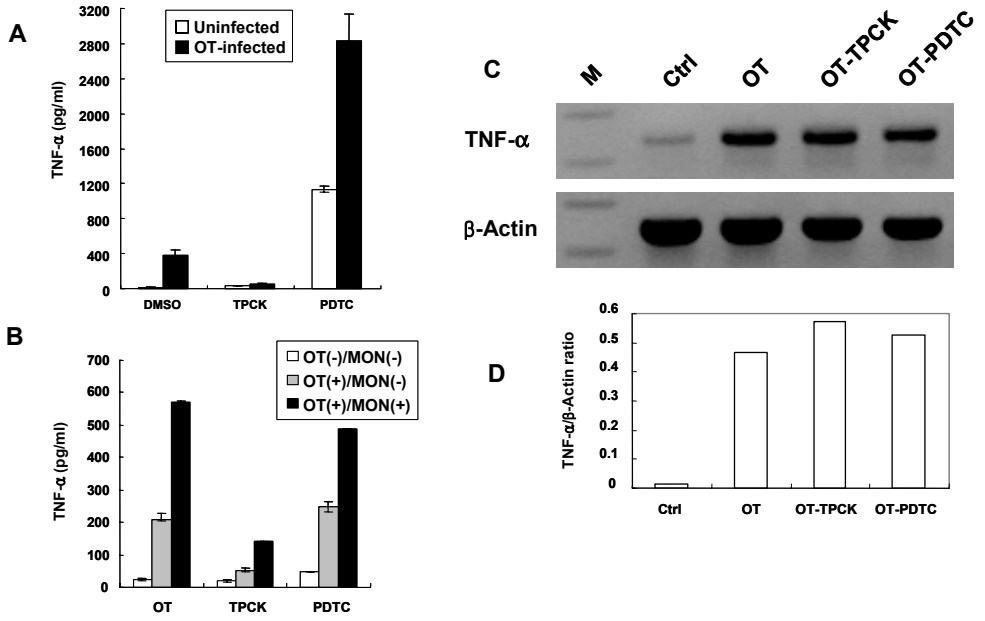

Figure 15. Effect of TPCK or PDTC on *O. tsutsugamushi*-stimulated TNF-α production in J774A.1. (A) Determination of TNF-α protein secretion in J774A.1 cells infected with *O. tsutsugamushi* with or without TPCK or PDTC. Cell-free supernatants were collected and tested using ELISA kit. Mean ± SD from three duplicate experiments. (B) Determination of intracellular TNF-α protein level by ELISA kit. Cells were infected with *O. tsutsugamushi* with or without TPCK or PDTC in the absence or presence of monensin. (C) Determination of TNF-α mRNA induction by RT-PCR analysis. Medium alone (Ctrl), *O. tsutsugamushi*-infected cells (OT), infected cells in the presence of TPCK (OT-TPCK) or PDTC (OT-PDTC), M, size marker. (D) The band intensities were determined and normalized with mRNA level of β-actin.

intracellular TNF-α protein synthesis, the cells were treated with monensin which blocked the intracellular protein transport processes. In both intracellular and extracellular parts, the level of TNF-α protein decreased in the cells treated with TPCK, whereas the level of TNF-α protein did not decrease in the cells treated with PDTC. In the cells treated with TPCK and monensin together, level of TNF-α protein decreased. In the cells treated with PDTC and monensin together, level of TNF-α protein was similar to that infected *O. tsutsugamushi* without monensin (Figure 15B). These results show that TPCK treatment did not affect secretion but blocked TNF-α protein synthesis, and PDTC treatment did not block TNF-α protein synthesis and facilitated TNF-α secretion. To examine TNF-α mRNA level, RT-PCR analysis was performed (Figure 15C). When the cells were infected with *O. tsutsugamushi* in the presence of TPCK or PDTC, mRNA level of TNF-α was not reduced, compared with that in the cell infected with *O. tsutsugamushi* alone (Figure 15C). These results show that both TPCK and PDTC treatment could not affect TNF-α gene expression. Decrease in production of TNF-α by treatment with TPCK seems to be caused by regulation through post-transcriptional or translational mechanism.

8. Bacterial invasion but not viability was required for *O. tsutsugamushi*-stimulated TNF-α production.

To evaluate effect of bacterial invasion on TNF-α production, J774A.1 cells were infected for 6 h with *O. tsutsugamushi* in the absence or presence of cytochalasin D (CD) which inhibits bacterial invasion. As shown in Figure 16A, TNF-α production decreased in cells treated with

CD as compared with that in CD-untreated cells. mRNA level of TNF-α decreased (Figure 16B). In addition, just attachment of *O. tsutsugamushi* did not fully activate TNF-α production fully. These results suggest that bacterial invasion was required for host TNF-α production.

It was investigated that heat inactivation of bacteria have effect on TNF-α production. When the cells were treated with heat-inactivated bacteria (HOT), TNF-α induction increased higher than that treated with live *O. tsutsugamushi*.

To ascertain whether blockage of NF-κB activation occurs in this experimental condition, western blot analysis was performed. NF-κB was activated in the cell treated with CD whereas it was not activated in the cells treated with HOT (Figure 16D).

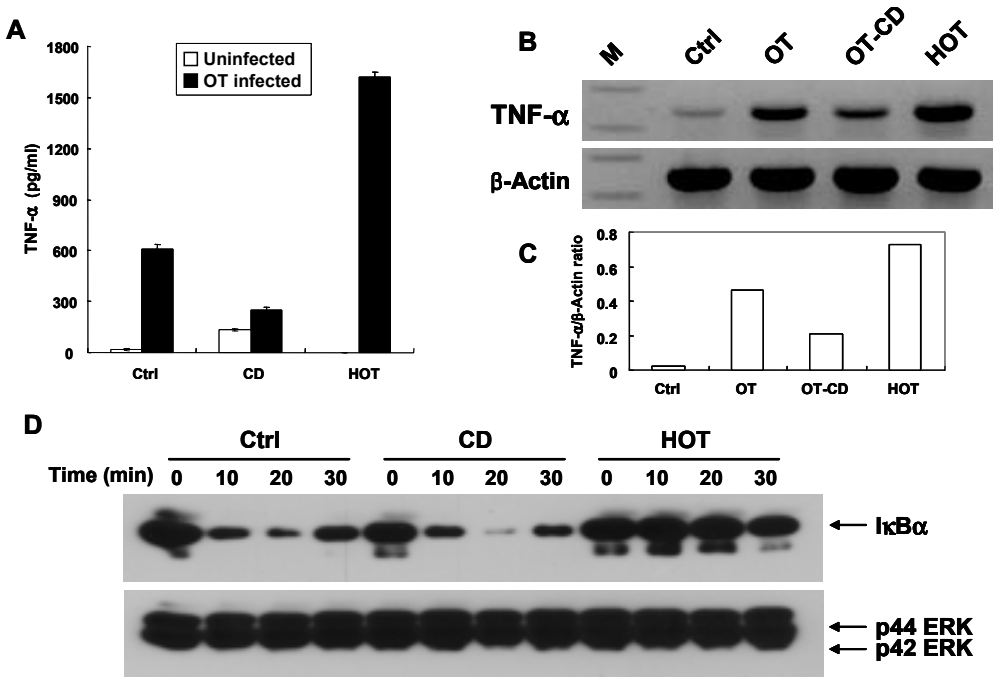

Figure 16. Effect of cytochalasin D (CD) or heat-inactivated *O. tsutsugamushi* (HOT) on *O. tsutusgamushi*-induced TNF-α production and IκBα degradation in J774A.1. (A) Determination of TNF-α protein secretion in J774A.1 cells infected with *O. tsutusgamushi* with or without CD or treated with HOT. Cell-free supernatants were collected and tested using ELISA kit. Mean ± SD from three duplicate experiments. (B) Determination of TNF-α mRNA induction by RT-PCR analysis. Medium alone (Ctrl), *O. tsutusgamushi*-infected cells (OT), infected cells in the presence of CD (OT-CD) and heat-inactivated *O. tsutusgamushi* (HOT), M, size marker. (C) The band intensities were determined and normalized with mRNA level of β-actin. (D) Effect of CD or heat inactivation of bacteria on IκBα degradation in J774A.1. J774A.1 cells were infected with *O. tsutusgamushi* with or without CD or treated with HOT. Total lysates were loaded on gels (25 ㎍ of protein per lane) and subjected to SDS-PAGE and immunoblotting using rabbit polyclonal antibodies to total I κBα and p44/p42 ERK.

DISCUSSION

TNF-α is one of the proinflammtory cytokines and plays a crucial role in host defense and inflammatory response. In this study, I showed that TNF-α gene expression was induced and TNF-α protein was produced during *O. tsutsugamushi* infection (Figures 1 and 2). It was reported that susceptible mouse strains (C3H/HeN) produced higher level of TNF-α in response to *O. tsutsugamushi* infection compared to resistant mouse strains (BALB/c) (Yun *et al.*, 2005). However, receptors and signal transducing molecules involved in *O. tsutsugamushi*-induced TNF-α production have not yet been elucidated.

This study showed that TLR4 and LPS is probably not involved in *O. tsutsugamushi*-stimulated TNF-α production (Figure 3). This result is consistent with that *O. tsutsugamushi* has little or no peptidoglycan or lipopolysaccharide components (Amano *et al.*, 1987). However, this result does not exclude the possibility that the other TLRs are involved in *O. tsutsugamushi*-induced TNF-α production. As macrophages from mice with the targeted disruption of the TLR-associated adaptor molecules MyD88 are not able to produce TNF-α to microbial stimuli (Kawai *et al.*, 1999; Henneke *et al.*, 2001), it should be verified whether *O. tsutsugamushi*-induced TNF-α production have relation with TLRs. If TLRs are not implicated in TNF-α production, another candidate is nucleotide-binding site and leucine-rich repeat (NBS-LRR) domains (NOD protein family). Since *O. tsutsugamushi* replicates in the cytoplasm after escape from phagosome, NOD family could recognize it in the cytoplasm. Response of receptor interacting protein 2 (RIP2) deficient mice suggest

involvement of NOD family, because RIP2 is a downstream molecule which is recruited by NOD. If both TLRs or NOD family are not pattern recognition receptors to *O. tsutsugamushi*, unknown receptor may be involved in bacterial recognition.

TNF-α have its own receptor, TNF receptor (TNFR) family, and can amplify the signals. Cycloheximide (CHX) can block eukaryotic protein synthesis. When the cells were infected with *O. tsutsugamushi* in the presence of CHX, the level of TNF-α protein decreased but the level of TNF-α mRNA did not decrease. Although treatment of CHX alone increase TNF-α mRNA expression because CHX induces sustained ERK activation through inhibition of the translation and activity of MAPK phosphatase-1 (MKP-1) (Lin *et al.*, 2000), these results indicate that *O. tsutsugamushi*-stimulated TNF-α production does not require eukaryotic protein synthesis.

O. tsutsugamushi activates MAPKs in macrophage cells (Figure 10). When the cells were treated with inhibitor of MAPK/Erk kinase 1/2 (MEK1/2), PD98059 (PD), ERKs phosphorylation was inhibited selectively (Figure 11). At the same time, the level of TNF-α mRNA was reduced and the secretion of TNF-α protein was completely prevented. TNF-α production was mainly regulated at transcriptional level and partly at post-transcriptional level. Treatment of SB203580 (SB), a p38 MAPK inhibitor, did not block p38 MAPK phosphorylation. SB-treatment rather increased slightly the level of p38 phosphorylation (Figure 11). SB treatment rather increased the level of TNF-α mRNA but prevented secretion of TNF-α protein completely (Figure 12). In addition, SB treatment rather increased phosphorylation of ERK. Because SB-stimulated Raf-1 activates MEK1 (Kalmes *et al.,* 1998), activation of ERK increased slightly in the cells treated with SB (Figure 11), and then

TNF-α mRNA expression increased by SB treatment (Figure 12C and D). SB203580 suppresses MK2 which is a downstream substrate and regulates biosynthesis of TNF-α at a post-transcriptional level (Kotlyarov *et al.,* 1999). It suggests that p38 MAPK regulates TNF-α production by translational mechanism. SP600125, a JNK inhibitor, could not inhibit TNF-α induction significantly but the level of TNF-α production was greatly reduced (Figure 13). This result indicates that TNF-α production via JNK pathway is mainly regulated by post-transcriptional machanism.

NF-κB activation also occurs at early time during *O. tsutsugamushi* infection (Figure 14A). Degradation of IκBα represented NF-κB activation indirectly, because degradation of IκBα and activation of NF-κB are concomitant event. PDTC, a NF-κB inhibitor, greatly inhibited activation of NF-κB. However, it did not affect the level of mRNA but increased TNF-α protein production (Figure 15). It suggests that PDTC inhibits NF-κB fully and may affect secretion of TNF-α protein. Although PDTC treatment blocked activation of NF-κB, the TNF-α mRNA did not decrease any more. This suggests that blockage of NF-κB activity only is not sufficient for complete reduction in TNF-α gene expression during *O. tsutsugamushi* infection, and the other transcription factors also play roles in transcription.

Cytochalasin D (CD) treatment downregulates significantly TNF-α mRNA expression and results in decrease in TNF-α production. This result suggests that TNF-α production may be attributed to attachment of bacteria and invasion. Heat-inactivated *O. tsutsugamushi* did not activate NF-κB but rather increased the mRNA and protein level. It means that *O. tsutsugamushi*-stimulated TNF-α production did not require live bacteria.

In conclusion, this study suggests model of signaling and gene regulatory mechanisms of TNF-α biosynthesis (Figure 17). *O. tsutsugamushi*

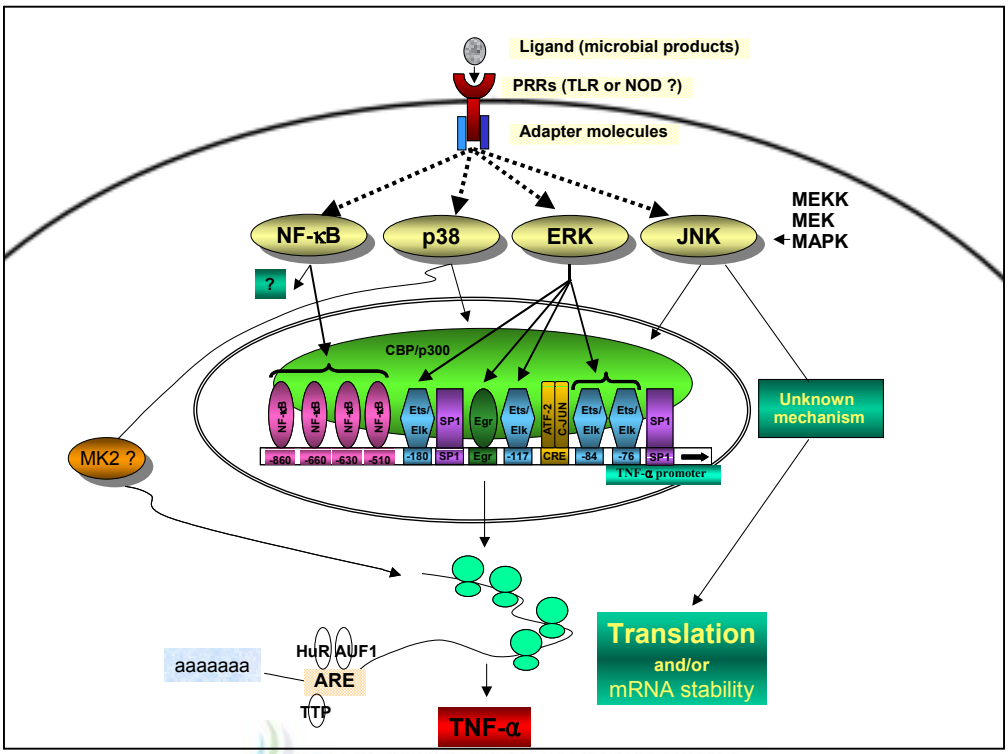

Figure 17. Model of signaling and gene regulatory mechanisms of TNF-α biosynthesis during *O. tsutsugamushi* infection. *O. tsutsugamushi* stimulates receptor(s) and activates host MAPK and NF-κB signaling pathways. Signals from NF-κB and ERK pathways are required to promote strong transcription. Formation of enhanceosome was followed. NF-κB and other transcription factors interact with DNA, co-activators such as CBP/p300 and the RNA polymerase II holoenzyme. Transport of TNF-α mRNA is facilitated by ERK pathway. Transported mRNA is stabilized by p38-MK2 pathway. JNK pathway possibly regulates translation of mRNA.

stimulates pattern recognition receptors and subsequently activates signal cascade (Akira *et al.*, 2004). These receptor complexes interact homophilically with adaptor molecules such as myeloid differentiation primary-response protein 88 (MyD88) and tumor necrosis factor receptor-associated factors (TRAFs). Following the interaction, MyD88 recruits IL-1R-associated kinase (IRAK) and TRAF6. Phosphorylated IRAK and TRAF6 then dissociated from the receptor and lead to the activation of the IKK complex and MAP kinases. These signals subsequently activate NF-κB and MAPK signaling pathways, respectively. Signals from NF-κB and ERK pathways are required to promote strong transcription. Formation of enhanceosome was followed (Lee *et al.*, 2005). Variation within the enhanceosome composition result in activation of specific genes (Tsai *et al.*, 1996; Falvo *et al.*, 2000). NF-κB and other transcription factors interact with DNA, co-activators such as CREB binding protein/a histone acetyltransferase (CBP/p300) and the RNA polymerase Ⅱ holoenzyme. Transport of the synthesized mRNA of TNF-α is regulated by ERK pathway (Dumitru *et al.*, 2000), and stability of mRNA is regulated by p38-MK2 pathway (Kotlyarov *et al.*, 1999). In addition, p38 and JNK pathways possibly regulate translation of TNF-α mRNA.

In conclusion, MAPKs pathways are required to induce maximal TNF-α production during *O. tsutsugamushi* infection. In addition, TNF-α production is regulated by transcriptional and post-transcriptional control. Further study is needed to verify the PRR and signal transduction mechanism during *O. tsutsugamushi* infection.

REFERENCES

Amano A, Tamura A, Ohashi N, Urakami H, Kaya S, Fukushi K. 1987. Deficiency of peptidoglycan and lipopolysaccharide components in *Rickettsia tsutsugamushi*. Infect Immun 55, 2290-2292.

Allen AC, Spitz S. 1945. A comparative study of the pathology of scrub typhus (tsutsugamushi disease) and other rickettsial disease. Am J Pathol 21, 603-681.

Ahmad-Nejad P, Hacker H, Rutz M, Bauer S, Vabulas RM. Wagner H. 2002. Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments. Eur J Immunol 32, 1958-1968.

Akira S, Takeda K. 2004. Toll-like receptor signalling. Nature Rev immunol 4, 499-511.

Ashkar S, Weber GF, Panoutsakopoulou V, Sanchirico ME, Jansson M, Zawaideh S, Rittling SR, Denhardt DT, Glimcher MJ and Cantor H. 2000. Eta-1 (osteopontin): an early component of type-1 (cell-mediated) immunity. Science 287, 860-864.

Baggiolini M, Dewald B, Moser B. 1994. Interleukin-8 and related chemotactic cytokines-CXC and CC chemokines. Adv Immunol 55, 97-179.

Beutler B, Cerami A. 1986. Cachectin and tumor necrosis factor as two sides of the same biological coin. Nature 320, 584-588.

Burnett JW. 1980. Rickettsioses: a review for the dermatologist. J Am Acad Dermatol 2, 359-373.

Chang WH, Kang JS, Lee WK, Choi MS, and Lee JH. 1990. Serological classification by monoclonal antibodies of *Rickettsia tsutsugamushi* isolated in Korea. J Clin Microbiol 28, 685-688.

Chen Z, Gibson TB, Robinson F, Silvestro L, Pearson G, Xu B, Wright A,

Vanderbilt C, MH Cobb. 2001. MAP kinases. Chem Rev 101, 2449-2476.

Chi WC, Huang JJ, Sung JM, Lan RR, Ko WC, Chen FF. 1997. Scrub typhus associated with multiorgan failure: a case report. Scand J Infect Dis 29, 634-635.

Cho NH, Seong SY, Huh MS, Han TH, Koh YS, Choi MS, Kim IS. 2000. Expression of chemokine genes in murine macrophage infected with *Orientia tsutsugamushi*. Infect Immun 68, 594-602.

Choi NJ, Kim MK, Park HJ, Lim BU, Kang JS. 1998. Apoptosis of murine macrophage-like cells infected with *Orientia tsutsugamushi*. J Korean Soc Microbiol 33, 399-406.

Dumitru CD, Ceci JD, Tsatsanis C, Kontoyiannis D, Stamatakis K, Lin JH, Patriotis C, Jenkins NA, Copeland NG, Kollias G, Tsichlis PN. 2000. TNF-α induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. Cell 103, 1071-1083.

Enatsu T, Urakami H, Tamura A. 1999. Phylogenetic analysis of *Orientia tsutsugamushi* strains based on the sequence homologies of 56-kDa type-specific antigen genes. FEMS Microbiol Lett 180, 163-169.

Falvo JV, Uglialoro AM, Brinkman BMN, Merika M, Parekh BS, Tsai EY, King HC, Morielli AD, Peralta EG, Maniatis T, Thanos D, Goldfeld AE. 2000. Stimulus-specific assembly of enhancer complexes on the tumor necrosis factor alpha gene promoter. Mol Cell Biol 20, 2239-2247.

Frodin M, Gammeltoft S. 1999. Role and regulation of 90 kDa ribosomal S6 kinase (RSK) in signal transduction. Mol Cell Endocrinol 151, 65-77.

Groves MG, Rosenstreich DL, Taylor BA, Osterman JV. 1980. Host defenses in experimental scrub typhus: mapping the gene that controls natural resistance in mice. J Immunol 125, 1395-1399.

Hedrick JA, Zlotnik A. 1999. Chemokines and chemokine receptors in T-cell develpoment. Chem Immunol 72, 57-68.

Heil F, Ahmad-Nejad P, Hemmi H, Hochrein H, Ampenberger F, Gellert T, Dietrich H, Lipford G, Takeda K, Akira S, Wagner H, Bauer S. 2003. The Toll-like receptor 7 (TLR7)-specific stimulus loxoribine uncovers a strong relationship within the TLR7, 8 and 9 subfamily. Eur J Immunol 33, 2987-2997.

Henneke P, Takeuchi O, van Strijp JA, Guttormsen JA, Smith A, Schromm AB, Espevik TA, Akira S, Niaet V, Kasper L, Golenbock DT. 2001. Novel engagement of CD14 and multiple Toll-like receptors by Group B Streptococci. J Immunol 167, 7069-7076.

Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, Akira S. 1999. Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: Evidence for TLR4 as the *Lps* gene product. J Immunol 162, 3749-3752.

Iwasaki H, Tskada N, Nakamura T, Ueda T. 1997. Increased levels of macrophage colony-stimulating factor, gamma interferon, and tumor necrosis factor alpha in sera of patients with infection. J Clin Microbiol 335, 3320-3322.

Jerrells TR and Geng P. 1994. The role of tumor necrosis factor in host defense against scrub typhus rickettsiae. Ⅱ. Differential induction of tumor necrosis factor-alpha production by *Rickettsia tsutsugamushi* and *Rickettsia conorii*. Microbiol Immunol 38, 713-719.

Jerrells TR, Osterman JV. 1981. Host defenses in experimental scrub typhus: inflammatory response of congenic C3H mice differing at the *Ric* gene. Infect Immun 31, 1014-1022.

Kalmes A, Deou J, Clowes AW, Daum G. 1999. Raf-1 is activated by the p38 mitogen-activated protein kinase inhibitor, SB203580. FEBS Letts 444, 71-74.

Kawai T, Adachi O, Ogawa T, Takeda K, Akira S. 1999. Unresponsiveness of MyD88-deficient mice to endotoxin. Immunity 11, 115-122.

Kawamura A Jr, Tanaka H, Tamura A, Kawamura AJ, Tanaka H, Tamura

A. 1995. Tsutsugamushi disease. University of Tokyo Press.

Kim IS, Seong SY, Woo, SG, Choi, MS, and Chang, WH. 1993. High-level expression of a 56-kilodalton protein gene (*bor*56) of *Rickettsia tustusgamushi* Boryong and its application to enzyme-linked immunosorbent assays. J Clin Microbiol 31, 598-605.

Koh YS. 2001. Exprssion of chemokine and tumor necrosis factor alpha genes in murine peritoneal macrophages infected with *Orientia tsutsugamushi*. J Microbiol 39, 186-194.

Koh YS, Yun JH, Seong SY, Choi, MS, Kim IS. 2004. Chemokine and cytokine production during *Orientia tsutsugamushi* infection in mice. Microbiol Immunol 36, 51-57.

Kotlyarov A, Neininger A, Shubtert C, Eckert R, Birchmeier C, Volk HD, Gaestel M. 1999. MAPKAP kinase 2 is essential for LPS-induced TNF-α biosynthesis. Nat Cell Biol 1, 94-97.

Kyriakis JM, and Avruch J. 2001. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev 81, 807-869.

Lee SB, Schorey JS. 2005. Activation and mitogen-activated protein kinase regulation of transcription factors Ets and NF-κB in mycobacterium-infected macrophages and role of these factors in tumor necrosis factor alpha and nitric oxide synthase 2 promoter function. Infect Immun 73, 6499-6507.

Lin WW, Hsu YW. 2000. Cycloheximide-induced $cPLA_2$ activation is via the MKP-1 down-regulation and ERK activation. Cell Signal 12, 457-461.

Medvedev AE, Flo T, Lugalls RR, Golenbock DT, Teti G, Vogel SN, Espevik T. 1998. Involvement of CD14 and complement receptors CR3 and CR4 in nuclear factor-κB activation and production induced by lipopolysaccharide and group B streptococcal cell walls. J Immunol 164, 4535-4542.

Medzhitov AE, Charles A, Janeway Jr. 1997. Innate immunity: The virtues of a nonclonal system of recognition. Cell 91, 295-298.

Matsumoto M, Funami K, Tanabe M, Oshiumi H, Shingai M, Seto Y, Yamamoto A, Seya T. 2003. Subcellular Localization of Toll-Like Receptor 3 in Human Dendritic Cells. J Immunol 171, 3154-3162.

Nacy CA, and Groves MG. 1981. Macrophages in resistance to rickettsial infection: early host defense mechanisms in experimental scrub typhus. Infect Immun 31, 1239-1250.

Ng FK, Oaks SC Jr, Lee M, Groves MG, Lewis Jr GE. 1985. A scanning and transmission electron microscopic examination of *Rickettsia tsutsugamushi*-infected human endothelial endothelial. MRC-5, and L-929 cells. Jpn J Med Sci Biol 38, 125-139.

Oaks SC Jr, Ridgway RL, Shirai A, Twartz JC. 1983. Scrub Typhus: Bulletin No. 21 from the Institute for Medical Research Malaysia, United States Army Medical Research Unit.

Ohashi N, Fukuhara M, Shimada M, Tamura A. 1995. Phylogenetic position of *Rickettsia tsutsugamushi* and the relationship among its antigenic variants by analyses of 16S rRNA gene sequence. FEMS Microbiol Lett 125, 299-304.

Pasparakis M, Alexopoulou L, Douni E, Kollias G. 1996. Tumor necrosis factors in immune regulation: everything that's interesting is...new!. Cytokine Growth Factor Rev 7, 223-229.

Patarca R, Freeman GJ, Singh RP, Wei F-Y, Durfee T, Blattner F, Regnier DC, Kozak CA, Mock BA, Morse HC Ⅲ, Jerrells TR and Cantor H. 1989. Structural and functional studies of the early T lymphocyte activation 1 (Eta-1) gene. Definition of a novel T cell-dependent response associated with genetic resistance to bacterial infection. J Exp Med 170, 145-161.

Qureshi TS, Larivière L, Leveque G, Clermont S, Moore JK, Philippe G, Malo D. 1999. Endotoxin-tolerant mice have mutations in Toll-like receptor

4 (*Tlr4*). J Exp Med 189, 615-625.

Rikihisa Y, Ito S. 1979. Intracellular localization of *Rickettsia tsutsugamushi* in polymorphonuclear leukocytes. J Exp Med 150, 703-708.

Roux PP, Blenis J. 2004. ERK and p38 MAPK-Activated Protein Kinases: a Family of Protein Kinases with Diverse Biological Functions. Microbiol Mol Biol Rev 68, 320-344.

Sanghera JS, Weinstein SL, Aluwalia M, Girn J, Polech SL. 1996. Activation of multiple proline-directed kinases by bacterial lipopolysaccharide in murine macrophages. J Immunol 156, 4457-4465.

Seong SY, Choi MS, Kim IS. 2001. *Orientia tsutsugamushi* infection: overview and immune responses. Microbes Infect 3, 11-21.

Siveke JT, Hamann A. 1998. T helper 1 and T helper 2 cells respond differentially to chemokines. J Immunol 160, 550-554.

Tamura A. 1988. Invasion and intracellular growth of *Rickettsia tsutsugamushi*. Microbiol Sci 5, 228-232.

Tamura A and Urakami H. 1981. Easy method for infectivity titration of *Rickettsia tustusgamushi* by infected cell counting. Nippon Saikingaku Zasshi 36, 783-785.

Tamura A, Urakami H, Ohashi N. 1991. A comparative view of *Rickettsia tsutsugamushi* and the other groups of rickettsiae. Eur J Epidemiol 7, 259-269.

Tsai EY, Yie J, Thanos D, Goldfeld A. 1996. Cell-type specific regulation of the human tumor necrosis factor alpha gene in B cells and T cells by NFATp and ATF-2/JUN. Mol Cell Biol 16, 5232-5244.

Utaisincharoen P, Arjcharoen S, Lengwehasatit I, Limposuwan K, Sirisinha

S. 2005. Burkholderia pseudomallei invasion and activation of epithelial cells requires activation of p38 mitogen-activated protein kinase. Microb Pathog 38, 107-112.

Vassalli P. 1992. The pathophysiology of tumor necrosis factors. Annu Rev Immunol 10, 411-452.

Yun JH, Koh YS, Lee KH, Hyun JW, Choi YJ, Jang WJ, Park KH, Cho NH, Seong SY, Choi MS, and Kim IS. 2005. Chemokine and cytokine production in susceptible C3H/HeN mice and resistance BALB/c mice during *Orientia tsutsugamushi* infection. Microbiol Immunol 49, 551-557.

국문초록

*Orientia tsutsugamushi*는 절대세포 내 기생세균으로 쯔쯔가무시 병의 원인 병원체이다. 이 균은 주로 모세혈관을 이루는 혈관내피세포를 감염하여 혈관염을 유발하는데 이는 orientiae가 염증반응을 증폭시키는 기전을 유도함을 암시한다. 쯔쯔가무시 병의 발병기전을 이해하기 위해 *O. tsutsugamushi* 감염 후 tumor necrosis factor-alpha (TNF-α)의 유전자 발현을 *in vivo*와 *in vitro*에서 조사하였다. 마우스에서는 감염 후 4 일과 8 일 사이에 TNF-α 유전자가 발현 유도되는 것이 확인되었다. macrophage cell line에서는 6 시간과 24 시간 사이에 TNF-α 유전자가 최대로 발현된 후, 48 시간까지 감소하였다. TLR4 결핍 쥐의 대식세포가 *O. tsutsugamushi*에 대하여 반응하지 않음을 증명하였다. *O. tsutsugamushi*를 감염시킨 대식세포에서 세포 추출물을 western blot 분석을 수행하였더니 ERK1/2와 JNK1/2 그리고 p38 MAPK의 인산화를 확인하였다. 특이 저해제를 사용하여 선택적으로 각 MAP kinase를 억제하였더니, TNF-α의 분비가 대부분 감소하였다. 그러나 이 저해제들은 *O. tsutsugamushi*의 숙주세포로의 침입을 방해하지 않았고, TNF-α의 가공과 분비에도 영향을 주지 않았다. p38과 JNK 경로를 통한 숙주 TNF-α 생성유도는 주로 전사 후 조절 기전-주로 번역단계 조절-으로 조절됨을 확인하였다. 반면, ERK 경로는 주로 TNF-α 유전자 발현의 전사 단계를 조절하였다. 열처리하여 사멸된 Orientia를 숙주세포에 처리하였을 때 TNF-α 생산유도가 감소하지 않았다. 균의 숙주세포 침입을 막는 cytochalasin D를 *O. tsutsugamushi*와 함께 처리하였을 경우 TNF-α 유전자의 발현은 감소하였는데 이는 세균에 의한 숙주세포 침입이 TNF-α 생산 유도에 요구됨을 시사한다. 결론적으로 이 결과들은 *O. tsutsugamushi* 감염 시 TNF-α 생성이 최적화되기 위해서는 MAPK 경로가 작동해야 한다는 것을 암시한다.

주요어: *Orientia tsutsugamushi,* TNF-α, ERK, JNK, p38, 신호전달, 유전자 발현 조절

감사의 글

그렇게도 아득하기만 하더니 어느덧 박사학위논문을 마무리하는 시점에 이르렀습니다. 지나온 시간들과 저의 연구결과물을 돌이켜보니, 다듬어지지 않은 부분이 많음을 깨달으며 더욱 노력하리라 다짐을 하게 됩니다.

이 논문이 있기까지 연구수행과 완성에 도움과 격려를 아끼지 않은 많은 분들에게 이 지면을 빌어 감사의 마음을 전하고 싶습니다. 본 논문이 완성되기까지 부족한 점을 꼼꼼하게 지적해 주시는 세심한 배려와 주말에도 시간을 내주신 고영상 교수님께 감사드립니다. 그리고 바쁘신 와중에도 많은 관심을 가져주시고 조언을 아끼지 않으신 박덕배 교수님, 유은숙 교수님, 현진원 교수님, 이근화 교수님께도 감사의 인사와 함께 앞으로도 많은 지도를 부탁드리며 항상 건강하시길 기원합니다. 더불어 오늘의 저를 올바른 배움의 길로 갈 수 있게 이끌어주신 배종면 교수님, 이봉희 교수님, 이영기 교수님, 강현욱 교수님, 강희경 교수님, 조문제 교수님, 홍성철 교수님, 김문두 교수님, 이상이 교수님, 정영배 교수님, 조수현 교수님, 김수영 교수님, 은수용 교수님 그리고 이창인 학장님을 비롯해 다른 여러 의대 교수님들께도 감사드립니다. 그리고 항상 애정을 가지고 저를 걱정해주시는 김세재 교수님께 감사의 인사와 함께 하시는 일이 더욱 발전하시길 기원합니다. 언제나 든든한 후원자인 우리 집 장남 원종이와 짜증 한 번 안내고 무리한 부탁도 다 들어주는 형복 오빠 그리고 항상 웃는 얼굴로 적극적으로 도와주는 혜자와 은희에게도 감사의 뜻을 전합니다. 또한 항상 열심히 하는 정은이와 시우, 약리학 실험실의 상철이, 재희, 정일이, 경진이, 엘비라 선생님, 은진이, 생리학실험실의 희경이, 임상교수실험실의 안춘산 선생님, 최수길 선생님, 생화학실험실의 김영미 선생님, 임희경 선생님, 경아, 진영이, 장예, 미경 선생님, 혜숙이, 예방의학실험실의 양영자 선생님, 고여주 선생님, 그리고 이정희 선생님, 경희 선생님, 한은혜 선생님, 최지강 선생님, 오근희 선생님과 명선이에게 감사의 뜻을 전합니다. 항상 모범이 되어주시던 강신해 선생님, 박지권 선생님 그리고 분자생물학 실험실의 최수연 선생님과 영준이, 경만이, 그리고 여러 후배들과 TIC의 현근희 선생님과 명옥이에게도 감사의 뜻을 전합니다. 그리고 항상 고민을 들어주고 휴식이 되는 나의 벗인 혜영이와 상렬이, 내가 불쑥 찾아가도 싫은 내색 없이 잘 대해주는 수영이에게도 감사의 뜻을 전합니다.

마지막으로 오늘이 있기까지 항상 사랑으로 지켜봐 주신 부모님과 잘 챙겨주지 못했던 동생들에게 말로 다 표현할 수 없는 고마움과 사랑을 전합니다.